MICROBLED IN DEMENTIA:
CONNECTING THE DOTS

Jeroen Goos
VRIJE UNIVERSITEIT

Microbleeds In Dementia

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‘Tobt niet, het komt toch anders....’

Opa Goos
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INTRODUCTION

1.1.1 General introduction

Dementia
Dementia is a syndrome that includes progressively affected memory, thinking and behaviour, and as such interferes with everyday activities. Recently, it has been estimated that 35.6 million people are living with dementia worldwide and the projections were that these numbers will double by 2030 and even triple by 2050 [http://www.who.int/features/factfiles/dementia/en/index.html]. As not only patients, but also caregivers and family greatly suffer from this devastating disease, these figures of epidemic proportions predict an important public health and economical problem, as no disease modifying therapies are available.

The most prevalent causes of dementia are Alzheimer disease (AD) and vascular dementia (VaD). Currently, two main theories explaining the pathogenesis of AD are popular the amyloid cascade hypothesis,¹ and the vascular hypothesis.²

Imaging of dementia
Clinical neuroimaging is being used increasingly in diagnosing neurodegenerative diseases and has become one of the most important diagnostic tools. According to current guidelines, neuroimaging, preferably magnetic resonance imaging (MRI), should be performed at least once during the diagnostic work-up of patients with suspected or definite dementia.³ MRI is able to exclude other causes mimicking dementia, and moreover support the clinical diagnosis by identifying certain patterns of atrophy and vascular damage. Recently, a multi-sequence MRI protocol for dementia screening has been recommended to include an axial T2*-weighted gradient echo sequence, in order to detect signs of past hemorrhages.⁴
Microbleeds

After the introduction of the T2*-weighted MRI sequence, sensitive for paramagnetic tissue properties, mysterious black dots became visible in the brains of selected human subjects. In the last year of the previous millennium, Fazekas and coworkers performed the first post-mortem radiologic neuropathologic correlation study and discovered that these black dots visible at T2*-weighted MRI, corresponded to small foci of hemosiderin, deposited in macrophages in brain parenchyma surrounding abnormal small cerebral vessels. The observed magnetic susceptibility signal loss on this specific MRI sequence, resulting in these black dots, was thus confirmed to arise from chronic blood breakdown products and have been considered since as radiologic evidence of past microscopic hemorrhage, referred to as cerebral microhemorrhages or microbleeds (MBs).

Clinical problem of MBs in dementia

The relevance of these clinically silent microscopic hemorrhages has long been unknown. Therefore, MBs have been considered to simply be shadows, or even mere artefacts, which technically speaking, may be true indeed, as they result from signal distortion due to increased magnetic susceptibility. As research on the subject intensified however, it became clear that MBs were associated with signs of small vessel disease and related to vascular risk factors. Moreover, they were more frequently found in patients with stroke and specific vessel diseases. From stroke populations, evidence has emerged that MBs could predict subsequent hemorrhagic and also ischemic strokes. Furthermore, MBs have been associated with worse cognitive outcomes and mortality in stroke populations. Moreover, our own group found that MBs were more frequent in patients with several stages of neurodegenerative diseases and vascular dementia (VaD) than in subjects with only subjective memory complaints. This suggested, that MBs may also be implicated in neurodegenerative disease. Although data suggested that MBs relate to cognitive impairment in several populations, in a memory clinic setting this was unclear.

Associated pathologies of MBs in dementia

The pioneering work of Fazekas et al. had shown that most MBs were related to hypertensive vasculopathy or arteriolosclerosis in patients that had died from intracerebral hemorrhage. Two patients in that study however, showed lesions originating from vessels with extensive amyloid angiopathy. The Rotterdam scan study provided epidemiologic evidence for an etiologic dichotomy based on location of MBs. Strictly lobar MBs were found more often in carriers of the APOE ε4 allele, and as such were hypothesized to be related to cerebral amyloid angiopathy (CAA), while MBs in deep brain regions were found to be related to hypertension and vascular risk factors, and hence hypertensive vasculopathy, in analogy to large intracerebral hematomas. Alzheimer disease (AD) is closely associated with CAA, but also frequently presents with cerebrovascular disease, both potential causes of MBs. The origin of MBs, the role of blood-brain barrier (BBB) herein, and their risk factors in a memory clinic population however, were not yet elucidated.

Imaging microangiopathy

MBs are detected with specific T2*-weighted MRI sequences. Not only pulse-sequence, but field strength, and other MRI parameters, such as slice thickness, in-plane resolution, echo times and flip angle have all been found to influence detection of MBs. Besides various acquisition options, a new way of post-processing appeared promising, showing improved contrast and more sensitive detection. Although the radiologic benefits of this new sequence have repeatedly been reported, not much was known about clinical relevance of this improved detection. Optimizing MB imaging has been found to increase MB prevalence. Moreover, the high sensitivity of MRI, also due to the larger “blooming” effects of the hemosiderin deposits with total brain coverage, offered by modern techniques, resulted in a far more frequent detection of MBs during life, than what has been observed at neuropathologic evaluation. This raises questions about the limits of ongoing advances in MB detection and about the interpretation, and relevance, of MBs with new techniques.

Cerebral microinfarcts (MIs), like MBs, are thought to result from small vessel disease. MIs however, seemingly mirror MBs as they do not result from
hemorrhage, but from ischemia. Furthermore, MIs are a frequent post-mortem neuropathologic observation, but are not visible with conventional in vivo MRI, whereas MBs are primarily a radiologic construct. Recently, these MIs have been associated with dementia and cognitive deficits during life, therefore in vivo detection of MIs currently is an important challenge in the imaging of dementia.

1.1.2 Aims of this thesis

The general aim of this thesis was to investigate the relevance of the radiologic construct of MBs in dementia and more specifically in AD. We aimed to assess this in relation to: I clinical relevance, II underlying vasculopathies and associated pathologies and III novel imaging techniques.

1.1.3 Outline of thesis

As the clinical relevance of MBs in dementia was unknown, we set out to study the relationship of MBs with cognition, as negative impact has been observed in other populations, but not in AD. In chapter 2.1 we cross-sectionally studied the cognitive performance of AD patients with many MBs compared with AD patients, matched for age and sex, without MBs. Subsequently, we present two longitudinal studies. The first one focuses on the predictive value of MBs at baseline regarding future cognitive decline in AD patients (chapter 2.2). In the second longitudinal study, we set out to investigate the natural occurrence of new, or incident MBs in a large memory clinic cohort, also aiming to identify risk factors of incident MBs (chapter 2.3).

The underlying and associated pathologies of MBs in dementia are presented in chapter 3. As both causes of MBs - hypertensive vasculopathy and CAA are commonly observed in AD - we investigated the vasculopathies underlying MBs and their associations. We aimed to explore the possibility that MBs in AD result from increased CAA related intravascular amyloid deposition, using clinical chemistry and PET imaging studies, which assessed different aspects of BBB function. First, we related MBs with different forms of amyloid beta in CSF and plasma, at the same time biochemically assessing BBB integrity (chapter 3.1) in AD and VaD patients. PET scanning using Verapamil allowed assessment of different aspects of BBB function (chapter 3.2). Finally, we conclude the clinical and pathophysiological parts of this thesis, by verifying the relation between MBs at MRI and cognitive functioning on the one hand, and (peri)vascular amyloid deposition on the other, with neuropathological evidence from an illustrative case in chapter 3.3.

After showing the clinical relevance of MBs and the possible capability of MRI to predict their neuropathological etiology, indicating the importance of sensitive detection, we describe several novel developments in imaging of microangiopathy in chapter 4, since several of these developments seemed radiologically promising, but clinical relevance was largely unknown. First, we investigated the radiologic and clinical relevance of susceptibility-weighted imaging (SWI) in a memory clinic setting (chapter 4.1). We proceed with the value of ultra-high field scanning in a young and relatively pure AD sample (chapter 4.2). In the last technical chapter, we attempted to detect currently invisible MIs in AD and non-AD cases, by using advanced quantitative methods at a regular field strength (chapter 4.3).

In the final chapter 5, the main findings are summarized and discussed, followed by recommendations for future research.
2. de la Torre JC. Is Alzheimer’s disease a neurodegenerative or a vascular disorder? Data, dogma, and dialectics. The Lancet Neurology. 2004;3:184-190
1.5 and 7T. AJNR Am J Neuroradiol. 2011;32:1043-1049


‘When I quote others I do so in order to express my own ideas more clearly.’

Michel de Montaigne

MICROBLEEDS IN DEMETIA - SINGING A DIFFERENT ARIA


Philip Scheltens and Jeroen Goos

VU University Medical Center, Alzheimer Center, Department of Neurology, De Boelelaan 1118, 1081 HZ Amsterdam, The Netherlands.
In 2011, researchers used imaging techniques to investigate brain microbleeds in patients with dementia and highlighted how lobar microbleeds could be used as a marker for amyloid pathology and for predicting mortality. New guidelines on the inclusion and exclusion of participants with microbleeds in anti-amyloid clinical trials were also published.

Following initial reports of small dot-like lesions on gradient-echo MRI in the brains of patients with dementia (Figure 1), many investigators set out to describe the prevalence and incidence of these lesions—later designated as microbleeds or microhemorrhages—in both healthy people and individuals with this condition. Prevalence rates varied from 10% in healthy elderly individuals to 60% in patients with vascular dementia. Interest in these lesions peaked when the first cases of incident microbleeds along with increased signal intensity on fluid-attenuated inversion recovery imaging, thought to represent vasogenic edema, were reported in patients receiving amyloid-lowering therapy. During the 2010 international Conference on Alzheimer Disease (AD), turmoil ensued over a cautionary letter from the FDA, which suggested drastic cut-offs in randomized clinical trials of amyloid-lowering drugs, both in terms of excluding patients with a single microbleed and terminating the participation of patients who developed a new microbleed during the study. In response, a series of important papers regarding the detection, prevalence and clinical relevance of microbleeds in patients with dementia-related disease appeared in 2011, including a consensus statement from an international working group that introduced new terminology.

At the beginning of 2011, Cordonnier and van der Flier reviewed the available literature on brain microbleeds in patients with AD. The authors suggested that these lesions were associated with amyloid pathology and may have a crucial role in the pathophysiology of AD. Microbleeds were proposed to represent a link between the amyloid cascade hypothesis and the vascular hypothesis—both popular explanatory models for the pathogenesis of AD. Furthermore, the location of the microbleeds was suggested to indicate their underlying etiology: lobar microbleeds would presumably be associated with cerebral amyloid angiopathy (CAA), whereas microbleeds in deep brain regions would be associated with hypertensive vasculopathy and increased risk of vascular complications. The clinical implications of microbleeds in patients with dementia were also stressed. Besides an association with cognition, microbleeds have been linked to mortality, especially in cases of multiple lesions, as described by Henneman et al. in a 2009 study. These investigators did not, however, assess the cause of death in their patients.

Throughout 2011—within months of publication of the review by Cordonnier and van der Flier—reports were published on several studies that have substantially extended our knowledge on microbleeds in patients with dementia-associated diseases. The suggestion that microbleeds were closely linked to amyloid pathology was supported by the findings of Yates et al. for the Australian imaging, Biomarkers and Lifestyle study of Ageing Research Group. They found that even in healthy controls, lobar microbleeds (detected using 3T susceptibility-weighted imaging [SWI]) were associated with higher amyloid burden, as seen on $^{11}$C-Pittsburgh compound B (PiB) PET imaging. Moreover, PiB-positive scans were more prevalent in participants with multiple lobar microbleeds (86%) than in those with only one lobar microbleed (67%). In agreement with these findings, results from a study by Goos et al., in which cerebrospinal fluid amyloid biomarkers were used to assess amyloid burden, confirmed the relationship between lobar microbleeds and amyloid pathology.

Altmann–Schneider and colleagues studied the relationship between microbleeds and mortality (with assessment of the cause of death) in a population of 435 elderly people with pre-existing vascular disease. Individuals with more than one microbleed had a sixfold increase in the risk of stroke-related death compared with those with no lesions. The location of the lesions also affected the risk of poor clinical outcomes: compared with individuals without any lesions, patients with nonlobar microbleeds had a twofold increase in the risk of cardiovascular death, and individuals with probable CAA type (lobar) microbleeds had a sevenfold increase in the risk of stroke-related death. These findings support the hypothesis that microbleeds have separate etiologies depending on their location in the brain.
Microbleeds in Dementia: Connecting the dots

The importance of detecting microbleeds before and during clinical trials was underlined in an extensive review on amyloid-related imaging abnormalities (ARIA) by Sperling et al. for the Alzheimer's Association Research Roundtable workgroup. These imaging abnormalities, seen on MRI, are thought to represent a spectrum of 'leaky vessels' that occur following anti-amyloid immunization therapy. The working hypothesis was that vasogenic edema (now called ARIA-E) caused leakage only of proteinaceous fluids from the vessels, whereas microbleeding—under the new umbrella term ARIA-H (for hemorrhage)—caused more-extensive leakiness of the vessels that allowed blood cells to cross the blood–brain barrier. For patients in clinical trials, the presence of microbleeds at baseline may be a risk factor for developing ARIA, as the number of lobar lesions is assumed to correlate with the presence and severity of CAA. Given the uncertainty regarding the risk of ARIA and concerns about CAA severity, Sperling et al. recommended a cut-off value for the exclusion of participants in trials of amyloid-modifying therapies for AD at four microbleeds. This new guideline allows for variability in imaging measurements and reflects the uncertainty regarding the clinical relevance of small numbers of microbleeds. The authors further stated that occurrence of new asymptomatic microbleeds in patients during trials should not automatically disqualify them from receiving further treatment; however, owing to a lack of data, no exact cut-off for disqualification could be given. As the authors stressed, counting of microbleeds is not an exact science, and the uncertainty is further complicated by the varying sensitivities of different MRI techniques for detecting these lesions.

Reporting in Stroke in May 2011, Goos et al. compared conventional gradient-echo imaging with SWI to detect microbleeds in 140 patients from a memory clinic, and also to determine whether microbleeds were associated with patient and clinical characteristics. As expected, use of the more-advanced SWI technique enabled identification of patients with microbleeds with a greater sensitivity than could be achieved with gradient-echo imaging (40% versus 23%). SWI also detected a higher number of microbleeds per patient than did gradient-echo imaging. However, the correlation between lesion numbers, clinical outcomes and other radiological outcomes was limited. The clinical relevance of microbleeds will probably depend more on their location and size, than on the total number of lesions per se. The authors concluded, therefore, that although new imaging techniques can show a higher number of lesions, conventional imaging methods can already detect the majority of clinically relevant lesions. New imaging methods might help in identifying any associations between lesions and clinical and radiological outcomes; however, these new techniques are in urgent need of validation.

De Reuck et al. made an interesting attempt to validate MRI findings with pathology, as reported in Cerebrovascular Disorders. The researchers investigated 20 postmortem brains from patients with AD with different cerebrovascular lesions. Images of 45 large sections of the cerebral hemispheres, brainstem and cerebellum obtained using 7.0T T2*-weighted MRI were paired with images showing histological detection of hematomas and microbleeds. In the cortico-subcortical regions, the sensitivity, specificity, and positive and negative predictive values of T2* imaging to detect microbleeds were excellent. However, analysis of MRI alone resulted in an overestimation of microbleeds in the striatum due to the presence of iron deposits that were, in fact, not related to real hemorrhages. Furthermore, 31% of T2* hyposignals in the deep white matter were shown to be vessels filled with postmortem thrombi. Judging from these findings, more studies are needed before we can fully understand the correlations between MRI and pathology in patients with AD.

From this selection of studies published in 2011, we can conclude that lobar microbleeds are a marker for underlying amyloid pathology, are associated with stroke-related mortality, and should be adequately investigated in patients participating in anti-amyloid clinical trials. Optimization of imaging methods to detect microbleeds will be of the utmost importance, and emerging techniques will need to be evaluated and calibrated using clinical and pathological correlates.
Brain microbleeds on MRI.

Numerous lobar microbleeds with sparing of the basal ganglia and thalamus, suggestive of severe cerebral amyloid angiopathy in a 71-year-old patient with dementia with Lewy bodies. Image obtained using susceptibility-weighted imaging at 3T.

CLINICAL ASSOCIATIONS OF MICROBLEEDS IN MEMORY CLINIC PATIENTS

‘You can’t connect the dots looking forward; you can only connect them looking backwards.’

Steve Jobs

ALZHEIMER’S DISEASE PATIENTS WITH MULTIPLE MICROBLEEDS; RELATION WITH CSF BIOMARKERS AND COGNITION


Authors: JDC Goos MD1, MI Kester MD1, F Barkhof MD PhD2, M Klein PhD3, MA Blankenstein PhD4, Ph Scheltens MD PhD1, and WM van der Flier PhD1,5.

Institutional affiliations: From the Alzheimer Center and 1Department of Neurology, 2Radiology, 3Medical Psychology, 4Clinical Chemistry, and 5Departments of Epidemiology and Biostatistics, VU University Medical Center, Amsterdam, the Netherlands.
Abstract

**Background:** Microbleeds (MBs) are commonly observed in AD. A minority of patients has multiple MBs.

**Purpose:** We aimed to investigate associations of multiple MBs in AD with clinical and MRI characteristics, and CSF biomarkers.

**Methods:** AD patients with multiple (≥8) MBs on T2*-weighted MRI were matched for age, sex and field strength with AD patients without MBs on a 1:2 basis. We included 21 patients with multiple MBs (73±7yrs, 33%F) and 42 patients without MBs (72±7yrs, 38%F). MMSE was used to assess dementia severity. Cognitive functions were assessed using neuropsychological tests. Medial temporal lobe atrophy (MTA; 0-4), global cortical atrophy (0-3), and white matter hyperintensities (WMH; 0-30) were assessed using visual rating scales. In a subset, APOE genotype and CSF amyloid beta 1-42 (Aβ42), total tau (tau) and tau phosphorylated at threonine 181 (ptau-181) were determined.

**Results:** Multiple MB patients performed worse on MMSE (multiple MB: 17±7; no MB: 22±4, p<0.05), despite similar disease duration. Atrophy was not related to presence of MBs, but multiple MB patients had more WMH (multiple MB: 8.8±4.8; no MB: 3.2±3.6, p<0.05). Adjusted for age, sex, WMH and MTA, the multiple MB group additionally performed worse on VAT object naming and animal fluency. Multiple MB patients had lower CSF Aβ42 levels (307±61) than patients without MBs (505±201, p<0.05). Adjusted for the same covariates, tau and ptau-181 were higher in the multiple MB group.

**Conclusion:** Microbleeds are associated with the clinical manifestation of AD. These results suggest that MBs are implicated in the neuropathogenesis of AD.

Introduction

Alzheimer’s disease (AD) is the main cause of dementia in the elderly. The pathological hallmarks are neuritic plaques (amyloid beta) and neurofibrillary tangles (tau). Furthermore, in 70-98% of AD patients intravascular amyloid beta deposition is found at autopsy. Atrophy, especially of the medial temporal lobes, constitutes the radiological hallmark of AD on MR imaging. In addition, white matter hyperintensities (WMH) have been shown to be more prevalent in AD patients than in controls. Microbleeds (MBs) can be observed on T2*-weighted gradient echo MRI. Histologically they represent hemosiderin laden macrophages resulting from leakage from cerebral small vessels.

Recent findings indicate that MBs in the general elderly population are relatively common and are even more frequently observed in AD patients. In our memory clinic population, prevalence of one or more MBs in AD patients was 18%. Among those patients, the majority had only one or few MBs. However, a subgroup of AD patients shows many MBs. Lobar MBs in cortico-subcortical locations with a posterior predilection, are thought to be an expression of underlying bleeding prone cerebral amyloid angiopathy (CAA), especially in AD patients. Since CAA has been related previously to low amyloid levels in cerebrospinal fluid (CSF), presumably resulting from increased intravascular amyloid deposition, we hypothesized that amyloid levels in CSF would be reduced in AD patients with multiple MBs as result of intra vascular amyloid pathology.

The occurrence of MBs seems to increase with age and is believed to coincide with hypertension, ischemic and hemorrhagic stroke, lacunes and the extent of white matter disease. MBs have been associated with cognitive decline in patients with stroke and subcortical vascular dementia, and in the general elderly population. In AD however, the clinical significance of MBs remains elusive. Former studies finding no correlation of MBs with clinical manifestation included many patients with one or just a few MBs. We took a proof-of-principle approach by comparing AD patients with many MBs with AD patients without any MBs,
maximizing possible differences between patient groups. We aimed to investigate the associations of multiple MBs in AD patients with clinical, neuropsychological and MRI characteristics and levels of cerebrospinal fluid (CSF) biomarkers.

Material and Methods

Patients
From our database of patients who underwent dementia screening at the Alzheimer Center of the VU University Medical Center Amsterdam (VUMC) memory clinic we retrospectively reviewed presence and number of MBs in patients with a diagnosis of probable AD, visiting between May 2001 and July 2008 (n=427). Of these, 350 patients (82%) had no MBs, 67 patients (18%) had one or more MBs present on baseline T2*-weighted gradient echo imaging. For the current study, we selected patients with eight or more MBs, resulting in 21 patients, representing the 5% of AD patients with the most severe MB-burden (multiple MB group; age 73±7 years, 33% female). The patients were matched on a 1:2 basis for age, gender and MRI field strength with AD patients without any MBs. These “control” AD patients without MBs were selected to have complete data of interest (no MB group n=42; age 72±7 years, 38% female). All patients underwent standardized dementia screening, including past medical history, physical, neurological and neuropsychological examination. Patients were considered as having arterial hypertension, diabetes mellitus, hypercholesterolemia if they had a known history of the disease or were receiving drug treatment. Furthermore, screening involved MRI and routine laboratory examinations. Diagnoses of probable AD were made in a multidisciplinary consensus meeting according to the clinical criteria of the National Institute of Neurological Disorders and Stroke-Alzheimer’s Disease and Related Disorders Association (NINCDS-ADRDA). The level of education was classified using the system of Verhage, ranging from 1 (low) to 7 (high). The study was approved by the ethical review board of the VUMC and all subjects gave written informed consent.

Neuropsychological Assessment
The neuropsychological test battery was designed to screen the major cognitive functions. Two experienced neuropsychologists were responsible for testing. All testing was done on the same day as MRI scanning as part of our screening routine, except for three patients, who were tested within 2 months of scanning. Dementia severity was assessed using the Mini-Mental State Examination (MMSE). For memory, the Visual Association Test (VAT) was used. Patients are shown cue cards with an object and association cards with the previously seen object plus an interacting object and are asked to name them. Subsequently, the cue cards are shown without delay and patients are asked to recall the now missing interacting objects [score 0–12]. VAT object naming was used as a measure for language (0–12). Language functions were additionally assessed using category fluency, where patients have to produce as many animals as possible in a time span of 60s. In addition, the Trail Making Test (TMT) was used. The simple part A provides a measure of psychomotor speed. It requires the connection by pencil of numbers (1–20) positioned randomly on a sheet of paper. The more complex part B requires patients to alternate between numbers and letters (e.g., 1–A–2–B–3–C– ...), and was used to evaluate executive functioning. For both conditions, the time required for completion is recorded. Additionally, digit span (forward and backward) was used to assess working memory. Ongoing efforts to optimize the neuropsychological screening protocol resulted in a slightly varying content of the neuropsychological battery over the years. As a result, availability of the data varied per test, from 43 patients on the TMT B test, digit span forward and backward (n=56), TMT A (n=57), VAT naming (n=58), animal fluency (n=59), MMSE (n=60), and VAT (n=61).

MRI protocol
The majority of scans (14 in the multiple MB group and 28 in the no MB group) were obtained on a 1.0T scanner (Magnetom Impact; Siemens, Erlangen, Germany). Twenty-one scans (7 in the multiple MB group and 14 in the no MB group) were obtained on a 1.5T platform (Siemens Sonata Syngo [n=17], or Siemens Avanto [n=4], Erlangen, Germany). Scan protocol included (1) axial T2*-weighted gradient echo images for MB detection (19-23 slices, field of view [FOV] 250mm, matrix 256x192-
Microbleeds in Dementia: Connecting the dots

MRI assessment

MRI rating was performed blinded to the patients’ clinical data. MBs were defined as rounded hypointense homogeneous foci up to 10mm in size in the brain parenchyma on T2*-weighted images. Lesions in sulci possibly representing flow voids from pial vessels and symmetrical lesions in the basal ganglia, supposedly representing iron or calcium deposits, were excluded. Hyposignals inside a lesion compatible with an infarct were not counted as MBs, but regarded to be probable hemorrhagic transformations. Cavernous angiomas were not taken into account. MBs were counted in four lobar regions: frontal, parietal, temporal and occipital and in two non-lobar regions: basal ganglia (including thalamus) and infratentorial. In addition, on the FLAIR sequence white matter hyperintensities (WMH) were assessed using the Age-Related White Matter Change (ARWMC) scale. The ARWMC scale has scores from 0-3 (none, punctuate, early confluent and confluent) given in 5 regions, each left and right, adding up to a total range from 0 to 30. In addition, the presence of large vessel and lacunar infarcts was assessed. Large vessel infarcts were rated as present or absent, based on hyperintensity of the lesion on both FLAIR and T2-weighted sequences. Lacunar infarcts were defined as deep lesions from 3 to 15mm, with low signal on FLAIR and T1 sequences and high signal on T2-weighted images. Lacunar infarcts were scored as present or absent. Furthermore, two widely used visual rating scales for the assessment of atrophy were used. Medial temporal lobe atrophy (MTA) was rated using a five point rating scale (0-4), using oblique reconstructions of the MP-RAGE sequences, perpendicular to the long axis of the hippocampus. In the analysis the average MTA score for the left and right side was used. Global cortical atrophy (GCA) was assessed on the FLAIR sequence. The GCA scale ranges from 0-3. On both scales maximal atrophy is represented by the highest score.

APOE genotyping

DNA was isolated from 10mL of ethylenediaminetetra-acetic acid/blood and was available from 55 of 63 patients (87%). APOE genotype was determined with the light cycler APOE mutation detection method (Roche Diagnostics GmbH, Mannheim, Germany). Patients were categorized according to APOE ε4 status [non-carriers, heterozygous and homoygous].

CSF analysis

In a subset of patients 51 of 63 patients (81%) cerebrospinal fluid (CSF) was obtained by lumbar puncture between the L3/L4 or L4/L5 intervertebral space, using a 25-gauge needle, and collected in 10mL polypropylene tubes. Within two hours, CSF samples were centrifuged at 1800 g for 10 minutes at 4°C. A small amount of CSF was used for routine analysis, including total cells (leucocytes and erythrocytes), total protein and glucose. CSF was aliquoted in polypropylene tubes of 0.5 or 1 ml and stored at -80°C until further analysis. CSF amyloid-beta 42 (Aβ42), total tau (tau) and tau phosphorylated at threonine 181 (ptau-181) were measured with Innotest sandwich ELISA as described previously. As the manufacturer does not supply controls, the performance of the assays was monitored with pools of surplus CSF specimens. In the study period multiple specimens with various concentrations which were included in 7-18 runs have been used for this purpose. The inter-assay coefficients of variation were (mean ± SD) 11.3±4.9% for Aβ42, 9.3±1.5% for tau and 9.4±2.5 for ptau-181. The team involved in the CSF analysis was not aware of the clinical diagnosis.
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Statistical analysis
For statistical analysis SPSS 14.0 for windows (Chicago, IL) was used. Categorical data were analysed by Chi-squared tests. Comparison between groups for continuous variables was executed by student t-tests. Analysis of variance (ANOVA) was used to adjust for age, sex, WMH and MTA. We used logistic regression to investigate the association between MBs and APOE ε4 status adjusted for age, sex, WMH and MTA. The correlation between number of MBs and MMSE was investigated using Spearman correlation coefficient. Significance was set at p<0.05.

Results
In the group of 21 patients with multiple MBs the total number of MBs was 491, with a median of 15 MBs (range: 8-104). Lobar locations accounted for 94% of the total number of MBs. Percentage of total MB count per lobe was: temporal 32%, parietal 24%, occipital 19% and frontal 19%. Only 6% of all detected MBs were in non-lobar locations, with 4% in the basal ganglia and thalamus and 2% in infratentorial regions. Eleven patients in the multiple MB group (52%) had strictly lobar MBs (example figure 1). Six patients (29%) had a few non-lobar MBs next to mainly lobar MBs, three patients (14%) had comparable numbers of non-lobar and lobar MBs, whereas only one patient (5%) presented more non-lobar than lobar MBs.

MRI characteristics, medical history, and medication use are shown in table 1. Patients with multiple MBs showed more severe WMH than those without MBs (p<0.001). The presence of lacunar or large vessel infarcts did not differ between groups. No difference between groups regarding atrophy (global cortical or medial temporal lobe) was found. There was no difference in disease duration, level of education or medical history between groups. Patients with multiple MBs more often used antiplatelet medication, while there were no differences in other medications.

Neuropsychological and laboratory results are shown in table 2. Patients with multiple MBs had lower MMSE than AD patients without MBs (17±7 versus 22±4, p<0.005). Furthermore, within the group of patients with multiple MBs only, we found a correlation between the total number of MBs and MMSE (Spearman r=-0.47; p<0.05, figure 2), with more MBs being associated with lower MMSE scores. After removal of one outlier with a MMSE of 2 and 38 MBs, the correlation remained of moderate strength, although significance was lost (Spearman r=-0.38, p<0.05). Unadjusted, there was no group difference for any of the neuropsychological tests. After adjustment for age, sex, MTA and WMH, patients with multiple MBs additionally performed worse on animal fluency, VAT object naming and digit span (forward and backward) than the group without MBs (p<0.05). There were no associations between age or sex and any of the neuropsychological measures. Medial temporal lobe atrophy was associated with lower VAT memory scores (Spearman r=-0.26, p<0.05), but there were no associations with WMH.

Patients with multiple MBs had lower CSF levels of Aβ42 than patients without MBs in univariate analysis (p<0.01). Adjusted for age, sex, MTA and WMH, patients with multiple MBs additionally had higher levels of CSF tau and ptau-181. The aforementioned covariates had no univariate association with CSF Aβ42 levels. CSF levels of tau and ptau, however were both associated with WMH (Spearman r = -0.36, p<0.01 and Spearman r = -0.34, p<0.05 respectively) and MTA (Spearman r = -0.30, p<0.05 and Spearman r = -0.28, p<0.05 respectively), albeit in the counter-intuitive direction as higher CSF (p)tau levels were observed in patients with relatively little MTA and WMH.

Patients with multiple MBs were more often homozygous for APOE ε4 (31% versus 17%), although this difference was not significant (p=0.55). When we used logistic regression to adjust for age, sex, MTA and WMH, we found that homozygous APOE ε4 carriers had an increased risk to have multiple MBs (OR(95% CI) = 16 [0.9-276]), almost reaching significance. After removing 4 patients with multiple deep MBs from analysis, we found an even higher odd’s ratio of 19 (CI: 1.0-377); p<0.05. On visual inspection, we found that all homozygous APOE ε4 carriers in the multiple MB group had no or only very little WMH (ARWMC score £ 4). There was no association between heterozygous APOE ε4 carriership and multiple MBs (OR(95% CI)=1.6 [0.1-19]).
Discussion

In this proof-of-principle case-control study, we showed the relationship between multiple MBs and more pronounced impairment in a number of cognitive domains. Moreover, we found that multiple MBs were associated with lower CSF levels of Aβ42 and higher levels of tau and ptau-181. Although MBs were associated with more severe WMH, this did not explain the more pronounced cognitive decline or abnormal CSF biomarker levels. Furthermore, our findings could not be explained by longer disease duration, level of education or more severe atrophy.

We found an association of multiple MBs with lower CSF Aβ42, suggesting a direct link between MBs and amyloid-beta, one of the key proteins involved in AD. A post mortem study showed a relationship between the severity of CAA and lower AB42 levels, which could not be explained by amyloid plaque or tangle burden.13 The authors hypothesized that low levels of Aβ42 may reflect increased deposition of amyloid beta not only in plaques, but also in cerebral vessels. Accumulation of Alzheimer related amyloid pathology in the vessels may lead to reduced vessel wall integrity, which in turn may result in MBs. Alternatively, the more abnormal CSF levels may have been caused indirectly, via vascular risk factors and associated WMH.29 This seems unlikely however, as results remained unchanged after adjustment for WMH. Moreover, when we inspected the univariate associations between CSF levels of [p]tau and WMH, we found that patients with higher [i.e. more abnormal] [p]tau levels had less WMH. A possible explanation for this seemingly counter-intuitive finding, could be that less WMH implies more pure AD, associated with more abnormal Alzheimer biomarkers. The combination of high CSF tau levels and low grade periventricular WMH has been described before in MCI patients converting to AD.30 We therefore feel that the association between MBs and tau after adjustment for WMH, provides additional support for the notion that MBs may have a central role in the pathogenesis of AD.

Earlier studies reporting on prevalence of MBs in AD (with prevalences ranging from 17-32%),6,7,10,12,15,20 were not able to show any relationship between MB occurrence and cognitive performance. In these studies most patients had only one or few MBs. We reasoned that having one or only a few microbleeds is not sufficient for any measurable clinical effect. Therefore, we took a different approach by selecting the 5% of AD patients with the most severe MB burden, and comparing these to AD patients without any MBs. In this way, we aimed to maximize the supposed deleterious effects associated with MBs and to increase the power to demonstrate clinical correlates of MBs. In this way we were able to show that AD patients with multiple MBs present with more severe cognitive impairment. This finding could not be explained by a more advanced disease stage, as illustrated by disease duration or degree of atrophy. Our findings are in line with a recent study that showed a relationship between number of MBs and cognitive impairment in subcortical vascular dementia.18 Like our study, the number of observed MBs was high (median number of 13). In contrast with our study, patients were of Asian origin and were diagnosed with subcortical vascular dementia, although the authors mention that underlying Alzheimer pathology could not be excluded.

The vast majority of MBs in our population was located in lobar regions. The locations of MBs in our study are in line with other studies that suggested CAA as underlying vasculopathy of lobar MBs in AD.6,7,10,12 Only a small minority of MBs was found in the basal ganglia, thalami or infratentorial regions. MBs in the deep gray matter structures have been associated with vascular risk factors and hypertensive microangiopathy at autopsy.3,4,5,31,32 Furthermore, lobar MBs have been associated with APOE ε4 status in community based elderly population studies, while pure non-lobar MBs lack an association with APOE ε4.3 In our current study, the relative prevalence of homozygous APOE ε4 was nearly twice as high in the multiple MB group as in the no MB group, although the observed difference did not reach significance, probably due to lack of power. Moreover, when patients with multiple deep MBs were excluded, the risk became even higher. These findings support the notion that strictly lobar MBs are indeed CAA related, and as a result have a stronger association with homozygous APOE carriership. Remarkably, all homozygous ε4 carriers with multiple MBs had low WMH scores, seemingly suggesting separate pathophysiological mechanisms for MBs presenting with and without WMH.

Limitations of the study include the relatively small sample size, cross-sectional...
design, and the retrospective nature of the study. The retrospective design resulted in varying extent of incomplete data. Furthermore, our ongoing efforts to optimize the neuropsychological screening protocol have resulted in a slightly varying order and content of the neuropsychological evaluation during the years. We did not account statistically for the missing data, in this way choosing to stay close to the original data. We feel that, given the relatively large amount of missing data, imputation of missing data would potentially have added too much noise.

Furthermore, different scanners may have induced variability in the results, as detection of MBs presumably depend largely on imaging parameters. The most prominent differences in MB detection however, are reported on comparing images with post-processing (i.e. susceptibility-weighted imaging, SWI) to regular T2*-weighted pulse sequences without post-processing, varying echo times to a greater extent than was the case in our study and doubling of field strengths, i.e. 1.5T versus 3T. Firstly, on all scanners T2*-weighted gradient-recalled echo was performed as opposed to SWI, that results in considerably higher prevalence of MBs. Secondly, regarding scanning parameters, slice thickness was constant on all machines, inter slice gaps were comparable (1-1.5mm), flip angle and most importantly echo time were of comparable order (22-25ms) in our protocols. Finally, by matching for field strength we minimized the supposed effect of higher field strengths. Moreover, field strengths did not include 3T, but only 1 and 1.5T.

According to our proof-of-principle approach, we compared patients with many MBs to patients without any MBs, to maximize supposed effects associated with MBs. Since, to our knowledge, a firm definition of “many MBs” does not exist, we had to use an arbitrary cut-off. We included the 5% of AD patients with the most severe MB burden, with the concomitant cut-off of eight or more MBs. The main outcomes remained unchanged however, when we used 5MBs or 10MBs as cut-off, illustrating the robustness of our findings. These findings must be confirmed in a larger cohort, ideally pathology confirmed, including AD patients with one or few MBs, to answer the question whether our findings are specific for the small subgroup of AD patients with many MBs, or that there is a dose-response relationship, with patients with one or a few MBs being in between. The critical question raised by these findings, is whether MBs are a mere marker of more severe changes in the process of dementia, or whether these findings represent a different pathology altogether.

Acknowledgements
J.D.C. Goos is supported by Stichting Dioraphte. The Alzheimer Center VUmc is supported by Alzheimer Nederland and Stichting VUmc fonds. The clinical database structure was developed with funding from Stichting Dioraphte.
### Table 1. MRI characteristics, medical history and medication.

<table>
<thead>
<tr>
<th>Variable</th>
<th>AD ≥8 MBs (n=21)</th>
<th>AD no MBs (n=42)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MRI characteristics</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MBs</td>
<td>24±24</td>
<td>0</td>
</tr>
<tr>
<td>ARWMC score</td>
<td>8.8±4.8</td>
<td>3.2±3.6*</td>
</tr>
<tr>
<td>Lacunar infarcts</td>
<td>5(24%)</td>
<td>6(14%)</td>
</tr>
<tr>
<td>Large vessel infarcts</td>
<td>2(3%)</td>
<td>1(2%)</td>
</tr>
<tr>
<td>Global cortical atrophy score</td>
<td>1.1±0.8</td>
<td>1.1±0.7</td>
</tr>
<tr>
<td>MTA score</td>
<td>2.0±0.9</td>
<td>1.6±0.9</td>
</tr>
<tr>
<td><strong>Medical History</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Level of education [1-7]</td>
<td>4.5±1.7</td>
<td>5.1±1.2</td>
</tr>
<tr>
<td>Duration of symptoms [years]</td>
<td>3±3</td>
<td>3±2</td>
</tr>
<tr>
<td>Hypertension</td>
<td>6(29%)</td>
<td>16(38%)</td>
</tr>
<tr>
<td>Stroke or TIA</td>
<td>31(14%)</td>
<td>25(5%)</td>
</tr>
<tr>
<td>Peripheral or cardiac vasculopathy</td>
<td>2(10%)</td>
<td>4(14%)</td>
</tr>
<tr>
<td>Diabetes Mellitus</td>
<td>2(10%)</td>
<td>3(7%)</td>
</tr>
<tr>
<td>Hypercholesterolemia</td>
<td>0(0%)</td>
<td>3(7%)</td>
</tr>
<tr>
<td><strong>Medication</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NSAIDS</td>
<td>0(0%)</td>
<td>1(2%)</td>
</tr>
<tr>
<td>Anticoagulation</td>
<td>0(0%)</td>
<td>0(0%)</td>
</tr>
<tr>
<td>Antiplatelet medication</td>
<td>6(29%)</td>
<td>7(17%)*</td>
</tr>
</tbody>
</table>

Data are represented as number of patients with the variable present (%), or mean±sd. *p<0.01, AD=Alzheimer’s disease, MBs=Microbleeds, ARWMC=Age Related White Matter Changes, MTA=Medial Temporal Lobe atrophy, TIA=transient ischemic attack, NSAIDS=non-steroidal anti-inflammatory drugs, ≈ Level of education according to Verhage.

### Table 2. Neuropsychology and laboratory results.

<table>
<thead>
<tr>
<th>Variable</th>
<th>AD multiple MBs (n=21)</th>
<th>AD no MBs (n=42)</th>
<th>Unadjusted</th>
<th>Adjusted*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Neuropsychological data</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMSE score</td>
<td>17±7</td>
<td>22±4</td>
<td>**</td>
<td>**</td>
</tr>
<tr>
<td>Visual Association Test</td>
<td>4±4</td>
<td>6±4</td>
<td>†</td>
<td></td>
</tr>
<tr>
<td>VAT object naming</td>
<td>10±3</td>
<td>12±1</td>
<td>**</td>
<td></td>
</tr>
<tr>
<td>Animal fluency</td>
<td>11±6</td>
<td>13±5</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>Trail Making Test A</td>
<td>127±78</td>
<td>97±88</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trail Making Test B</td>
<td>401±335</td>
<td>331±251</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Digit span forward</td>
<td>10±2</td>
<td>11±2</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>Digit span backward</td>
<td>6±3</td>
<td>7±2</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td><strong>Laboratory results</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CSF Aβ42 [pg/ml]</td>
<td>307±61</td>
<td>505±201</td>
<td>**</td>
<td>**</td>
</tr>
<tr>
<td>CSF tau pg/ml</td>
<td>940±708</td>
<td>597±298</td>
<td>**</td>
<td></td>
</tr>
<tr>
<td>CSF ptau-181</td>
<td>110±64</td>
<td>85±41</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>APOE ε4: non-carriers</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>heterozygous</td>
<td>23%</td>
<td>32%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>homozygous</td>
<td>46%</td>
<td>51%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>APOE ε4: heterozygous</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>APOE ε4: homozygous</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>APOE ε4: homozygous</td>
<td></td>
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</tbody>
</table>

Data are represented as mean±sd unless indicated otherwise. Availability for incomplete data for multiple MB patients: blood pressure measurements 17/21, MMSE and VAT naming 18/21, VAT 19/21, animal fluency 17/21, TMT A 15/21, TMT B 11/21, APOE 13/21, CSF 9/21. a: adjusted for WMH, MTA, age and sex. †p<0.10, *p<0.05, **p<0.01. MMSE=mini mental status examination, Aβ42= amyloid-beta 42, tau= total tau, ptau-181= tau phosphorylated at threonine 181, APOE=apolipoprotein E.
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Figure 1
Example of axial T2*-weighted gradient echo image of a patient with multiple MBs. Multiple MBs in the occipital and temporal lobes, mainly on cortico-subcortical junctions, with left side more affected. The MBs were strictly lobar in location, suspect for probable CAA.

Figure 2
Scatterplot demonstrating the association with total number of MBs per patient and MMSE scores in AD patients. Correlation between the total number of MBs and MMSE in the multiple MB group (Spearman r=-0.47; p<0.05). For two patients with more than 40 MBs MMSE data were missing.


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‘Not everything that can be counted counts, and not everything that counts can be counted.’

William Bruce Cameron

MICROBLEEDS DO NOT AFFECT RATE OF COGNITIVE DECLINE IN ALZHEIMER’S DISEASE

Abstract

Objective: To investigate the relationship between brain microbleeds (MBs) and the rate of cognitive decline in Alzheimer’s Disease (AD).

Methods: In this cohort study, we studied 221 AD patients with available baseline MRI (1.0 or 1.5T) and at least two Mini Mental State Examinations (MMSE) obtained more than one year apart from our memory clinic. Mean ±standard deviation] follow-up time was 3±1 years and patients had a median of 4 MMSEs [range 2-17]. We used linear mixed models with sex and age as covariates to investigate whether MBs influenced the rate of cognitive decline.

Results: Mean age was 68±9, 109 (49%) patients were female and baseline MMSE was 22±4. There were 39 patients (18%) with MBs (median = 2, range 1-27) and 182 without.

Linear mixed models showed that overall, patients declined 2 MMSE points per year. We found no association of the presence of MBs with baseline MMSE or change in MMSE. Adjustment for atrophy, white matter hyperintensities, lacunes and vascular risk factors did not change the results, nor did stratification for MB location, APOEε4 carrierrship or age-at-onset (≤65 years vs >65 years). Repeating the analyses with number of MBs as predictor rendered similar results.

Conclusion: MBs did not influence the rate of cognitive decline in AD patients. The formerly reported increased risk of mortality in patients with MBs seems not to be attributable to a steeper rate of decline per se, but might be due to vascular events, including [hemorrhagic] stroke.
Microbleeds (MBs) are small rounded regions of hypointensities on gradient echo (GRE) T2*-weighted MRI which frequently occur in AD patients. Histologically, MBs represent hemosiderin, likely from leakage through cerebral small vessels, contained within surrounding macrophages in the brain parenchyma. In the setting of AD, especially lobar MBs are believed to arise from leakage from fragile amyloid laden vessel walls, defined as cerebral amyloid angiopathy (CAA). The relationship between MBs or CAA and cognition in AD is unclear. Cross-sectionally, some studies find that AD patients with CAA or MBs are more severely cognitively impaired, while others find no such relationship. Several cross-sectional studies have reported a relationship between MBs and cognition in the elderly with or without increased vascular risk and in patients with small and large vessel disease. Previous studies have shown that patients with MBs have a higher risk of mortality. It is not known, however, whether AD patients with MBs are prone to a more rapid rate of cognitive decline. In the present cohort study, we hypothesized that the presence of MBs reflects a heavier burden of pathology in AD, resulting in a steeper rate of cognitive decline. The aim of this study was to assess the predictive value of baseline MBs on cognitive decline over time in patients with AD.

Methods

Patients, design and setting
In this cohort study, we included consecutive patients with AD who presented between 2000 and 2008 at the outpatient memory clinic of the Alzheimer Center of the VU University Medical Center (VUmc), with baseline MRI with GRE sequence on 1.0T or 1.5T and a minimum duration of follow-up of one year. At baseline, all patients underwent a standardized dementia assessment including medical history, informant-based history, physical and neurological examination, laboratory tests, neuropsychological testing, electroencephalogram (EEG) and magnetic resonance imaging (MRI) of the brain. Furthermore, we obtained information on smoking habits, current use of Alzheimer medication anti-thrombotics, and medical history. Hypertension, diabetes mellitus, hypercholesterolemia and myocardial infarction were defined based on self-reported medical history and medication use. Diagnoses of probable AD were made in a multidisciplinary consensus meeting according to the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer’s Disease and Related Disorders Association (NINCDS-ADRDIA) diagnostic criteria. None of the patients had symptomatic brain haemorrhage. Autopsy was available for 5 patients (2 with MBs, 3 without MBs). The diagnosis AD was confirmed in all cases. In one case (no MBs) significant co-existent vascular pathology was mentioned.

We followed patients clinically, with (semi-)annual assessment of their general level of cognitive functioning. The outcome measure was Mini Mental State Examination (MMSE). To be included in this study, patients had to have a minimum of two MMSE’s at least one year apart. The resulting data set included 1021 MMSE scores from 221 patients. Follow-up time varied between one and seven years (mean±sd: 3±1) and patients had a median of 4 MMSE scores (range: 2-17) (table 1).

Standard Protocol Approvals, Registrations, and Patient Consents
The study was approved by the local Medical Ethical Committee. All patients gave written informed consent for their clinical data to be used for research purposes.

MRI
Baseline MRI was performed on a 1.0 Tesla machine (n= 179; Magnetom Impact Expert Siemens AG, Erlangen, Germany) or 1.5 Tesla machine (n= 42; Siemens Sonata Syngo, Erlangen, Germany). The scan protocol included T1-weighted, T2-weighted, Flair and GRE images and has been described previously. Scan parameters for the axial GRE images used for MB detection were as follows: Impact: 19 slices, field of view 250 mm, matrix 256x256, slice thickness: 5 mm, interslice gap: 1 mm, repetition time: 800 ms, echo time: 22 to 25 ms, flip angle 20°. Sonata: 21 slices, field of view 250 mm, slice thickness: 5 mm, interslice gap: 1.5 mm, repetition time: 415 ms, echo time: 25 ms, flip angle 15°.
MRI rating was performed blinded to clinical data. MBs were defined as rounded hypointense homogeneous foci measuring up to 10 mm in the brain parenchyma on GRE images. Lesions in sulci possibly representing flow voids from pial vessels and symmetrical lesions in the basal ganglia, supposedly representing iron or calcium deposits, were not taken into account. Hypointensities inside infarcts were not counted as MBs, but regarded to be probable hemorrhagic transformations. Cavernous angiomas were not taken into account. We counted MBs in four lobar regions [frontal, parietal, temporal and occipital] and in two non-lobar regions [basal ganglia including thalamus, and infratentorial]. The main determinant was presence of at least one MB. In additional analyses, number of MBs and MB by location were used as determinants.

We performed visual rating of medial temporal lobe atrophy (MTA) on coronal T1-weighted images according to the 5-point (0-4) Scheltens scale. Global cortical atrophy (GCA, range 0-3) and WMH severity (Fazekas, range 0-3) were rated visually on axial FLAIR images; the highest scores represent maximal pathology. We counted lacunar infarcts, defined as well-demarcated lesions from 3 to 15 mm, with a cerebrospinal fluid-like signal on all sequences.

APOE and CSF biomarkers

DNA was isolated from 10 ml EDTA blood. APOE genotype was determined with the Light Cycler APOE mutation detection method (Roche Diagnostics GmbH, Mannheim, Germany). APOE genotype was available for 186 patients. CSF biomarkers were assessed as markers of Alzheimer neuropathology. CSF was obtained by a lumbar puncture. Amyloid b 1-42 (Aβ42), total tau and tau phosphorylated at threonine-181 (Ptau-181) were measured by sandwich ELISA (Innotest b-amyloid[1-42], Innotest hTAU-Ag, and Innotest Phosphotau(181P); Innogenetics, Gent, Belgium). CSF was available for 158 patients.

Statistical analysis

We used SPSS 15.0 to perform statistical analyses. Baseline differences between groups were studied using Student’s t-test, Mann-Whitney U-test or x²-test where applicable. We used linear mixed models to assess associations between presence of MBs and the rate of cognitive decline as measured by MMSE. This approach has increased statistical power as it accounts for within-person correlations over time, allows different numbers of assessments, and accounts for varying time intervals between assessments. A random intercept and a random slope with time (in years) were assumed, i.e. baseline MMSE [main effect of MBs] and change in MMSE over time (interaction effect of MBs*time) were allowed to vary between patients. The first model included terms for presence of MBs, time, and the interaction between presence of MBs and time, with sex and age as covariates and MMSE score as dependent variable. Second, we used a model additionally adjusting for MTA, GCA, WMH and presence of lacunes. A third model also adjusted for the following potential confounders: smoking, hypertension, diabetes, hypercholesterolaemia, myocardial infarction, use of anti-thrombotics or Alzheimer medication. Furthermore, we repeated the analyses after stratification according to age-at-onset (≤65 years vs >65 years) and according to APOE ε4 carriership (non-carriers vs carriers). The same models were run with a term for the number instead of the presence of MBs. Finally, we run the models again to assess the influence of the location of MBs on the rate of MMSE change over time in two ways: first with a categorical variable based on presumed underlying etiology, defined as: (i) no MBs; (ii) strictly non-lobar MBs; (iii) strictly lobar MBs; (iv) both lobar and non-lobar MBs and second with a categorical variable based on laterality: (i) no MBs; (ii) left sided MBs; (iii) right sided MBs; (iv) bilateral MBs.

Results

Demographic and clinical characteristics of the study sample are presented in table 1. Eighteen percent of the patients had one or more MBs and 82% had no MBs. Patients with MBs were older than patients without MBs. Groups did not differ in sex, education, APOEε4 carriership, follow-up time or number of follow-ups. Patients with MBs more often died within the study period, although this difference did not reach significance. Patients with MBs more often had a history of hypertension and they more often used anti-thrombotic medication, but there were no differences in other vascular risk factors. Furthermore, patients with MBs had lower CSF levels of
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52 but there were no differences in tau or ptau. Relative to normal values, both groups showed decreased CSF levels of Aβ42 and increased CSF levels of total tau and Ptau-181 [normal values: ³ 550, Aβ42>550, total tau< 375, Ptau-181 <52].25 Patients with MBs had more MTA and WMH than patients without MBs. We found no differences between groups on GCA or number of lacunes.

Linear mixed models with random intercept and slope showed that across groups, patients declined two MMSE points per year (β [SE] = -2.10 [0.31], p= 0.00). No association of MB presence with baseline MMSE (β [SE] = 0.26 [0.72], p= 0.72) or rate of cognitive decline (β [SE] = 0.01 [0.34], p= 0.97) was found [Figure 1]. Adjustment for MTA, GCA, WMH and number of lacunes did not change the results [baseline MMSE: β [SE] = 0.34 [0.76], p= 0.65; rate of decline: β [SE] = -0.09 [0.34], p= 0.79], nor did further adjustment for smoking, hypertension, diabetes, hypercholesterolaemia, myocardial infarction, use of anti-thrombotics or Alzheimer medication. Also, stratification according to age at onset [Figure 2] and APOE ε4 carriernesship [Figure 3] did not reveal any associations between MBs and cognition. We repeated all analyses with MBs as a continuous measure to study the associations with the number, rather than the presence, of MBs which did not reveal any associations with baseline MMSE or rate of cognitive decline either. Furthermore, restricting the sample to patients with MBs only showed no associations of number of MBs with baseline MMSE (β [SE] = -0.15 [0.13], p= 0.23) or rate of cognitive decline (β [SE] = 0.00 [0.05], p= 0.98). When the location of the MBs [no MBs; strictly non-lobar MBs; strictly lobar MBs; both lobar and non-lobar MBs] was taken into account, we observed no association of location of MBs with baseline MMSE or rate of cognitive decline [all p’s > 0.05] [Figure 4]. Similarly, location of MBs in terms of laterality [no MBs; left sided MBs; right sided MBs; bilateral MBs] did not reveal any associations [data not shown].

Discussion

The main finding of this longitudinal study is that presence and number of MBs are not associated with rate of cognitive decline in AD. Neither stratification for age-at-onset or APOE-genotype, nor taking into account MB location [deep vs lobar; left hemisphere vs right hemisphere], nor restricting the analysis to patients with MBs only revealed any significant relation between MBs and rate of cognitive decline in this clinical sample of AD patients.

There are two former longitudinal studies that have looked into the relationship between MBs and rate of cognitive decline in Mild Cognitive Impairment (MCI) which are in line with our findings. One study found no difference in MBs between stable and progressive MCI patients after one year.26 The other study found that the presence of MBs predicts conversion from MCI to dementia, but the significance of this effect was lost after adjustment for age.27

Previous studies have suggested that the presence of one or a few MBs does no real harm, but having multiple MBs is indicative of a more malignant outcome. Despite a modestly – though nonsignificantly – increased mortality rate, we were not able to demonstrate an association between the presence or number of MBs and rate of cognitive decline. In AD, it seems that downstream phenomena such as loss of synapses and neurodegeneration are largely responsible for cognitive decline. Our results suggest that MBs do not affect these downstream pathological Alzheimer-processes. Two previous studies showed that patients with multiple MBs have a higher risk of mortality.17,18 Our current results support the notion that the increased risk of mortality in AD patients with MBs is not related to the Alzheimer process itself, but rather to vascular events, including [hemorrhagic] stroke.

Variability in rate of decline on MMSE in our sample of AD patients was large. The determinants of the rate of decline in AD are largely unknown, as we are presently unable to predict which patients will show faster progression than others. The current study shows that MBs are not an important determinant of rate of decline in AD. We cannot exclude the possibility that MBs have a subtle effect on rate of cognitive
of linear mixed models for statistical analyses. These models take into account all available data points, allowing patients to have variable numbers of follow-up measurements. In this way, patients with only two available MMSE scores could also be included in the study, as the statistical model appropriately takes into account that the estimate of cognitive decline is less precise in these patients. A potential limitation is the relatively small number of AD patients with MBs, as despite the large sample size of 221 patients with clinical follow up, only 39 had MBs. Still, this number is in agreement with previously reported prevalence of MBs in AD and the large group of patients without any MBs adds power to the statistical analyses. A second limitation is that although information on the use of Alzheimer medication and other types of medication at baseline was available, use of medication in the course of the disease was not recorded. Nonetheless, although the use of cholinesterase inhibitors and memantine may have influenced the rate of cognitive decline, we do not suspect that this effect would be different for patients with MBs than for those without.

Third, our outcome measure was the MMSE, a crude measure of cognitive decline, that does not capture all aspects of disease severity. Still, the MMSE is a generally accepted and widely used test for the evaluation of cognition in elderly patients. A future study should investigate the impact of MBs on the decline of specific cognitive domains and on the relationships between MBs and neuropsychiatry symptoms, also in nondemented populations where subtle effects of MBs may still be discerned.

Recently, the interest in the clinical consequence of MBs has risen, since amyloid related imaging abnormalities (ARIA) including cerebral MBs have occurred in patients participating in clinical trials with therapeutic agents to lower amyloid-β burden in AD. In this context, our finding of a lack of association between MBs and the rate of cognitive decline may be of importance. If the rate of cognitive decline — often a primary outcome measure in clinical trials — is not influenced by the presence and number of MBs, excluding patients with MBs may not be necessary. However, it should be noted that the prognosis of ARIA-hemosiderin deposition (ARIA-H) may be different from that of spontaneously occurring MBs. Therefore, further research is needed regarding the risk of accelerated cognitive decline in patients with ARIA-H.
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Table 1. Baseline demographic and clinical characteristics.

<table>
<thead>
<tr>
<th></th>
<th>No Microbleeds</th>
<th>≥ 1 Microbleeds</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>182</td>
<td>39</td>
</tr>
<tr>
<td>Sex, n (%) female</td>
<td>94 (52)</td>
<td>15 (39)</td>
</tr>
<tr>
<td>Age, mean (sd)</td>
<td>67 (9)</td>
<td>71 (8)*</td>
</tr>
<tr>
<td>APOE ε4 carriers, n (%)</td>
<td>111 (71)</td>
<td>20 (69)</td>
</tr>
<tr>
<td>MMSE at baseline, mean (sd)</td>
<td>22 (4)</td>
<td>22 (4)</td>
</tr>
<tr>
<td>Follow-up time (years), mean (sd)</td>
<td>3 (1)</td>
<td>3 (1)</td>
</tr>
<tr>
<td>Number of MMSE’s, median [range]</td>
<td>4 (2-17)</td>
<td>4 (2-10)</td>
</tr>
<tr>
<td>Mortality, n(%)</td>
<td>24 (13%)</td>
<td>8 (21%)</td>
</tr>
</tbody>
</table>

| Smoking, n(%)          | 26 (16%)       | 3 (8%)          |
| Hypertension, n(%)     | 48 (27%)       | 20 (51%)*       |
| Diabetes mellitus, n(%) | 12 (7%)       | 3 (8%)          |
| Hypercholesterolaemia, n(%) | 28 (16%) | 9 (23%)         |
| Myocardial infarction, n(%) | 6 (3%)    | 3 (8%)          |
| Use of anti-thrombotics, n(%) | 32 (18%) | 13 (33%)*       |
| Use of Alzheimer medication, n(%) | 13 (7%) | 2 (5%)          |
| CSF amyloid-beta 1–42, pg/mL * | 459 (166) | 406 (153)*     |
| Total tau, pg/mL *     | 639 (399)     | 739 (497)       |
| Tau phosphorylated at threonine 181, pg/mL * | 87 (34) | 94 (44)         |
| Microbleeds, median (range) | --- | 2 (1-27)*     |
| MTA, mean (sd)*        | 1.4 (0.9)     | 1.9 (1.0)*      |
| GCA, mean (sd)*        | 1 (1)         | 1 (1)           |
| WMH, mean (sd)*        | 0.8 (0.8)     | 1.5 (1.0)*      |
| Lacunes, mean (sd)     | 0 (0)         | 0 (1)           |

Acknowledgements
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Annual MMSE change for patients with and without MBs, stratified according to age at onset.

Annual MMSE change was calculated as last MMSE score minus first MMSE score, divided by follow up time in years. Note that for the statistical analysis linear mixed models were used, which showed no association between MBs and rate of cognitive decline.
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Figure 3  Annual MMSE change for patients with and without MBs, stratified according to APOE ε4 genotype.

Annual MMSE change was calculated as last MMSE score minus first MMSE score, divided by follow up time in years. Note that for the statistical analysis linear mixed models were used, which showed no association between MBs and rate of cognitive decline for either APOEε4 carriers or noncarriers.

Figure 4  Annual MMSE change according to the presence and location of MBs. Linear mixed models showed no association between the location of MBs and the rate of cognitive decline.
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15. Werring DJ, Frazer DW, Coward LJ et al. Cerebral microbleeds on T2*-weighted gradient-echo MRI. *Brain* 2004;127:2265-75.
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It will have blood, they say: blood will have blood."

William Shakespeare

2.3 THE INCIDENCE OF CEREBRAL MICROBLEEDS: A LONGITUDINAL STUDY IN A MEMORY CLINIC POPULATION


Authors: JDC Goos MD1, WJP Henneman MD2, JD Sluimer MD2, H Vrenken PhD2, IC Sluimer PhD2, F Barkhof MD PhD2, MA Blankenstein PhD2, Ph Scheltens MD PhD2, and WM van der Flier PhD3,5.

Institutional affiliations: From the Alzheimer Center and 1Department of Neurology, 2Department of Radiology, 3Department of Physics and Medical Technology, 4Department of Clinical Chemistry, 5Departments of Epidemiology and Biostatistics, VU University Medical Center, Amsterdam, the Netherlands.
Abstract

**Background:** Cerebral microbleeds (MBs) are commonly observed in memory clinic patients. Little is known about occurrence of and risk factors for developing new MBs in this population.

**Objective:** To investigate incidence of lobar and non-lobar MBs in a memory clinic population. Furthermore, to assess risk factors for the development of new MBs and their associations with other MRI changes.

**Methods:** 254 patients visiting our memory clinic, with repeat gradient-recalled echo T2*-weighted MRI, were included (scan interval 1.9±0.9 years). Baseline and incident MBs were regionally counted. White matter hyperintensities (WMH) and progression of WMH were assessed using visual rating scales. Baseline brain volume and whole-brain atrophy rate were estimated automatically. In a subset, APOE was determined.

**Results:** Thirty-one (12%) patients developed new MBs (range 1-19). Both multiple strictly lobar and non-lobar MBs at baseline predicted incident MBs (OR 8.4, 95% CI 2.2-33.2 and OR 33.8, 95% CI 8.1-140.8). Furthermore, baseline WMH grade (OR 1.2, 1.1-1.3), lacunar infarcts (OR 2.8, 1.3-6.0) and APOE ε2 carriersonship (OR 4.2, 1.4-12.5) predicted MB incidence. Incident MB patients had more progression of WMH (p<0.01) and incident lacunar infarcts (p<0.05). These relations were most prominent for incident non-lobar MBs. Incident strictly lobar MBs were associated with baseline systolic tension and smoking.

**Conclusion:** In addition to APOE genotype, presence and progression of small vessel disease and vascular risk factors were predictors of new MBs. The latter are potentially modifiable, suggesting the possibility of preventing incident MBs, hopefully resulting in slower clinical decline.

Introduction

Cerebral microbleeds (MBs), small rounded regions of signal loss on gradient-recalled echo (GRE) T2*-weighted MRI, are frequently observed in a memory clinic population. Histologically MBs represent hemosiderin, likely from leakage through cerebral small vessels, contained within surrounding macrophages in the brain parenchyma. Important risk factors associated with MBs are higher age and arterial hypertension. Clinically they have been associated with cognitive impairment, higher stroke risk, lower CSF amyloid levels and higher mortality. Radiologically, MBs have been associated with white matter hyperintensities (WMH) and lacunar infarcts. MBs may occur in lobar or deep locations. It has been suggested that risk factors for MBs differ according to their location.

The aforementioned associations are largely based on cross-sectional studies. Therefore, little is known about frequency of and risk factors for development of new MBs. Only a few longitudinal studies have been performed in specific populations with relatively small sample sizes. Most studies, undifferentiated for MB location, found that the baseline number of MBs and WMH predicted incidence of MBs. In addition to baseline MBs, one study found systolic blood pressure as a predictor of new MB development in ischemic stroke patients.

The aim of this study was to evaluate the frequency of newly occurring MBs and to assess possible risk factors for incident MBs in a large sample of memory clinic patients. In addition, we investigated whether risk factors differed according to the location of new MBs. Finally, we evaluated associations of incident MBs with other radiological brain changes over time.
Material and Methods

Patients
Patients were included from the memory clinic of the Alzheimer Center of the VU University Medical Center Amsterdam (VUMC). Between 2004 and 2007 all patients attending our memory clinic were invited for a repeat MR scan. For this study, we retrospectively selected all patients who underwent two MRI scans of acceptable quality, on the same scanner using an identical imaging protocol, including T2*-weighted GRE sequences. There were 254 patients who fulfilled these criteria. All patients underwent standardized dementia screening, including past medical history, physical, neurological and neuropsychological examination. Patients were considered as having a history of arterial hypertension, diabetes mellitus, and hypercholesterolemia if they had a known history of the disease or were receiving drug treatment. Furthermore, screening involved routine laboratory examinations. Diagnoses were made in a multidisciplinary consensus meeting. Probable AD (n=74) was diagnosed according to the clinical criteria of the National Institute of Neurological Disorders and Stroke-Alzheimer’s Disease and Related Disorders Association (NINCDS-ADRDA),19 and a diagnosis of MCI (n=62) was based on Petersen criteria.20 When all clinical investigations were normal, patients were considered to have subjective complaints. The subgroup of controls (n=47) consisted of 33 patients with subjective complaints and 14 volunteers without cognitive complaints, who underwent the same screening procedure as patients attending our memory clinic. The subgroup of other dementia (n=39) included vascular dementia (n=2),21, frontotemporal lobar degeneration (n=24), dementia with Lewy bodies (n=6), corticobasal degeneration (n=4), progressive supranuclear palsy (n=2) and CADASIL (n=1). The subgroup of other disorders (n=32) included patients with other neurological disorders including stroke), psychiatric disorders and unclear diagnoses. Dementia severity was assessed using the Mini-Mental State Examination (MMSE).22

Standard Protocol Approvals, Registrations, and Patient Consents
The study was approved by the local ethical review board and all subjects gave written informed consent for their clinical data to be used for research purposes.

MRI protocol
All scans were obtained on the same 1.0T scanner (Magnetom Impact; Siemens, Erlangen, Germany). The scan protocol was identical for all patients at both time points (scan interval 1.9±0.9 years; range of 0.5-5.6) and included axial GRE images (19 slices, field of view (FOV) 250mm, matrix 256x256, slice thickness: 5mm, interslice gap: 1mm, repetition time (TR): 800ms, echo time (TE): 22ms, flip angle 20 degrees).

MRI assessment
MRI rating was performed blinded to the clinical data. Side-by-side comparison of baseline and follow-up scans was used to assess incidence of MBs and change in WMH over time. MBs were defined as rounded hypointense homogeneous foci measuring up to 10mm in the brain parenchyma on GRE images. Lesions in sulci possibly representing flow voids from pial vessels and symmetrical lesions in the basal ganglia, supposedly representing iron or calcium deposits, were excluded. Hypointensities inside infarcts were not counted as MBs, but regarded to be probable hemorrhagic transformations. MBs were counted in four lobar regions (frontal, parietal, temporal and occipital) and in two non-lobar regions (basal ganglia including thalamus, and infratentorial). Patients with MBs were considered to have strictly lobar MBs, when MBs occurred in lobar regions exclusively. In all other cases, i.e. non-lobar MBs only or MBs in both locations, MBs were regarded as non-lobar MBs.

WMH were assessed using the Age-Related White Matter Change (ARWMC) scale.23 The ARWMC scale has scores from 0-3 (none, punctuate, early confluent and confluent) given in 5 regions, each left and right, adding up to a total range from 0 to 30. For WMH progression we used a modified Rotterdam Progression Scale,24 in which absence (0) or presence (1) of progression of WMH is rated for three periventricular regions and four subcortical regions, resulting in a total range from 0 to 7. In addition, the presence at baseline and occurrence of new large vessel infarcts and lacunar infarcts were assessed. Large vessel infarcts were rated as present or absent. Lacunar infarcts were defined as well demarcated lesions from 3 to 15mm, with cerebrospinal fluid-like signal on all sequences. Percentage of brain volume change (PBVC) between the two time points and
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normalized baseline brain volume (NBV) were measured from the MPRAGE images by using Structural Image Evaluation, using Normalisation, of Atrophy (SIENA) and the cross-sectional processing counterpart SIENAX, a fully automated technique part of FSL 4.0 [detailed explanation see: (http://www.fmrib.ox.ac.uk/analysis/research/siena/)]. A description of this method and our modifications to remove non-brain tissue can be found in a previous paper.

All individual scans, registration results, and SIENA output were reviewed by a rater, who was blinded to the diagnosis.

**APOE genotyping**

DNA was isolated from 10mL of ethylenediaminetetra-acetic acid/blood and was available from 197 of 254 patients (78%). APOE genotype was determined with the light cycler APOE mutation detection method (Roche Diagnostics GmbH, Mannheim, Germany). Patients were categorized according to APOE ε4 and to APOE ε2 status.

**Statistical analysis**

For statistical analysis SPSS 14.0 for windows (Chicago, IL) was used. Risk factors for incident MBs were assessed using logistic regression analysis. In the first model, univariate associations with incident MBs were assessed. Associations were adjusted for age, sex, scan interval and WMH in the second model. Data are represented as odds ratio (OR) with accompanying 95% confidence interval (95% CI), we did not account statistically for. Incident MBs [1 or more new MBs in any region] were used as primary outcome measure. Analyses were then repeated for incident strictly lobar MBs and for incident MBs in non-lobar or mixed location, and for patients with MBs at baseline only.

**Results**

**MRI characteristics at baseline and follow-up**

Mean age was 66±10 years, 52% were males. Forty-nine (19%) patients had one or more MBs at baseline. The number of baseline MBs ranged from 1-47. Of the patients with MBs at baseline, 27 (55%) patients had strictly lobar MBs and 22 (45%) patients had non-lobar MBs.

Among all 254 patients, 31 (12%) patients showed incident MBs at follow-up (range 1-19 new MBs per patient). The overall prevalence of one or more MBs rose from 19% at baseline to 24% at follow-up. A small subgroup of 6 (2%) patients with MBs present at baseline, had less MBs visible on follow-up. A schematic overview of MB distribution at baseline and follow-up is presented in figure 1.

Baseline demographics, clinical and MRI characteristics by MB incidence are shown in table 1. Age, sex and diagnosis were not related to incident MBs. Patients developing new MBs had higher MMSE scores than the group without incident MBs. Patients with incident MBs were more frequently former and current smokers. Other vascular risk factors were comparable between groups. There was no difference in APOE ε4 status between groups. However, patients with incident MBs had higher prevalence of the APOE ε2 allele. Baseline prevalence and number of MBs were both higher in the patients who developed new MBs. The burden of WMH and lacunar infarcts was more severe in the group with incident MBs. No differences between groups were found for large vessel infarcts or baseline brain volume.

Of the 31 patients with incident MBs, 18 patients had new strictly lobar MBs (example upper panel figure 2) and 13 patients had new MBs in non-lobar (example lower panel of figure 2) or mixed locations. When we separately compared patients with strictly lobar incident MBs and patients with non-lobar incident MBs to the patients without incident MBs (Table 1), results were largely comparable, except for the following results. Patients with non-lobar incident MBs were older than patients without new MBs. Patients with new strictly lobar MBs had a higher prevalence of former (33%) and current [40%] smokers. Patients with new strictly lobar MBs consumed less alcohol than patients without new MBs. The group with incident non-lobar MBs had the highest prevalence [85%] and number of MBs present at baseline. The higher WMH loads and lacunar infarct counts in patients with incident MBs were attributable to the group with incident non-lobar MBs.
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Risk factors for incident microbleeds

Logistic regression showed that there was no relation between age, sex, diagnosis or MMSE and incidence of MBs (Table 2). There was a dose-dependent relationship between smoking (former smoker, current smoker) and the risk of developing new MBs. Higher systolic, but not diastolic, blood pressure predicted incident MBs. No other vascular risk factors were associated with an elevated risk for incident MBs. APOE ε4 carriership did not predict incident MBs. Carriership of the APOE ε2 allele however, inferred a more than fourfold elevated risk of incident MBs. To gain more insight in the influence of number and location of baseline MBs on the development of new MBs, we constructed a new variable. This variable contained 5 baseline MB categories: no MBs, single strictly lobar, single non-lobar, multiple strictly lobar, and multiple non-lobar. Logistic regression showed that having one MB present at baseline, regardless of location, was associated with a non-significantly elevated risk of developing new MBs. Multiple MBs were associated with an elevated risk of 8.4(2.2-33.2) for strictly lobar MBs and 33.8(1.1-140.8) for non-lobar MBs. Finally, the degree of WMH and number of lacunar infarcts at baseline predicted the occurrence of new MBs. After adjustment for age, sex, scan interval and WMH (Model 2), significance was lost for smoking, systolic blood pressure and lacunar infarcts, but all other associations remained comparable. Subsequently, we restricted the analyses to the 49 patients with at least one MB present at baseline, consisting of 33(67%) patients without incident MBs versus 16(33%) patients with incident MBs. Results remained largely comparable, and can be found online in Table e-1.

To assess risk factors for new MBs by location, we repeated analyses restricted for the prediction of strictly lobar and non-lobar incident MBs. APOE ε2 carriership (OR 4.8, 1.1-20.8), multiple MBs (OR 105.6, 20.0-554.5), WMH grade (OR 1.3, 1.2-1.5), and lacunar infarcts (OR 5.3, 2.2-12.8) were specifically related to development of new non-lobar MBs, while there was no significant association with new strictly lobar MBs. In addition, age (OR 1.1, 1.0-1.2) predicted new non-lobar MBs. New strictly lobar MBs were associated with smoking (OR 3.5, 0.9-13.6 for former and OR 6.5, 1.7-24.6 for current smoking) and a threefold, but non-significant, increased risk of APOE ε2 carriership (OR of 3.2[0.8-13.1]).

Relations of longitudinal variables with incident MBs are shown in Table 3. Patients with incident MBs showed more progression of WMH, and had a higher incidence of lacunar infarcts. Incidence of large vessel infarcts and progression of atrophy, and MMSE change were not associated with MB incidence.

Discussion

We found that 12% of memory clinic patients showed incident MBs during two year follow-up. Multiple MBs at baseline, regardless of location, as well as possession of the APOE ε2 allele independently predicted new MBs in this population. WMH and lacunar infarcts, were also related to incident MBs, together with vascular risk factors systolic blood pressure and smoking.

Recent cross-sectional studies have indicated the clinical relevance of MBs in a memory clinic setting, and the current longitudinal study adds to our understanding of risk factors for the development of new MBs. There are only a few longitudinal studies on incidence of MBs, describing relatively small samples of specific populations.12,14-16,27 In CAA and CADASIL patients, MBs were very frequent and numerous. These studies identified baseline MBs as the most important predictor for MB incidence. We also found baseline MBs as the most important predictor of new MBs. We extended these findings by taking location into account. Though both locations predicted development of new MBs, the predictive value of non-lobar MBs exceeded that of strictly lobar MBs.

The cross-sectional association between MBs and other MRI markers of small vessel disease has been repeatedly reported.1,5,10,28 Moreover, a large prospective population based study associated MBs in deep locations (basal ganglia, thalami and infratentorial) with arterial hypertension, WMH and lacunar infarcts.5 This is in line with the cross-sectional and temporal relation we found between new non-lobar MBs and progression of WMH and lacunes. We found no association between strictly lobar incident MBs and progression of WMH. In line with our findings, a former
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A longitudinal study in CAA patients did not find a relation between incident lobar MBs and progression of WMH either. These findings might further support the notion that etiology of MBs may differ according to MB location, with deep MBs caused by hypertensive vasculopathy, more closely associated with vascular risk factors and other markers of small vessel disease and lobar MBs caused by CAA and supposedly related to APOE ε4 status.

The APOE ε4 and APOE ε2 genotypes have been associated with CAA and (macro) hemorrhages. CAA presumably causes strictly lobar MBs. Some studies showed associations of homozygous carrierrship of the APOE ε4 allele with lobar MBs. We did not confirm these findings in our longitudinal study, but we found patients with incident MBs to be more frequent carriers of the APOE ε2 allele. We are not aware of any study directly associating only the APOE ε2 genotype to MBs. Surprisingly, APOE ε2 had a stronger association with the development of new non-lobar MBs, although APOE ε2 carrierrship also conferred a threefold (though nonsignificant) increased risk of lobar MBs.

Based on cross-sectional studies, reporting the relation of burden of MBs and impaired cognition, we expected to find an association between incidence of MBs and MMSE change. In this study however, we did not find such a relationship. This lack of association may be due to the heterogeneity of our population, relatively low numbers of incident MBs, or crude cognitive measurement.

Strengths of the current study are the relatively large sample size of patients with repeat MRI on one single scanner with identical scan protocol. All patients underwent an extensive standardized diagnostic workup. Limitations are the heterogeneity of our sample which may limit the generalisability and power of our findings. Additionally, although our total population was large, the group of patients with incident MBs was relatively small, especially when we took location into account. A possible selection bias and source of underestimation of new MBs in our sample could be if patients with new MBs were less likely to undergo a second scan, due to rapid deterioration, including death. We feel however, that the resulting sample, reflects a 'normal' memory clinic sample fairly well and as such represents an important selection of patients. We used a scanner with relatively low field strength. Furthermore, slices were relatively thick (5.0mm) and the echo time of 22ms was relatively short, diminishing the susceptibility effect, possibly underestimating the total number of MBs. In our study, we found a 2% loss of MBs. Whether this represents a biological phenomenon of resorption, or results from methodological issues is unclear. However, the loss of MBs at follow-up of 2% is comparable with an earlier report, finding a MB loss of 2.3% in a CAA population, with slightly thicker slices of 6mm, but higher field strength of 1.5T. Moreover, it is far lower than the 20% of patients who were reported to show less MBs on follow-up in a recent study using SWI, which is a more sensitive method of MB detection.

Our data suggest that developing new MBs in a memory clinic setting is predominantly related to vascular risk factors and markers of small vessel disease. Furthermore, MBs were not related to baseline total brain volume or progressive loss of brain volume. This suggests that in this heterogeneous sample, MBs are not linked to neurodegeneration. Former studies have suggested that MBs in AD are closely linked to accumulation of amyloid-beta in the vessels (CAA) based on the reported lobar predominance of MBs in AD. We therefore feel that CAA indeed may be an important cause of MB incidence in AD patients. Our sample however, also contained patients with subjective complaints or MCI. The etiology of MBs in these patients is less clear. We recently showed that MBs in MCI patients were closely related to small vessel disease and associated with a nearly twofold risk of progression to non-Alzheimer’s dementia, e.g. VaD. Our results suggest that strict management of vascular risk factors in memory clinic patients may reduce the development of new MBs in the future and as a result may slow down clinical decline.

Acknowledgements
J.D.C. Goos is supported by Stichting Dioraphte. The Alzheimer Center VUmc is supported by Alzheimer Nederland and Stichting VUmc fonds. The clinical database structure was developed with funding from Stichting Dioraphte. Lieza Exalto has assisted in the SIENA analyses.
## Table 1: Baseline demographics, clinical and MRI characteristics by MB incidence category

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<th>Baseline variable</th>
<th>No incident MBs (n=223)</th>
<th>Total incident MBs (n=31)</th>
<th>Lobar incident MBs (n=18)</th>
<th>Non-lobar incident MBs (n=13)</th>
</tr>
</thead>
<tbody>
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<td><strong>Patient characteristics</strong></td>
<td></td>
<td></td>
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<tr>
<td>Age at first visit (y)</td>
<td>66±10</td>
<td>69±9</td>
<td>68±11</td>
<td>72±6*</td>
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<tr>
<td>Sex, male</td>
<td>117(53%)</td>
<td>16(52%)</td>
<td>7(39%)</td>
<td>9(69%)</td>
</tr>
<tr>
<td>Diagnosis</td>
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<tr>
<td>Controls</td>
<td>42(89%)</td>
<td>5(11%)</td>
<td>3(17%)</td>
<td>2(4%)</td>
</tr>
<tr>
<td>MCI</td>
<td>49(79%)</td>
<td>13(21%)</td>
<td>5(9%)</td>
<td>8(13%)</td>
</tr>
<tr>
<td>AD</td>
<td>65(88%)</td>
<td>9(12%)</td>
<td>6(9%)</td>
<td>3(4%)</td>
</tr>
<tr>
<td>Other dementia</td>
<td>38(97%)</td>
<td>1(3%)</td>
<td>1(3%)</td>
<td>0(0%)</td>
</tr>
<tr>
<td>Other disorders</td>
<td>29(91%)</td>
<td>3(9%)</td>
<td>3(9%)</td>
<td>0(0%)</td>
</tr>
<tr>
<td>MMSE</td>
<td>25±4</td>
<td>26±3*</td>
<td>26±3</td>
<td>26±2</td>
</tr>
<tr>
<td>Hypertension</td>
<td>58(26%)</td>
<td>11(36%)</td>
<td>6(33%)</td>
<td>5(39%)</td>
</tr>
<tr>
<td>Diabetes Mellitus</td>
<td>14(6%)</td>
<td>2(7%)</td>
<td>0(0%)</td>
<td>2(15%)</td>
</tr>
<tr>
<td>Hypercholesterolemia</td>
<td>35(16%)</td>
<td>4(13%)</td>
<td>2(11%)</td>
<td>2(15%)</td>
</tr>
<tr>
<td>Smoking</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never</td>
<td>121(64%)</td>
<td>41(22%)</td>
<td>9(33%)</td>
<td>9(33%)</td>
</tr>
<tr>
<td>Former</td>
<td>41(22%)</td>
<td>26(14%)</td>
<td>4(27%)</td>
<td>5(42%)</td>
</tr>
<tr>
<td>Current</td>
<td>26(14%)</td>
<td>9(33%)*</td>
<td>5(33%)</td>
<td>4(33%)</td>
</tr>
<tr>
<td>Alcohol Units/Day</td>
<td>1.4±1.2</td>
<td>1.1±1.1</td>
<td>0.7±0.5*</td>
<td>1.8±1.4</td>
</tr>
<tr>
<td>Systolic tension</td>
<td>149.6±21</td>
<td>160.2±22*</td>
<td>160.0±22</td>
<td>160.5±22</td>
</tr>
<tr>
<td>Diastolic tension</td>
<td>88.0±10</td>
<td>90.4±11</td>
<td>89.6±12</td>
<td>91.5±11</td>
</tr>
<tr>
<td>APOE ε4 carrier</td>
<td>92(54%)</td>
<td>14(64%)</td>
<td>9(75%)</td>
<td>5(50%)</td>
</tr>
<tr>
<td>APOE ε2 carrier</td>
<td>14(8%)</td>
<td>6(27%)*</td>
<td>3(25%)</td>
<td>3(30%)*</td>
</tr>
</tbody>
</table>

**Baseline variable**

<table>
<thead>
<tr>
<th>MRI Characteristics</th>
<th>No incident MBs (n=223)</th>
<th>Total incident MBs (n=31)</th>
<th>Lobar incident MBs (n=18)</th>
<th>Non-lobar incident MBs (n=13)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total MB prevalence</td>
<td>34(15%)</td>
<td>16(52%)*</td>
<td>5(28%)</td>
<td>11(85%)*</td>
</tr>
<tr>
<td>Number of MBs</td>
<td>0 [0-8]</td>
<td>1 [0-47]*</td>
<td>0 (0-7)</td>
<td>3 [0-47]**</td>
</tr>
<tr>
<td>ARWMC</td>
<td>3.3±4</td>
<td>7.1±6**</td>
<td>4.2±4</td>
<td>11.1±5**</td>
</tr>
<tr>
<td>Lacunar infarct</td>
<td>13(6%)</td>
<td>6(19%)*</td>
<td>18(7.2%)</td>
<td>5(39%)**</td>
</tr>
<tr>
<td>Large vessel infarct</td>
<td>13(6%)</td>
<td>1(3%)</td>
<td>0(0%)</td>
<td>1(8%)</td>
</tr>
<tr>
<td>NBV(mL)</td>
<td>1532±101</td>
<td>1535±110</td>
<td>1535±87</td>
<td>1534±140</td>
</tr>
</tbody>
</table>

Data are represented as number of patients with variable present, n(%), mean±sd, and for number of MBs as median (range). *p<0.05, **p<0.01 [*,**] compared with the no incident MB group. MBs = microbleeds, MCI = mild cognitive impairment, AD = Alzheimer disease, MMSE = mini mental state examination, ARWMC = age related white matter changes, NBV = normalized brain volume. Availability for incomplete data: MMSE 242/254, Smoking 215/254, Alcohol 168/254, blood pressure measurements 191/254, APOE 198/254.
Table 2. Associations between baseline risk factors and incident microbleeds

<table>
<thead>
<tr>
<th>Baseline variables</th>
<th>Model 1</th>
<th>Model 2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Patient characteristics</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>1.0(1.0-1.1)</td>
<td>1.0 (1.0-1.1)</td>
</tr>
<tr>
<td>Male sex</td>
<td>1.0(0.5-2.1)</td>
<td>0.9(0.4-2.0)</td>
</tr>
<tr>
<td><strong>Diagnosis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td>2.2(0.7-6.8)</td>
<td>2.2(0.7-6.8)</td>
</tr>
<tr>
<td>MCI</td>
<td>1.2(0.4-3.7)</td>
<td>1.2(0.4-3.7)</td>
</tr>
<tr>
<td>AD</td>
<td>0.2(0.03-2.0)</td>
<td>0.2(0.03-2.0)</td>
</tr>
<tr>
<td>Other dementia</td>
<td>0.9(0.2-3.9)</td>
<td>0.9(0.2-3.9)</td>
</tr>
<tr>
<td>MMSE</td>
<td>1.1(1.0-1.2)</td>
<td>1.1(1.0-1.2)</td>
</tr>
<tr>
<td>Hypertension</td>
<td>1.6(0.7-3.5)</td>
<td>1.6(0.7-3.5)</td>
</tr>
<tr>
<td>Diabetes</td>
<td>1.0(0.2-4.8)</td>
<td>1.0(0.2-4.8)</td>
</tr>
<tr>
<td>Hypercholesterolemia</td>
<td>0.8(0.3-2.4)</td>
<td>0.8(0.3-2.4)</td>
</tr>
<tr>
<td>Smoking</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never</td>
<td>3.0(1.1-7.9)**</td>
<td>3.0(1.1-7.9)**</td>
</tr>
<tr>
<td>Former</td>
<td>4.7(1.7-12.9)**</td>
<td>4.7(1.7-12.9)**</td>
</tr>
<tr>
<td>Current</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alcohol Units/Day</td>
<td>0.8(0.6-1.3)</td>
<td>0.8(0.6-1.3)</td>
</tr>
<tr>
<td>Systolic tension</td>
<td>1.3(1.0-1.6)*</td>
<td>1.3(1.0-1.6)*</td>
</tr>
<tr>
<td>Diastolic tension</td>
<td>1.3(0.8-1.9)</td>
<td>1.3(0.8-1.9)</td>
</tr>
<tr>
<td>APOE ε4</td>
<td>1.5(0.6-3.8)</td>
<td>1.7(0.7-4.6)</td>
</tr>
<tr>
<td>APOE ε2</td>
<td>4.2(1.4-12.5)**</td>
<td>3.6(1.2-11.3)*</td>
</tr>
<tr>
<td><strong>MRI Characteristics</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Microbleeds</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>1.7(0.4-8.1)</td>
<td>1.7(0.4-8.1)</td>
</tr>
<tr>
<td>One strictly lobar</td>
<td>2.8(0.6-14.2)</td>
<td>2.8(0.6-14.2)</td>
</tr>
<tr>
<td>Multiple strictly lobar</td>
<td>8.4(2.2-33.2)**</td>
<td>8.4(2.2-33.2)**</td>
</tr>
<tr>
<td>Multiple non-lobar</td>
<td>33.8(8.1-140.8)**</td>
<td>33.8(8.1-140.8)**</td>
</tr>
<tr>
<td><strong>Other</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ARWMC</td>
<td>1.2(1.1-1.3)**</td>
<td>1.2(1.1-1.3)**</td>
</tr>
<tr>
<td>Lacunar infarct</td>
<td>2.8(1.3-6.0)**</td>
<td>2.8(1.3-6.0)**</td>
</tr>
</tbody>
</table>

Table 3. Longitudinal MMSE and brain changes associated with incident MBs

<table>
<thead>
<tr>
<th></th>
<th>No incident MBs</th>
<th>Incident MBs</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMSE change</td>
<td>-4.3±6</td>
<td>-3.8±5</td>
</tr>
<tr>
<td>progression of WMH [0-7]</td>
<td>0.6±1</td>
<td>1.2±1**</td>
</tr>
<tr>
<td>Incident lacunar infarcts</td>
<td>5(2%)</td>
<td>4(13%)*</td>
</tr>
<tr>
<td>Incident large vessel infarcts</td>
<td>4(2%)</td>
<td>1(3%)</td>
</tr>
<tr>
<td>% brain volume change</td>
<td>-2.2±2</td>
<td>-2.0±2</td>
</tr>
</tbody>
</table>

Data are represented as odds ratio (95% confidence interval) for total incident MBs. ∞ odds ratios per 10mmHg. *p<0.05, **p<0.01. MCI = mild cognitive impairment, AD = Alzheimer disease, MMSE = mini mental state examination, ARWMC = age related white matter changes, NBV = normalized brain volume, Model 1 was unadjusted, Model 2 was adjusted for age, sex, scan interval and white matter hyperintensities. Availability for incomplete data: MMSE 242/254, Smoking 215/254, Alcohol 168/254, blood pressure measurements 191/254, APOE 198/254.
Microbleeds in Dementia: Connecting the dots

Figure 1
Flow chart of MB distribution at baseline and follow-up

<table>
<thead>
<tr>
<th>Baseline</th>
<th>Follow-up</th>
</tr>
</thead>
<tbody>
<tr>
<td>No MBs</td>
<td>No MBs</td>
</tr>
<tr>
<td>(n=205)</td>
<td>(n=100)</td>
</tr>
<tr>
<td></td>
<td>Strictly lobar MBs</td>
</tr>
<tr>
<td></td>
<td>(n=13)</td>
</tr>
<tr>
<td></td>
<td>Non-lobar MBs</td>
</tr>
<tr>
<td></td>
<td>(n=2)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Strictly lobar MBs</th>
</tr>
</thead>
<tbody>
<tr>
<td>(n=27)</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
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<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Non-lobar MBs</th>
</tr>
</thead>
<tbody>
<tr>
<td>(n=22)</td>
</tr>
</tbody>
</table>

Figure 2
Axial T2*-weighted gradient-recalled echo of an 84 year old female patient with delayed diagnosis at baseline (A) and at follow-up (B). An incident lobar MB is visible in the left parietal lobe on the follow-up scan. Axial T2*-weighted gradient-recalled echo of a 76 year old female MCI patient at baseline (C) that progressed to probable Alzheimer disease at follow-up (D). An incident MB is visible in the pons on the follow-up scan.
Microbleeds in Dementia: Connecting the dots


‘Blood. Sometimes it sets my teeth on edge, other times it helps me control the chaos.’

Dexter Morgan

MICROBLEEDS RELATE TO ALTERED AMYLOID-BETA METABOLISM IN ALZHEIMER DISEASE


Authors: Jeroen DC Goos MD¹, Charlotte E Teunissen PhD², Robert Veerhuis PhD², Nicolaas A Verwey MD PhD³, Frederik Barkhof MD PhD³, Marinus A Blankenstein PhD², Philip Scheltens MD PhD¹, and Wiesje M van der Flier PhD¹,4.

Institutional affiliations: From the Alzheimer Center and ¹Department of Neurology, ²Department of Clinical Chemistry, ³Department of Radiology, ⁴Departments of Epidemiology and Biostatistics, VU University Medical Center, Amsterdam, the Netherlands.
Abstract

Objective: Microbleeds (MBs) in dementia relate to cerebral amyloid angiopathy (CAA). We studied the relation between MBs and amyloid-beta peptides in CSF and plasma in Alzheimer disease (AD) and vascular dementia (VaD).

Methods: We selected 26 probable AD patients with MBs, 26 age and sex matched AD patients without MBs, 11 VaD patients and 22 subjective complainers. We measured amyloid beta 1-42 [Aβ42] and 1-40 [Aβ40] in CSF and plasma and blood-brain barrier (BBB) function using albumin ratios.

Results: CSF Aβ42 was lowest in AD with MBs, whereas Aβ40 was selectively decreased in VaD. In plasma, amyloid-beta was non-significantly elevated in VaD compared to controls. Higher albumin ratios in VaD, suggested BBB dysfunction. A MB pattern indicating CAA, related to lower CSF Aβ42, while a non-CAA specific MB pattern showed higher plasma Aβ40.

Conclusions: Amyloid-beta is differentially implicated in AD with MBs and VaD. MB distribution related to different amyloid profiles, supporting distinct etiologies. Our results suggest that Aβ42 is retained in cerebrovasculature of AD patients with MBs, contrary to VaD, possibly draining amyloid.

Introduction

Alzheimer disease (AD) is the most common cause of dementia and is pathologically characterized by the combination of plaques, mainly consisting of amyloid beta 1-42 [Aβ42] and to a lesser extent amyloid beta 1-40 [Aβ40], and tau-positive neurofibrillary tangles.1 In the majority of AD patients Aβ accumulates in the small cerebral vessels, commonly referred to as cerebral amyloid angiopathy (CAA).2 In CAA, Aβ40 affects vessel walls more than Aβ42 does.3

Microbleeds (MBs) are defined as small round foci of signal loss on T2*-weighted MRI and are frequently found in AD patients (15-32%).4-7 Due to their predominantly lobar distribution, they are supposed to represent underlying concomitant CAA. MBs in deep gray matter regions have been associated with hypertensive vasculopathy,8, 9 a frequent cause of VaD, although it may also occur in AD.

In AD, reduced CSF Aβ42 levels are consistently observed, supposedly reflecting deposition of these proteins in plaques, although other explanations have been proposed.10, 11 In contrast, levels of the more soluble CSF Aβ40 are normal in AD patients.12-15 We recently found that AD patients with many MBs have even lower CSF Aβ42 levels than AD patients without MBs, potentially due to additional amyloid deposition in cerebral vessel walls.16 In sporadic non-demented CAA patients, presenting with lobar hemorrhage, lowered CSF levels of both Aβ42 and Aβ40 compared to controls and even to AD have been reported.17

In contrast to CSF, plasma levels of Aβ42 and Aβ40 currently have no diagnostic value in AD.18, 19 Nevertheless, high Aβ40 plasma levels have been associated with diffuse cerebral small vessel disease in patients with lacunar stroke, mild cognitive impairment, AD, CAA and VaD.20-22

We hypothesized that AD patients presenting with predominantly lobar MBs would have lower CSF levels of Aβ42 and Aβ40, providing evidence that MBs are related to CAA. In addition, we hypothesized that MBs may be associated with compromised BBB function, resulting in increased plasma levels of Aβ40 and Aβ42 resulting from leakage of these proteins.
Therefore, we assessed the relation between the presence and location of MBs and CSF and plasma levels of Aβ40 and Aβ42 in patients with AD. For comparison, we included a group of controls and VaD patients.

Material and Methods

Patients

Patients were included from the memory clinic of the Alzheimer Center of the VU University Medical Center Amsterdam (VUMC). Inclusion criteria were presence of a 3T MRI scan and availability of paired CSF and plasma samples, acquired on the same screening day. We selected 26 patients with probable AD with MBs and matched these patients for age and sex with 26 AD patients without MBs. For comparison, we selected a group of 12 VaD patients. A group of 22 patients with subjective memory complaints free of MBs, matched for age and sex as closely as possible to the AD patients served as controls.

All patients underwent abovementioned examinations on the same day, as a part of our standardized dementia screening process, further including medical history, physical, neurological and neuropsychological examination. Dementia severity was assessed using the Mini-Mental State Examination (MMSE). The level of education was classified using the system of Verhage, ranging from 1 (low) to 7 (high). Diagnoses were made in a multidisciplinary consensus meeting. Probable AD was diagnosed according to the clinical criteria of the National Institute of Neurological Disorders and Stroke-Alzheimer’s Disease and Related Disorders Association (NINCDS-ADRDA), and a diagnosis of VaD was based on National Institute of Neurological Disorders and Stroke and Association Internationale pour la Recherche et l’Enseignement en Neurosciences (NINDS-AIREN) criteria. One VaD patient received hemodialysis and was excluded. Nine out of 11 (82%) VaD patients fulfilled radiological criteria for vascular dementia based on small vessel disease (confluent WMH and/or multiple lacunar infarcts), the remaining 2 patients fulfilled the criteria based on strategically located large vessel infarcts. Finally, patients were considered to have subjective complaints when all clinical and neuropsychological investigations were normal. The local ethical review board approved the study and all subjects gave written informed consent for their clinical data to be used for research purposes.

MRI acquisition

MRI was performed on a whole-body 3T MR system (Signa HDxt, General Electric, Milwaukee, Wisconsin) using an eight-channel head coil. The MRI protocol included the following pulse sequences: (1) axial 2D gradient-echo echo-planar imaging (EPI matrix: 256x480, field of view [FOV] 25x19cm², slice thickness 3.0mm, repetition time [TR] 5300ms, echo time [TE] 25ms, 2 excitations), (2) sagittal 3D fluid-attenuated inversion-recovery (FLAIR matrix 224x224, FOV 25x25cm², slice thickness 1.2mm, TR 8000ms, TE 140ms) (3) axial 2D proton-density/T2-weighted fast spin echo (matrix 384x384, FOV 25x19cm², slice thickness 3.0mm, TR 9100ms, TE 23/114ms) (4) oblique reconstructions of a 3D fast spoiled gradient recalled echo-based sequence (FSPGR matrix 256x256, FOV 25x25cm² slice thickness 1mm, TR 708ms).

MRI assessment

MBs were counted by a single rater (J.D.C.G.), blinded for clinical information. MBs were defined as rounded hypointense homogeneous foci up to 10mm in brain parenchyma on the EPI images. Lesions in sulci possibly representing flow voids from pial vessels and symmetrical lesions in the basal ganglia, supposedly representing iron or calcium deposits, were excluded. Hypointensities inside infarcts were regarded to be probable hemorrhagic transformations. MBs were counted in four lobar regions (frontal, parietal, temporal and occipital) and in three non-lobar regions (basal ganglia including thalamus, brain stem and cerebellum).

WMH were assessed using the Fazekas scale with scores ranging from 0-3 (none, punctate, early confluent and confluent). In addition, the presence of large vessel infarcts and lacunar infarcts was assessed. Lacunar infarcts were defined as well demarcated lesions from 3 to 15mm, with CSF-like signal on all sequences. Furthermore, two widely used visual rating scales for the assessment of atrophy were used. Medial temporal lobe atrophy (MTA) was rated using a five point rating scale (0-4),
using oblique reconstructions of the 3D FSPGR sequences, perpendicular to the long axis of the hippocampus. In the analysis average MTA bilateral scores were used. Global cortical atrophy (GCA) was assessed on the FLAIR sequence. The GCA scale ranges from 0-3. The highest score represents maximal atrophy on both scales.

**CSF and blood measurements**

CSF was obtained by lumbar puncture between the L3/L4 or L4/L5 intervertebral space, using a 25-gauge needle, and collected in 10mL polypropylene tubes. A small amount of CSF was used for routine analysis, including total cells (leucocytes and erythrocytes), total protein and glucose. Within two hours, CSF samples were centrifuged at 1800 g for 10 minutes at 4°C and aliquoted in polypropylene tubes of 0.5 or 1ml and stored at -80°C until further analysis. CSF Aβ42, total tau (tau) and p-tau-181 (ptau) were measured with Innotest sandwich ELISA. The performance of the assays was monitored with internal quality controls consisting of pools of surplus CSF specimens. In the study period multiple internal quality controls with various concentrations, have been used. The interassay coefficient of variation (CV) (mean±SD) was 11.3±4.9% for Aβ42, 9.3±1.5% for tau, and 9.4±2.5% for ptau. CSF Aβ40 levels were determined with an in-house ELISA, described elsewhere. CSF Aβ40 levels were determined in one run with a mean intra-assay CV of 2.1%, based on duplo CVs of the 86 CSF samples. Interassay CVs were calculated using MultiQC and were 1.3% for the high (11.0ug Aβ40/L) and 4.3% for the low (5.9ug Aβ40/L) standard sample. Plasma samples were analyzed for Aβ42 and Aβ40 using a multi-parameter fluorimetric bead-based immunoassay using xMAP® technology (INNO-BIA plasma AB forms, innogenetics NV, Ghent), according to the manufacturer instructions. Intra- and interassay coefficients were 6% and 8% for Aβ42 and 8% and 25% for Aβ40, respectively.

CSF and serum albumin concentrations were determined by nephelometry in a Beckman Coulter Immage 800 immunochemistry system, previously described in more detail. Serum and CSF samples were tested in the same run and results were expressed as mg/L for CSF and g/L for serum. Subsequently, to estimate BBB function, CSF/serum albumin ratios were calculated.

**Statistical analysis**

We used SPSS 15.0 for windows (Chicago, IL) for statistical analysis. Biochemical markers were log-transformed because they mostly did not have a normal distribution. Groups were compared with one-way ANOVA and post-hoc LSD analyses for continuous variables and with Chi-squared tests for categorical data. In an additional analysis we attempted to circumvent clinical diagnosis by creating subgroups based on MB distribution. Patients with MBs were divided in a strictly lobar and a non-lobar group, irrespective of clinical diagnosis, in order to study the influence of spatial distribution of MBs on Aß levels. We compared these two groups using ANOVAs with the biomarkers as outcome measures, first without adjustment and subsequently with age, sex, diagnosis and total MB number as covariates.

**Results**

Patient and MRI characteristics are shown in Table 1. The AD groups were well matched for age and sex. The other groups did not differ with respect to age and sex and level of education either. As a result of our patient sampling, all AD patients in the MB group had MBs, as opposed to subjective complainers and AD patients without MBs. Nine (82%) VaD patients had at least one MB. Prevalence of multiple MBs (2 or more) tended to be higher in VaD patients (73%, example Figure 1B) than in AD patients with MBs (42%, p=0.09; example Figure 1A). The regional distribution of MBs differed between VaD and AD. Strictly lobar MBs tended to be more frequent in AD patients with MBs than in VaD patients (65% versus 33%, p=0.09). Conversely, MBs in the basal ganglia were more frequent in VaD patients than in AD patients with MBs (36% versus 8%, p=0.03). No differences in MB prevalence for the infratentorial regions were found. Large vessel infarcts, WMH and lacunar burden were higher in the VaD group compared to all other groups. AD patients with MBs had more severe WMH than AD patients without MBs (p=0.05), but they did not differ regarding lacunar or large infarcts. Global cortical and medial temporal lobe atrophy were more severe in all groups with dementia versus controls.
CSF biomarkers differed between groups (all $p<0.05$). Raw data and results of post-hoc comparisons are shown in Table 2. AD patients with MBs had lower CSF levels of Aβ42, but not of Aβ40, than AD patients without MBs (Figure 2A and B). CSF Aβ42 levels in VaD patients did not differ from AD patients without MBs, but were lower than in controls. Furthermore, CSF Aβ40 levels were selectively decreased in VaD patients compared to all other groups. There were no differences in CSF Aβ40 between AD patients and controls. CSF levels of tau and p-tau were highest in AD patients, regardless of MB presence, but did not differ between VaD patients and controls.

Plasma levels of neither Aβ42 nor Aβ40 differed between groups ($p=0.22$ and $p=0.20$). Visually however, Aβ42 and Aβ40 appeared higher in VaD (Figure 2C and 2D) and direct comparison of VaD with controls using t-tests, showed trends for plasma Aβ42 ($p=0.08$) and Aβ40 ($p=0.06$).

There was a group difference in CSF/serum albumin ratio ($p=0.05$), attributable to a selectively increased ratio in the VaD group. CSF/serum albumin ratios were not elevated in AD patients, regardless of MB presence (Figure 2E). When these analyses were repeated with age and sex as covariates, results remained virtually unaltered.

Subsequently, we created new subgroups in patients with MBs only ($n=35$), based on MB distribution to circumvent the inherent arbitrariness of clinical diagnosis. Two groups were defined: a strictly lobar group with at least one strictly lobar MB ($n=20$) and a non-lobar group with one or more MBs in non-lobar regions (either strictly non-lobar or in combination with lobar MBs; $n=15$). Age, sex and MMSE did not differ between groups. The strictly lobar group tended to contain relatively more AD patients (65% versus 35%, $p=0.09$). Besides a higher prevalence of lacunar infarcts in the non-lobar MB group (53% versus 10%, $p=0.006$), MRI parameters did not differ. Patients with strictly lobar MBs had lower CSF Aβ42 levels than patients with non-lobar MBs ($p=0.003$; Table 3). No difference was found between groups regarding CSF Aβ40 ($p=0.17$), although on visual inspection levels appeared lower in the non-lobar group. Additionally, patients with strictly lobar MBs had higher levels of tau and ptau. In plasma, Aβ42 levels did not differ, but Aβ40 plasma levels were higher in the non-lobar group ($p<0.05$). Albumin ratios were non-significantly elevated in patients with non-lobar MBs ($p=0.11$). After adjustment for age, sex, and education level results remained essentially unaltered.

**Discussion**

When AD patients with and without MBs were compared, those with MBs had reduced CSF Aβ42 CSF levels, whereas CSF Aβ40, plasma amyloid levels and BBB function did not differ. Another pattern was observed in VaD patients with only modestly reduced Aβ42, but markedly lower Aβ40 CSF levels. Additionally, VaD patients showed a tendency towards elevated Aβ40 plasma levels, possibly related to BBB dysfunction, suggested by a modestly elevated CSF/serum albumin ratio. These findings imply that different vasculopathies are associated with specific types of MBs, which may also explain the preference of lobar MBs for AD and non-lobar MBs for VaD. Further support was provided by our additional analyses based on MB location, as strictly lobar MBs predisposed for lower levels of Aβ42 in CSF, while Aβ40 CSF levels and BBB function were unaltered. Conversely, non-lobar MBs were associated with increased Aβ40 levels in plasma.

The current finding of extremely low CSF Aβ42 levels in AD patients with MBs on 3T MRI, corroborates our previous finding, in a completely independent sample. In the current study however, this association was present in AD patients with any number of MBs, while in the previous proof-of-principle study only AD patients with many MBs (arbitrarily defined as 8 or more), were included. Based on the predominantly lobar MB pattern and high prevalence of CAA in AD, we hypothesize that CAA explains our findings in AD with MBs. Moreover, when defining a subgroup with a strictly lobar MB pattern, most likely reflecting CAA, we observed even lower CSF Aβ42 levels. These findings are in line with findings of decreased CSF Aβ42 levels in non-demented CAA patients compared to AD patients, supporting the hypothesis that this amyloid peptide is not only deposited in senile plaques, but also in CAA affected vessel walls. This notion was further support by findings of a recent study, in which coregistered
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Positron emission tomography (PET) with Pittsburgh Compound B (PiB) and MRI images were used to analyze the spatial relationship between CAA and MBs. Furthermore, high MB counts have been associated with thickened amyloid positive vessels on pathological examination. Given the fact that Aβ40 is predominantly found in vessel walls on autopsy, and the recent observation of low CSF Aβ40 levels in non-demented sporadic CAA patients, we also expected to find decreased CSF Aβ40 levels in AD patients with MBs. However, CSF Aβ40 levels were unaltered in these patients. Moreover, analysing patients with strictly lobar MBs only, maximizing the CAA likelihood, no abnormal Aβ40 levels were observed either. A possible explanation for the unchanged CSF Aβ40 levels of our AD patients with MBs may be that their MBs reflect an earlier stage, or milder form of CAA compared to the sporadic CAA patients presenting with large intracerebral hemorrhages (ICH), fulfilling the Boston criteria. This explanation is in line with pathological studies describing early CAA with vessels positive for Aβ42 but negative for Aβ40. An alternative explanation may be a different subtype of CAA, as it has been suggested that MBs and ICHs, observed in CAA patients, may arise from different pathomechanisms. A specific subtype of CAA, the capillary form, has been associated with AD pathology. In capillary CAA, more prominent involvement of Aβ42 deposits in pericapillary spaces or in the glia limitans has been reported, possibly leading to specifically decreased Aβ42. Taking all together, our data provide evidence for the notion that MBs in AD reflect underlying CAA, which may differ however from CAA as defined by the Boston criteria in patients primarily presenting with ICH.

The distribution of MBs differed between VaD and AD patients with MBs, implicating that different pathomechanisms are involved. VaD patients presented relatively more often with non-lobar MBs in the basal ganglia, not considered to be typical for CAA, but probably represent hypertensive vasculopathy. Nevertheless, low levels of CSF Aβ40 and Aβ42 were found in VaD patients, implicating that this group, biochemically, resembled sporadic CAA more closely than our AD patients with MBs did. Although CSF Aβ42 levels were decreased in our VaD group, mean tau and ptau levels were not elevated, in contrast to both AD patient groups in our sample, as expected. Notably however, three patients with a clinical diagnosis of VaD presented with strictly lobar MBs and they all showed an AD-like CSF profile, including increased tau and ptau CSF levels, suggesting mixed dementia or even misdiagnosis. Furthermore, we found that VaD patients, in addition to lowered CSF Aβ40 levels, tended to have higher Aβ40 plasma values. This may imply transport, or rather leakage across the BBB of these proteins, as elevated CSF/serum albumin ratios were observed in these patients. In contrast, neither plasma amyloid levels, nor albumin ratios in AD patients with MBs were elevated. Studies on BBB dysfunction in AD have been inconclusive. Varying types of concomitant vessel pathology in AD patients may explain this controversy. Additionally, the relatively young age of our AD patients may also contribute to their unaltered BBB function.

Among the limitations of the current study is the relatively small sample size, containing few VaD patients. Another limitation is the lack of pathological confirmation of the supposedly different microangiopathies and clinically diagnosed dementias, especially since these disease states tend to overlap. Furthermore, our hypothesis that all MBs in AD are CAA related, in contrast to MBs in VaD, may not be entirely correct, for example indicated by some VaD patients showing strictly lobar MBs. In attempt to address this issue, we repeated our analyses disregarding clinical diagnosis, based on the MB pattern (strictly lobar, most likely to reflect CAA etiology, versus non-lobar). In doing so, probably approaching the underlying vasculopathy more closely than analysing clinical diagnosis alone, we found further and more direct evidence of the relation between strictly lobar MBs and Aβ42 levels in CSF. In addition, the non-lobar MB group, more likely to be associated with ischemic small vessel disease, showed higher Aβ40 plasma levels, further supporting the notion of different pathomechanisms. Strictly lobar MBs in dementia patients seem to be related to cerebrovascular deposition of Aβ42, as seen in AD-related CAA. Non-lobar MBs in patients with dementia, on the other hand, seem to be more closely related to Aβ40 and BBB function alterations and more likely reflect underlying ischemic small vessel disease, as seen in VaD and mixed dementia.
Acknowledgements

J.D.C. Goos is supported by Stichting Dioraphte. The Alzheimer Center VUmc is supported by Alzheimer Nederland and Stichting VUmc fonds. The clinical database structure was developed with funding from Stichting Dioraphte. Argonde van Harten is acknowledged for collecting the stored samples. Sisi Durieux-Liu and Harry Twaalfhoven are acknowledged for performing CSF analyses. The kits for plasma amyloid beta were gently provided by Innogenetics NV, Ghent.

Table 1: Patient and MRI characteristics by diagnosis

<table>
<thead>
<tr>
<th>Variable</th>
<th>SMC Patients (n=22)</th>
<th>AD patients without MBs (n=26)</th>
<th>AD Patients with MBs (n=26)</th>
<th>VaD Patients (n=11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at first visit (y)</td>
<td>65±6</td>
<td>67±7</td>
<td>67±7</td>
<td>70±6</td>
</tr>
<tr>
<td>Sex, male</td>
<td>12(55%)</td>
<td>16(62%)</td>
<td>16(62%)</td>
<td>8(67%)</td>
</tr>
<tr>
<td>MMSE</td>
<td>28±2</td>
<td>21±5</td>
<td>19±5&lt;sup&gt;a,d&lt;/sup&gt;</td>
<td>23±4</td>
</tr>
<tr>
<td>Education level (1-7)∞</td>
<td>5.2±1.3</td>
<td>5.0±1.5</td>
<td>4.8±1.5</td>
<td>4.9±1.3</td>
</tr>
<tr>
<td>MB Prevalence</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 or more</td>
<td>0(0%)</td>
<td>0(0%)</td>
<td>26(100%)</td>
<td>9(82%)</td>
</tr>
<tr>
<td>2 or more</td>
<td>-</td>
<td>-</td>
<td>11(42%)</td>
<td>8(73%)</td>
</tr>
<tr>
<td>Strictly Lobar</td>
<td>-</td>
<td>-</td>
<td>17(65%)</td>
<td>3(33%)</td>
</tr>
<tr>
<td>Basal Ganglia</td>
<td>-</td>
<td>-</td>
<td>2(8%)</td>
<td>4(36%)&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>Brain Stem</td>
<td>-</td>
<td>-</td>
<td>3(12%)</td>
<td>2(18%)</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>-</td>
<td>-</td>
<td>7(27%)</td>
<td>4(36%)</td>
</tr>
<tr>
<td>MBs, number</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median (range)</td>
<td>-</td>
<td>-</td>
<td>1(1-71)</td>
<td>14(0-103)</td>
</tr>
<tr>
<td>WMH score</td>
<td>0</td>
<td>8(36%)</td>
<td>5(19%)</td>
<td>0(0%)</td>
</tr>
<tr>
<td>1</td>
<td>13(59%)</td>
<td>18(69%)</td>
<td>10(39%)</td>
<td>2(18%)</td>
</tr>
<tr>
<td>2</td>
<td>1(5%)</td>
<td>3(12%)</td>
<td>8(31%)</td>
<td>0(0%)</td>
</tr>
<tr>
<td>3</td>
<td>0(0%)</td>
<td>0(0%)</td>
<td>3(12%)</td>
<td>9(82%)*&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td>Lacunar infarct</td>
<td>0(0%)</td>
<td>1(4%)</td>
<td>3(12%)</td>
<td>9(82%)*&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>Large vessel infarct</td>
<td>0(0%)</td>
<td>0(0%)</td>
<td>0(0%)</td>
<td>4(36%)*&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>GCA score</td>
<td>0.5±0.4</td>
<td>1.3±0.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.2±0.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.0±0.6&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Mean MTA score</td>
<td>0.3±0.4</td>
<td>1.6±0.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.4±0.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.3±0.8&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Data are represented as number of patients with variable present, n(%), mean±sd, and for number of MBs as median [range]. Chi-squared tests and one-way ANOVA and LSD were performed respectively. MBs = microbleeds, SMC = patients with subjective memory complaints, AD = Alzheimer disease, VaD = Vascular dementia, MMSE = mini mental state examination, WMH= white matter hyperintensities, GCA= global cortical atrophy, MTA=medial temporal lobe atrophy. <sup>a</sup>compared to SMC, <sup>b</sup>compared to AD without MBs, <sup>c</sup>compared to AD patients with MBs, <sup>d</sup>compared to VaD. <sup>a,b,c</sup>posthoc p values<0.05, **p<0.01. ∞Level of education according to Verhage.
Table 2: CSF and blood measurements by patient group

<table>
<thead>
<tr>
<th>Laboratory variables</th>
<th>SMC Patients (n=22)</th>
<th>AD Patients without MBs (n=26)</th>
<th>AD Patients with MBs (n=26)</th>
<th>VaD Patients (n=11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSF</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aß42 pg/ml</td>
<td>851±244</td>
<td>550±212</td>
<td>429±119</td>
<td>565±252</td>
</tr>
<tr>
<td>Aß40 pg/ml</td>
<td>12024±2882</td>
<td>11569±3517</td>
<td>11043±3478</td>
<td>8451±2819</td>
</tr>
<tr>
<td>tau pg/ml</td>
<td>282±139</td>
<td>585±346</td>
<td>707±478</td>
<td>303±179</td>
</tr>
<tr>
<td>ptau pg/ml</td>
<td>52±17</td>
<td>87±51</td>
<td>103±58</td>
<td>51±25</td>
</tr>
<tr>
<td>Plasma</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aß42 pg/ml</td>
<td>35.3±10</td>
<td>34.4±7</td>
<td>34.4±6</td>
<td>40.6±8</td>
</tr>
<tr>
<td>Aß40 pg/ml</td>
<td>177.3±52</td>
<td>186.8±43</td>
<td>189.8±36</td>
<td>216.8±56</td>
</tr>
<tr>
<td>Albumin Ratio</td>
<td>5.7±3</td>
<td>7.4±4</td>
<td>6.7±3</td>
<td>10.6±8</td>
</tr>
</tbody>
</table>

Raw data are presented as mean±sd, statistics were performed with log-transformed values using one-way ANOVA and LSD. MBs = microbleeds, SMC = subjective memory complaints, AD = Alzheimer disease, VaD = Vascular dementia. a= compared to SMC, b= compared to AD without MBs, c= compared to AD patients with MBs, d= compared to VaD.

Table 3: CSF and blood measurements and their ratios by MB group

<table>
<thead>
<tr>
<th>Laboratory variables</th>
<th>strictly lobar MBs (n=20)</th>
<th>non-lobar MBs (n=15)</th>
<th>Unadjusted</th>
<th>Adjusted∞</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSF</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aß42 pg/ml</td>
<td>382±103</td>
<td>526±150</td>
<td>**</td>
<td>**</td>
</tr>
<tr>
<td>Aß40 pg/ml</td>
<td>10963±2991</td>
<td>9737±3993</td>
<td></td>
<td></td>
</tr>
<tr>
<td>tau pg/ml</td>
<td>652±253</td>
<td>557±634</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>ptau pg/ml</td>
<td>95±30</td>
<td>83±79</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>Plasma</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aß42 pg/ml</td>
<td>35.1±6</td>
<td>36.8±7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aß40 pg/ml</td>
<td>185±39</td>
<td>214.8±47</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>Albumin Ratio</td>
<td>6.5±3</td>
<td>9.1±6</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Raw data are presented as mean±sd, statistics were performed with log-transformed values using ANOVA. MB = cerebral microbleed. +p<0.1, *p<0.05, **p<0.01. Missing data: CSF Aß40 n=1, albumin ratio CSF/serum n=1. ∞: adjusted for sex, age and education.
Figure 1

A. 87-year old AD patient with multiple lobar MBs, sparing the basal ganglia.

B. 71-year old VaD patient with 3 MBs in the right thalamus, but no lobar MBs.

Figure 2

Scatterplots of biomarker results.

A. CSF levels of amyloid beta 1-42
B. CSF levels of amyloid beta 1-40
C. plasma levels of amyloid beta 1-42
D. plasma levels of amyloid beta 1-40
E. ratio of CSF/serum albumin

SMC = subjective memory complaints, AD = Alzheimer disease, MBs = Microbleeds, VaD = vascular dementia. *p<0.05, + = direct comparison with SMC p<0.1.
Literature


‘Our life is frittered away by detail. Simplify, simplify.’

Henry David Thoreau

NO EVIDENCE FOR ADDITIONAL BLOOD-BRAIN BARRIER P-GLYCOPROTEIN DYSFUNCTION IN ALZHEIMER’S DISEASE PATIENTS WITH MICROBLEEDS


Daniëlle M.E. van Assema1,2 MD, Jeroen D.C. Goos2 MD, Wiesje M. van der Flier2,3 PhD, Mark Lubberink1,4 PhD, Ronald Boellaard1 PhD, Albert D. Windhorst1 PhD, Philip Scheltens2 MD PhD, Adriaan A. Lammertsma1 PhD, Bart N.M. van Berckel1 MD PhD

1 Department of Nuclear Medicine & PET Research, VU University Medical Center, PO Box 7057, 1007 MB Amsterdam, The Netherlands
2 Department of Neurology & Alzheimer Center, VU University Medical Center, PO Box 7057, 1007 MB Amsterdam, The Netherlands
3 Department of Epidemiology & Biostatistics, VU University Medical Center, PO Box 7057, 1007 MB Amsterdam, The Netherlands
4 PET Centre, Uppsala University Hospital, 751 85 Uppsala, Sweden

Abstract

Decreased blood-brain barrier P-glycoprotein function has been shown in Alzheimer’s disease patients using positron emission tomography with the radiotracer (R)-[11C]verapamil. Decreased P-glycoprotein function has also been hypothesized to promote cerebral amyloid angiopathy development. Here, we used positron emission tomography and (R)-[11C]verapamil to assess P-glycoprotein function in eighteen Alzheimer’s disease patients, of which six had microbleeds, presumably reflecting underlying cerebral amyloid angiopathy. No differences were found in binding potential and non-specific volume of distribution of (R)-[11C]verapamil between patient groups. These results provide no evidence for additional P-glycoprotein dysfunction in Alzheimer’s disease patients with microbleeds.
Introduction

Pathological hallmarks of Alzheimer’s disease (AD) are fibrillary amyloid-beta depositions in brain parenchyma and amyloid-beta accumulation in cerebral blood vessel walls, known as cerebral amyloid angiopathy (CAA).\(^1\) CAA is present in nearly all AD brains, although severity amongst individuals varies strongly.\(^2\) Microbleeds (MBs), which can be observed using gradient echo weighted magnetic resonance imaging (MRI) supposedly reflect underlying CAA.\(^3\) Prevalence of MBs in AD is reported to be around 23\%.\(^4\)

The mechanisms behind intraparenchymal and intravascular amyloid-beta depositions in sporadic AD still are largely unknown. A widely held hypothesis states that impaired clearance of amyloid-beta from the brain is the main problem in AD.\(^5,\)\(^6\)

There are several pathways for clearance of amyloid-beta, including degradation by proteolytic enzymes, perivascular drainage pathways and active transport over the blood-brain barrier (BBB).\(^7\)

A major transporter at the BBB is P-glycoprotein (Pgp), a 170 kDa transmembrane protein, which is highly expressed at the endothelial cells that line the brain blood vessel walls. Pgp functions as an efflux transporter for a wide variety of substrates,\(^8\) and is involved in amyloid-beta transport.\(^9\) Recently, decreased BBB Pgp function in sporadic AD patients was found using positron emission tomography (PET) with the radiolabelled Pgp substrate \([\text{I}^-\text{C}^-]\text{verapamil}.\(^{10}\) Decreased Pgp function has also been hypothesized to promote CAA development.\(^11\)

To date, no studies have been performed to assess differences in BBB Pgp function between AD patients with and without signs of advanced CAA in the brain. The purpose of the present study was to investigate global and regional associations between MBs and Pgp function in AD patients.

Materials and Methods

Patients

Eighteen patients with probable AD in a mild to moderate disease stage (Mini-Mental State Examination (MMSE) scores ≥ 20) were included in this study. Six of these patients had MBs on brain MRI. Patients were recruited from the outpatient Memory Clinic of the Alzheimer Center of the VU University Medical Center (VUmc) in Amsterdam, where they received a standard dementia screening that included medical history, physical and neurological examinations, screening laboratory tests, and MRI. Diagnosis was established by consensus in a multidisciplinary meeting according to the criteria for probable AD as proposed by the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer’s Disease and Related Disorders Association. To confirm presence of AD pathology in the brain, increased cortical accumulation of \([\text{I}^-\text{C}^-]\text{PIB PET was required}.\(^{12}\) \([\text{I}^-\text{C}^-]\text{PIB PET scans were evaluated by an experienced nuclear medicine physician (BvB) and all classified into being PIB positive or PIB negative. All eighteen AD patients were PIB positive at visual assessment of the \([\text{I}^-\text{C}^-]\text{PIB PET scan. Exclusion criteria were major psychiatric or neurological disorders (other than AD), history of alcohol and/or drug abuse and use of medication that could possibly interfere with Pgp function.}\(^{13}\) The study was approved by the Medical Ethics Review Committee of the VUmc. Written informed consent was obtained from all patients after a complete written and verbal description of the study.

MRI

Patients underwent structural MRI scanning using a 1.5 T Sonata scanner (Siemens Medical Solutions, Erlangen, Germany). The scan protocol included a coronal T1-weighted 3-D MPRAGE (magnetization prepared rapid acquisition gradient echo), which was used for co-registration and region of interest (ROI) definition. To assess presence of MBs and superficial siderosis, which is also a common MRI finding associated with CAA,\(^{14}\) a susceptibility weighted imaging (SWI) sequence was performed in all patients.
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MBs were detected on the SWI sequence by an experienced neuroradiologist and defined as rounded, hypointense homogeneous foci up to 10 mm in size.

**PET**

PET scans were acquired using an ECAT EXACT HR+ scanner (Siemens/CTI, Knoxville, USA) and data were corrected for attenuation, randoms, dead time, scatter and decay. (R)-[11C]verapamil and [11C]PIB PET data acquisition and analysis were performed as described previously [Tolboom et al. 2010;van Assema et al. 2011]. In brief, after acquiring a 10 min transmission scan in 2D mode, a dynamic 60 min emission scan in 3D mode was started simultaneously with the intravenous injection of 340±37 MBq (R)-[11C]verapamil. During the scan, arterial blood was withdrawn continuously through a radial artery cannula and, at set times, manual samples were taken to obtain a metabolite corrected plasma input curve. Images were reconstructed using a standard filtered back projection (FBP) algorithm, applying a Hanning filter with a cut-off at 0.5 times the Nyquist frequency. A zoom factor of 2.123 and a matrix size of 256x256x63 were used, resulting in a voxel size of 1.2x1.2x2.4 mm and a spatial resolution of approximately 6.5 mm full width at half maximum at the centre of the field of view. Images were also reconstructed using a partial volume corrected ordered subset expectation maximization (PVC OSEM) reconstruction algorithm, reducing partial volume effects (PVE). Kinetic analysis was performed using nonlinear regression of the standard two-tissue compartment model, including a blood volume component, and fixing the non-specific volume of distribution (= K1/k2) to the mean whole brain grey matter value. Possible differences in K1/k2 ratio and distribution volume (VT) (K1/k2·(1+k3/k4)) were investigated. The non-displaceable binding potential (BPND) was used as outcome measure. These were obtained for the global cortical brain region, frontal, parietal, temporal and occipital regions, posterior and anterior cingulate cortices, medial temporal lobe and cerebellum.

**Statistical analysis**

Data are presented as mean ± standard deviation, unless otherwise stated. Statistical analysis was performed using SPSS version 15.0. Group differences were calculated using non-parametric Mann-Whitney U tests. A p-value < 0.05 was considered significant.

**Results**

Twelve of the 18 patients (of which five females) included did not have microbleeds or superficial siderosis on brain SWI MRI scan (AD MB- group), while six males had single or multiple MBs in the brain (AD MB+ group). In the AD MB+ group, three patients had a single lobar MB, the other three multiple lobar MBs (three, four and nine, respectively). The patient with nine MBs also had superficial siderosis. There were no significant differences between AD MB- and AD MB+ groups with respect to age, MMSE scores, injected dose and specific activity of (R)-[11C]verapamil (Table 1). As shown in Table 2, there were no significant differences in BPND between AD MB- and AD MB+ groups for any of the regions investigated. Results were essentially the same after PVE correction [data not shown]. Distribution volume (VT) did not differ between the AD MB- and AD MB+ group for any of the regions investigated (for the global cortical brain region: AD MB- 0.84±0.16; AD MB+ 0.89±0.17, p 0.35).

In addition, the non-specific volume of distribution (K1/k2 ratio) did not differ (for the global cortical brain region: AD MB- 0.27±0.08; AD MB+ 0.29±0.07, p 0.71) between the AD MB- and AD MB+ group for any of the regions investigated.

**Discussion**

No differences were found in BPND of (R)-[11C]verapamil between AD patients with MBs and those without. These results indicate that there is no evidence of additional Pgp dysfunction at the BBB in support of the hypothesis of additionally impaired Pgp function in AD MB+ patients compared with AD MB- patients. There may be multiple explanations for these findings. First, it is possible that decreased Pgp function in AD MB+ patients could not be demonstrated because of a lack of power due to small sample size of groups. Second, it could be due to the inclusion criteria used in this study. Autopsy studies have shown that nearly all AD patients show some degree of vascular amyloid-beta. Still, only a minority of AD patients shows signs of CAA on MRI such as microbleeds or superficial siderosis during life. It is possible that...
only patients with severe CAA show MBs on MRI, but conclusive evidence is missing. An alternative explanation would be that severity of CAA pathology is only weakly related to the presence and number of MBs on MRI. In addition, it is also possible that additional Pgp dysfunction does occur, but at a more locoregional level, e.g. directly around MB locations, which would be beyond the spatial resolution of PET. However, it is also possible that AD MB+ and AD MB- patients really do not differ from each other in terms of Pgp function, since almost all AD patients do have some degree of CAA and all have amyloid-beta accumulation in the brain. Because both groups suffer from the same pathology, which very likely is present in the brain for several years or even decades before clinical symptoms occur, the possible destructive effects of these pathological processes will have taken place already. In a previous study, decreased Pgp function in AD patients compared with age-matched healthy controls was demonstrated. The order of events regarding Pgp dysfunction and amyloid deposition has not been unravelled yet. BBB Pgp dysfunction could contribute to accumulation of intraparenchymal and intravascular amyloid, while, on the other hand, Pgp dysfunction could also be the result of these amyloid depositions having a destructive effect on the blood vessel walls.

Negative findings were not due to different patterns of atrophy between groups, as volumes of the various cortical brain regions, including the global cortical brain region, did not differ between groups (for the global cortical region: AD MB- 192±24 mL; AD MB+ 193±17 mL, p 0.85). Additionally, applying a PVE correction did not alter results. A limitation of this study is the small sample size of the two groups. Strengths are the full dynamic scanning and quantitative kinetic modelling procedures. This is the first study that directly compares in vivo BBB Pgp function between AD patients with and without CAA characteristics, showing that Pgp function is comparable in AD patients with and without microbleeds.

**Acknowledgements**

The authors would like to thank the PET radiochemistry and technology staff of the Department of Nuclear Medicine & PET Research for tracer production and acquisition of PET data, and the technology staff of the Department of Radiology for acquisition of MRI data.

The research leading to these results has received funding from the European Community’s Seventh Framework Programme [FP7/2007-2013] under grant agreement n° 201380.

**Table 1. Characteristics of patient groups.**

<table>
<thead>
<tr>
<th></th>
<th>AD MB-</th>
<th>AD MB+</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>12</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Male/Female (% male)</td>
<td>7/5 (58%)</td>
<td>6/0 (100%)</td>
<td>0.07</td>
</tr>
<tr>
<td>Age (years)</td>
<td>63 ± 8</td>
<td>66 ± 2</td>
<td>0.12</td>
</tr>
<tr>
<td>MMSE</td>
<td>23 ± 3</td>
<td>23 ± 3</td>
<td>0.81</td>
</tr>
<tr>
<td>Injected dose (MBq)</td>
<td>348 ± 30</td>
<td>324 ± 48</td>
<td>0.35</td>
</tr>
<tr>
<td>Specific activity (GBq/μmol)</td>
<td>60 ± 36</td>
<td>51 ± 34</td>
<td>0.45</td>
</tr>
</tbody>
</table>

Group differences were calculated using Mann-Whitney U tests.

AD MB- = Alzheimer’s disease patient group without microbleeds
AD MB+ = Alzheimer’s disease patient group with microbleeds
MMSE = Mini-Mental State Examination
Injected dose = injected dose of [R]-[11C]verapamil
Specific activity = specific activity of [R]-[11C]verapamil
### Table 2. Binding potential (BP_{ND}) of (R)-[11C]verapamil for the several cortical brain regions.

<table>
<thead>
<tr>
<th>Region</th>
<th>AD MB-</th>
<th>AD MB+</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Global</td>
<td>2.15 ± 0.34</td>
<td>2.20 ± 0.34</td>
<td>0.85</td>
</tr>
<tr>
<td>Frontal</td>
<td>2.10 ± 0.33</td>
<td>2.15 ± 0.42</td>
<td>0.85</td>
</tr>
<tr>
<td>Parietal</td>
<td>2.15 ± 0.36</td>
<td>2.16 ± 0.33</td>
<td>0.78</td>
</tr>
<tr>
<td>Temporal</td>
<td>2.23 ± 0.39</td>
<td>2.31 ± 0.35</td>
<td>0.78</td>
</tr>
<tr>
<td>Occipital</td>
<td>2.20 ± 0.33</td>
<td>2.23 ± 0.27</td>
<td>0.51</td>
</tr>
<tr>
<td>Posterior Cingulate</td>
<td>2.12 ± 0.44</td>
<td>2.17 ± 0.44</td>
<td>0.51</td>
</tr>
<tr>
<td>Anterior Cingulate</td>
<td>2.14 ± 0.36</td>
<td>1.95 ± 0.42</td>
<td>0.45</td>
</tr>
<tr>
<td>Medial Temporal Lobe</td>
<td>2.82 ± 0.48</td>
<td>3.12 ± 0.52</td>
<td>0.30</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>2.03 ± 0.27</td>
<td>1.94 ± 0.28</td>
<td>0.51</td>
</tr>
</tbody>
</table>

Group differences were calculated using Mann-Whitney U tests.

AD MB- = Alzheimer’s disease patient group without microbleeds
AD MB+ = Alzheimer’s disease patient group with microbleeds
BP_{ND} = binding potential of (R)-[11C]verapamil

### Literature

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2010;81:882-884


‘We are each an experiment of one.’
George Sheehan

DEMENTIA WITH MICROBLEEDS AND TRANSIENT NEUROLOGICAL EPISODES: A CASE REPORT OF CAPILLARY CAA

Submitted

Authors: Jeroen DC Goos MD\textsuperscript{1,2}, Anna Carrano\textsuperscript{3}, Jeroen J.M. Hoozemans PhD\textsuperscript{3}, Frederik Barkhof MD PhD\textsuperscript{1,4}, Philip Scheltens MD PhD\textsuperscript{1,2}, Charlotte E. Teunissen PhD\textsuperscript{1,5}, Wiesje M van der Flier PhD\textsuperscript{1,2,4}, Yolande AL Pijnenburg MD PhD\textsuperscript{1,2} and Annemieke M Rozemuller MD PhD\textsuperscript{3}.

Institutional affiliations: From the \textsuperscript{1}Alzheimer Center, \textsuperscript{2}Department of Neurology, \textsuperscript{3}Department of Pathology, \textsuperscript{4}Department of Radiology, \textsuperscript{5}Department of Clinical Chemistry, \textsuperscript{6}Department of Epidemiology and Biostatistics, Neuroscience Campus Amsterdam, VU University Medical Center, Amsterdam, the Netherlands.
Case Presentation

In January 2005 a 62-year old male patient was referred to our memory clinic for a second opinion regarding a rapidly progressive dementia with acute episodes of deterioration, suspected of possible Alzheimer disease (AD) or vascular dementia (VaD).

In the preceding 3 years the patient had shown a minimal and gradual decline of initiative and emotional responsiveness. The past 6 months however, he had experienced 7 heterogeneous episodes with focal symptoms, but also more generalized behavioural and cognitive and disturbances, that all resolved within hours. Once, right-sided weakness was observed, while another episode was characterized by language and planning difficulties. In other episodes jerking of the head and neck was observed, as were headaches or vegetative symptoms (i.e. paleness and sweating). Paralleling the onset of these episodes, the patient also showed rapidly progressive cognitive decline, with memory and language difficulties and disorientation in time and place. He could no longer independently operate the telephone, or make coffee. His apathy also further increased.

The patient’s medical history reported chronic obstructive pulmonary disease. He had recently been prescribed acetylsalicylic acid and simvastatin for suspected strokes, salbutamol and formoterol for his respiratory complaints, paroxetine for presumed depression and rivastigmine for possible AD. He smoked 7 cigarettes and used up to 3 alcoholic consumptions per day. His maternal grandmother suffered from dementia before the age of 65. Dementia after 80 years of age occurred in his mother’s brother. The patient’s mother however, showed no signs of dementia as she died aged 74 from a cerebral tumour. His mother and daughter both suffered from migraine. He had followed higher professional training in biochemistry and worked as a chemist until 7 years ago. He was married with two children.

Neurologic Examination

On general physical examination, his blood pressure was 170/90 mmHg and his pulse regular at 52/minute. Neurological examination of this right-handed man revealed no abnormalities, apart from symmetrically reduced ankle jerks. In particular, there were no focal, or extrapyramidal signs, pathological reflexes or neglect phenomena. On cognitive and behavioural examination, he displayed restlessness and behavioural disinhibition, for example speaking very loud. He demonstrated little, but fluent, spontaneous speech with common semantic paraphasias. His episodic memory, temporary and spatial orientation and executive functioning were impaired. He also had mild apraxia and visuospatial dysfunctioning. His insight was impaired. He scored 14/30 on the MMSE. Baseline neuropsychological evaluation confirmed the observed impairments in memory, language, executive and visuospatial domains.

Investigations

Blood tests were unremarkable. MRI of the brain showed ventricular enlargement, notably without cortical or hippocampal atrophy. T2-weighted and FLAIR images showed beginning confluent white matter hyperintensities (WMH), likely of vascular origin, almost symmetrically distributed periventricular and throughout the deep white matter, also involving the anterior temporal lobes. Furthermore, WMH also extended towards the cortex, involving the U-fibers (figure 1A). Most remarkable however, were prominent abnormalities on T2*-weighted images, consisting of hundreds of punctuate hypointensities, or cerebral microbleeds (MBs), affecting all lobes, with a slight posterior predominance (figure 1B), sparing the basal ganglia and brainstem. No large intracerebral hemorrhages (ICHs) and large or small vessel infarcts were observed. In retrospect, the MRI data additionally revealed subtle signs of vascular edema, with effacement of the sulcal pattern, possibly due to leptomeningeal effusion, with or without additional swelling, appearing more prominent in the posterior lobes (figure 1A and 1C). EEG showed signs of diffuse and focal encephalopathy, with a dominant posterior frequency of 6.5-7Hz, with decreased responsiveness to eye-opening. Focal abnormalities, more prominent in the left parieto-temporal region, consisted of intermittent irregular delta activity, with sharp waves and biphasic waves, considered suggestive for a vascular origin. No specific epileptiform activity or triphasic wave complexes were seen.
Diagnostic considerations consisted of vascular dementia (VaD), cerebral autosomal dominant/recessive arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL/CARASIL), or vasculitis. Also metabolic, infectious, and (para)neoplastic causes were considered. Due to the rapid decline, Creutzfeldt Jakob Disease (CJD) was also considered. Over the next few months, he showed further decline and recurrent episodes. Therefore, EEG was repeated but remained unchanged. To further differentiate between CJD, AD and infectious causes, we performed a lumbar puncture. CSF examination revealed a normal cell count and slightly elevated total protein (0.97 g/l). HIV, borreliosis and lues antibody titers were negative, while CSF amyloid beta 1-42 levels were lowered (<500 pg/ml), however, total tau, ptau and the 14-3-3 protein were all normal. No APP (CAA) or NOTCH-3 (CADASIL) mutations were found on genetic testing.

After a few months, the patient was admitted to daycare and further declined cognitively (MMSE=11/30), despite rivastigmine treatment, with multiple recurring episodes. In June 2005 the patient was institutionalized to recover from a right-sided hemiplegia after an assumed ischemic stroke. He subsequently developed myoclonic jerks. In 2008 he developed contractures, urinary incontinence and severe cognitive impairment. He died from pneumonia in January 2011, aged 68 years.

Neuropathologic Findings
At macroscopy, the brain weight was 1500g, with a symmetrical appearance, without significant cortical and hippocampal atrophy, but markedly enlarged ventricles, most severely parieto-occipitally, with evidence of white matter loss. The circle of Willis was intact, with only moderate atherosclerosis. At microscopy, we observed widespread and severe amyloid angiopathy in all lobar cortical regions, with extensive capillary involvement and prominent dyshoric changes without classic senile plaques (Figures 2A-C). The dyshoric changes were generally accompanied by tau pathology, which was absent in the hippocampus (overall Braak stage 2). Frequently, these dyshoric changes were associated with reactive astrocytes and increased presence of microglia, but lymphocytic activation was not observed. A few recent hemorrhagic microinfarcts were found (Figures 2D-F), more frequently however, remnants of old strictly cortical microinfarcts (all <2mm) with and without iron deposition in macrophages were observed (Figures 2G-I). Many iron deposits surrounding vessels were observed in all cortical regions, however, many did not relate to vascular amyloid (Figure 2G, H). Alpha synucleinopathy was limited (Braak stage 1-2) and Lewy bodies were absent in the cortex. A neuropathological diagnosis of capillary CAA, with dyshoric changes and multiple cortical (hemorrhagic) microinfarcts was made.

Discussion
To our knowledge, this is the first case of rapidly progressive dementia accompanied by transient focal and more generalized neurological episodes, with multiple lobar MBs on MRI, which was confirmed to be capillary CAA (capCAA) with dyshoric changes at neuropathologic examination.

Transient focal neurological episodes, or “amyloid spells” are clinical CAA related phenomena, present in 15% of CAA patients. The symptoms may be positive and present as aura-like phenomena, or as partial motor seizure-like episodes, (i.e. limb shaking), but negative focal, TIA-like, symptoms have also been described. Typically, these episodes last several minutes and are stereotyped. They may relate to superficial siderosis, although MBs on MRI have also been hypothesized to play a role. Notably, our patient had no siderosis, but showed innumerable MBs on MRI. The exact pathophysiologic mechanism (seizure-like activity, spreading cortical depression or directly amyloid or ischemic/bleeding related) remains unclear however. In a stroke cohort studying CAA patients, the focal episodes were associated with subsequent intracerebral hemorrhage (ICH). Our patient clinically differed from those patients by the nature and duration of his episodes and a notable absence of any ICHs during his entire course.

Our autopsy findings may support a relationship between the transient symptoms with cognitive decline and the extensive capCAA with dyshoric changes. We hypothesized previously, that amyloid seemingly extending from capillaries into the neuropil...
Microbleeds in Dementia: Connecting the dots

Amyloid-lowering trials are also related to capCAA, supported by their shared overrepresentation of APOE ε4 homozygosity.[13] Possibly, the MBs observed at MRI also resulted from the capCAA, although we did not perform direct post-mortem correlation and arterioles were also affected. Additionally, the microinfarcts we observed in this case may not be typical of dyshoric capCAA.[4] We found both recent and old microinfarcts with and without hemorrhage. Intriguingly, recent hemorrhagic microinfarcts were frequently associated with amyloid positive vessels, but not with increased activation of astrocytes or microglia, or iron deposits. Conversely, older microinfarcts commonly showed less amyloid and more iron deposits in macrophages, with increased presence of activated microglia and astrocytes. These findings may suggest that microinfarcts or MBs are associated with inflammatory responses that may promote amyloid removal. Interestingly, in amyloid-lowering trials, it has been found that regions with radiologic signs of exaggerated immune responses, i.e. vasogenic edema and MBs on MRI, supposedly due to temporary “leaky vessels”, possibly by an effector cell/microglia-mediated mechanism, showed the most amyloid clearance.[14, 15]

In AD patients, MBs have been associated with decreased CSF levels.[16, 17] In this case, amyloid plaques were virtually absent. Therefore, his CSF abnormalities likely reflected the observed abundance of amyloid in vessels and capillaries. This neuropathologic evidence, further supports the notion that additional CSF amyloid abnormalities in AD patients with MBs may rather reflect deposition in vessels than plaques.[17]

Earlier, we observed that AD patients with multiple MBs had lower MMSE scores and were twice as often APOE ε4 homozygotes, strongly associated with capCAA, especially with dyshoric changes.[4] compared to AD patients without MBs.[16] Furthermore, post-mortem case series have associated capCAA with rapidly progressing dementia, lacking ante-mortem imaging however.[4, 18] Taken together, findings from this clinico-pathological study may suggest that demented patients, especially with many MBs and rapid decline, suffer from concomitant capillary or dyshoric CAA rather than large vessel CAA only.
To conclude, an atypical dementia presenting with multiple lobar MBs, without ICHs, or AD-like atrophy, may identify underlying capCAA (with dyshoric changes), which may be further supported by a CSF profile with amyloid, but no tau changes. CapCAA may also present with inflammation, but signs of vasogenic edema may be diffuse and relatively subtle.

**Acknowledgements**

The patient’s wife, Dr. Vroegindeweij and dr. van Eck are cordially acknowledged for providing additional clinical information and their cooperation. Research of the VUmc Alzheimercenter is part of the neurodegeneration research program of the Neuroscience Campus Amsterdam. The Alzheimer Center VUmc is supported by Alzheimer Nederland and Stichting VUmc fonds. The clinical database structure was developed with funding from Stichting Dioraphte.

**Figure 1**  
A: ante-mortem MRI findings  
Axial fluid-attenuated inversion recovery (FLAIR) image, showing subtle leptomeningeal signal increase due to effusion (arrows), with effacement of sulcal pattern (encircled), compared to frontal regions were preserved normal sulci with low signal. Axial T2*-weighted gradient recalled echo MRI at a comparable level as (A), showing numerous microbleeds in all lobar regions, with a preference for the posterior lobes. Microbleeds spared the basal ganglia and brain stem, suggestive for cerebral amyloid angiopathy. (C) FLAIR image of our patient one day after presentation at our memory clinic, showing asymmetric juxtacortical WMH in the left hemisphere, with possible leptomeningeal effusion.
Microbleeds in Dementia: Connecting the dots

Figure 2

Neuropathological examination by immunohistochemical analysis.

- Figure A: Immunohistochemical staining for amyloid beta (brown) showing small vessels and capillaries with dense staining of the vessel walls and extensive dystrophic changes in the absence of significant plaque load.
- Figure B: Congo red staining of an adjacent slice (of A&C), showing (pinkish/red) congophilic vessels.
- Figure C: Perl’s Prussian blue iron staining, showing intracellular mild iron deposition (light blue) surrounding the congophilic vessels.
- Figure D: Amyloid beta (Abeta) staining (brown) showing Abeta in and surrounding the vessel.
- Figure E: Congo red staining of an adjacent slice (of D&F), showing congophilic vessel walls (pinkish/red), with intra- and extravascular erythrocytes.
- Figure F: Perl’s Prussian blue iron staining, showing no signs of iron deposits (lack of blue) surrounding the lesion. Extravasations of erythrocytes can be observed, indicative of a recent hemorrhage.
- Figure G: Amyloid beta (Abeta) staining showing no presence of Abeta in or near the vessel. The vessel is surrounded by pigmented macrophages.
- Figure H: Congo red staining of an adjacent slice (of G&I), showing no congophilic vessel walls (pinkish/red). Pigment macrophages can be observed. I) Perl’s Prussian blue iron staining, showing increased iron deposition (blue) in the pigmented macrophages associated with the lesion.

Scale bars represent 100 micrometer.

Literature

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CHAPTER 04 - PART III

DEVELOPMENTS IN IMAGING SIGNS OF MICROANGIOPATHY

‘Method is much, technique is much, but inspiration is even more.’
Benjamin Cardozo

PATIENT AND RADIOLOGICAL CHARACTERISTICS ASSOCIATED WITH IMPROVED MB DETECTION USING SUSCEPTIBILITY-WEIGHTED IMAGING.


Authors: JDC Goos MD¹, WM van der Flier PhD¹,², DL Knol PhD², PJW Pouwels PhD³, P Scheltens MD PhD¹, F Barkhof MD PhD¹ and MP Wattjes MD⁴.

Institutional affiliations: From the Alzheimer Center and ¹Department of Neurology, ²Department of Epidemiology and Biostatistics, ³Department of Physics and Medical Technology and ⁴Department of Radiology, from the VU University Medical Center, Amsterdam, the Netherlands.
Abstract

**Background:** Susceptibility-weighted imaging (SWI) has been shown to be more sensitive in detecting cerebral microbleeds (MBs) than conventional T2*-weighted gradient recalled echo imaging (GRE). However, clinical relevance of this improved detection in terms of associations with clinical measures and risk factors is unclear. **Objective:** To investigate if associations of MBs with clinical characteristics, risk factors, white matter hyperintensities (WMH) and lacunes were different using GRE or SWI in memory clinic patients. **Methods:** 141 patients presenting at our memory clinic were included and underwent clinical evaluation and an MRI protocol including both GRE and SWI. Images were analyzed for number and location of MBs and WMH. In a subset of patients APOE was determined. Negative binomial regression was used to assess clinical and radiological associations with MB number. **Results:** MB prevalence was 23% with GRE and 40% with SWI. A total of 219 and 284 MBs were detected on GRE and SWI, respectively. Within groups with MBs, median MB count was 1 (range 1-144) on GRE and 2(1-129) on SWI (p<0.001). The increase of MBs on SWI was equally distributed among brain regions. Strengths of the associations with age, sex, WMH and presence of lacunes with higher MB numbers were comparable for GRE and SWI (all p<0.05); no differential independent associations were detected. **Conclusion:** SWI detected more MBs in more patients, irrespective of MB location. However, this enhanced detection hardly improved the association with vascular risk factors or radiologic markers of small vessel disease.

Introduction

Microbleeds (MBs), seen on T2*-weighted gradient-recalled echo (GRE) magnetic resonance imaging (MRI) are small rounded dotlike hypointense foci. Histologically, MBs represent hemosiderin, likely occurring from leakage through small cerebral vessels, contained by surrounding macrophages in the brain parenchyma. Clinically, MBs are associated with hypertension, signs of small vessel disease, ischemic and moreover hemorrhagic stroke, cognitive decline and mortality in different populations. However, these associations have not been conclusively found across all studies, probably partly due to differences in scanning techniques.

Technical developments such as new imaging sequences and higher magnetic field strengths have improved MB detection on MRI in recent years. The recently introduced sequence technique of Susceptibility-Weighted Imaging (SWI) is increasingly being used in the clinical routine setting and maximizes sensitivity to susceptibility effects by combining a long TE and fully flow-compensated 3D gradient-echo sequence. Furthermore, it uses filtered phase information to enhance the contrast in magnitude images and add a new source of information, i.e., difference in susceptibility between tissues. As a result, MBs are more sensitively detected by SWI compared to GRE.

A recent study showed that all hypointense lesions visible on post-mortem SWI corresponded to angiopathy related abnormalities (most commonly acute microhemorrhage, hemosiderin residua of old hemorrhages, and small lacunes ringed by hemosiderin). However, data dealing with the clinical relevance of this improved MB detection in terms of associations with clinical outcome measures and risk factors are rather limited. On MRI, MBs are associated with radiological signs of small vessel disease, white matter hyperintensities (WMH) and lacunar infarcts. Clinically, MBs have been quite consistently associated with the vascular risk factors, higher age and chronic hypertension. Apart from hypertensive vasculopathy, MBs are associated with cerebral amyloid angiopathy (CAA). Although both microangiopathies probably occur frequently in a memory clinic population, CAA has been found in the vast majority of AD patients and may play an important role in our population. As a result, MBs are commonly detected in memory clinic patients.
In this population, MBs have been associated with mortality. The relationship of MBs with cognition in Alzheimer disease (AD) however, has not yet been determined. Nevertheless, when MBs are numerous in AD patients they may contribute to cognitive decline, similar to patients with vascular dementia and stroke.

In this study, we aimed to investigate if the associations of MBs with clinical characteristics, risk factors, and associated MRI changes were different between GRE and SWI in a memory clinic population.

**Methods**

**Patient population**

From November 2007 to September 2008 a total number of 156 consecutive patients presenting at our memory clinic received a 1.5T-MRI scan including both GRE en SWI. Of these patients, 15 patients were excluded because of missing scans or scans of unacceptable quality for one or both sequences. This resulted in a total number of 141 patients with both GRE and SWI sequences of acceptable quality (mean age 62±9, 57% male).

All patients underwent standardized dementia screening including medical history, physical, neurological, neuropsychological examination and MRI. Dementia severity was assessed using the Mini-Mental State Examination (MMSE). Patients were considered as having arterial hypertension, diabetes mellitus, and hypercholesterolemia if they had a known history of the disease or were receiving drug treatment. Furthermore, screening involved routine laboratory examinations. Diagnoses were made in a multidisciplinary consensus meeting. Diagnoses of probable AD (n=49) diagnosis were made according to the clinical criteria of the National Institute of Neurological Disorders and Stroke-Alzheimer’s Disease and Related Disorders Association (NINCDS-ADRDA), and a diagnosis of Mild Cognitive Impairment (MCI) (n=16) was based on Petersen criteria. When all clinical investigations were normal, patients were considered to have subjective complaints (n=20). The subgroup of other dementia (n=18) included various diagnoses such as frontotemporal lobar degeneration (FTLD) (n=7), dementia with Lewy bodies (DLB) (n=5), and other neurodegenerative disorders (n=6). The subgroup of other disorders (n=38) included patients with other neurologic disorders (including stroke) (n=9), psychiatric disorders (n=18), and unclear diagnoses (n=11). The study was approved by the ethical review board of the VUMC Amsterdam and all subjects gave written informed consent for their clinical data to be used for research purposes.

**MRI protocol**

MRI was performed on a 1.5T whole-body MRI system (Sonata Syngo, Siemens Medical Systems, Erlangen, Germany), with an eight channel phased-array head coil. The imaging protocol included the following pulse sequences: (1) axial T2*-weighted GRE (21 slices, field of view (FOV) 250mm, voxel size in-plane: 1x1mm, slice thickness: 5mm, interslice gap: 1.5mm, repetition time (TR): 415ms, echotime (TE): 25ms, flip angle 15 degrees); (2) axial SWI (44 slices per slab, FOV 250mm, voxel size 1x1x2mm, TE 40ms, TR 48ms, flip angle 15°). SWI images were constructed, by multiplying magnitude images with filtered phase images to enhance the susceptibility effect and then performing a minimum intensity projection (mIP) reconstruction with a slice thickness of 6mm and an interslice gap of 2mm. (3) coronal T1-weighted three-dimensional magnetization-prepared rapid acquisition gradient-echo volumes (MPRAGE; single slab, 176 sections; voxel size 1x1x1.5mm; TR 2700ms, TE 5.2ms, inversion time (TI) 950ms; flip angle 8°); (4) axial 2D FLAIR (Fluid Attenuated Inversion Recovery; 42 slices, in-plane voxel size 1x1 mm, slice thickness 5mm, interslice gap 1.5mm, TE 108ms, TR 9000ms, TI 2500ms); (5) axial T2-weighted turbo spin echo (23 slices, voxel size in-plane: 0.6x0.6 mm, slice thickness 5mm, interslice gap 1.5mm, TE 114ms, TR 4590ms).

**Image analysis**

MBs were rated by one observer. MBs were defined as rounded hypointense homogeneous foci up to 10mm in size on GRE and SWI sequences. Lesions in sulci probably representing flow voids from vessels and in the globus pallidus, supposedly representing iron or calcium deposits were not considered. Choroid plexus and pineal calcifications were also not considered, as were lesions suggestive of partial volume effects.
Microbleeds in Dementia: Connecting the dots

Results

Microbleed detection

The inter-rater agreement concerning the detection of MBs was excellent on both modalities, which is reflected by weighted Cohen’s kappas for the three observers of at least 0.82 for MBs on GRE and 0.87 on SWI. Intra-rater agreement was also excellent with weighted Cohen’s kappa of 0.83 for GRE and 0.86 for SWI.

In 32 (23%) patients, one or more MBs were found on conventional GRE imaging. On SWI, at least one MB was detected in 56 (40%) patients. A total of 219 MBs and 284 MBs were detected on GRE and SWI respectively. In the groups with MBs, the median MB count was 1 (1-144) on GRE and 2 (1-129) on SWI (Wilcoxon signed rank test p<0.001). In 43 patients, more MBs were detected on SWI than on GRE (example figure 1), equal numbers of MBs were detected in 90 patients, and in 8 patients more MBs were detected on GRE (example figure 2) than on SWI. The overall increase of MBs on SWI was equally distributed among all lobes and non-lobar regions (data not shown). Multiple strictly lobar MBs were detected in 6% of patients using GRE and 14% using SWI (p<0.01).

Statistical analysis

For statistical analysis SPSS 15.0 for windows (Chicago, IL, USA) and Stata version 11 (StataCorp, College Station, TX, USA) were used. The degree of agreement was defined according to the method of Landis and Koch using weighted Cohen’s kappas,35 as follows: slight agreement, the kappa value ranged from 0.00 to 0.20; fair agreement, the kappa value ranged from 0.21 to 0.40; moderate agreement, the kappa value ranged from 0.41 to 0.60; substantial agreement, the kappa value ranged from 0.61 to 0.80; and excellent agreement, the kappa value ranged from 0.81 to 1.00. Categorical data were analysed by Chi-squared tests. Comparison between groups for continuous variables was executed by Student t-tests or Mann-Whitney tests when appropriate. For the difference in MB detection between the two sequences Wilcoxon signed rank tests were used. Negative binomial regression was used to investigate associations between number of MBs [dependent variable] and different clinical and imaging variables [independent variables], to account for the non-normal distribution of MBs with an over-representation of zero values. Analyses were executed for both sequences separately. In a second model, the negative binomial regression analysis was adjusted for age and sex. Negative binomial regression data are represented as negative binomial regression coefficients and their 95% confidence intervals. These coefficients are multiplicative effect estimates of the variable of interest per unit increase. Statistical significance was set at p<0.05.

In addition, white matter hyperintensities (WMH) were visually assessed based on the FLAIR sequence according to the modified Fazekas rating scale.34 The scale ranges from 0 to 3 (none, punctuate, early confluent and confluent). Furthermore, the presence of large vessel and lacunar infarcts was assessed. Large vessel infarcts were rated as present or absent, based on hyperintensity of the lesion on both FLAIR and T2-weighted sequences. Lacunar infarcts were defined as well demarcated lesions from 3 to 15mm, with cerebrospinal fluid-like signal on all sequences.

The assessment of MBs was performed according to their anatomical location [lobar or non-lobar]. Lobar MBs were allocated to one of four lobes: frontal, parietal, occipital and temporal. MBs in the basal ganglia including the thalamus were scored as non-lobar. Patients with multiple strictly lobar MBs were considered as probable CAA patients, in analogy to the Boston criteria.33 Due to the different coverage of scanning between the two sequences at the brain stem level, infratentorial MBs were not taken into account. In a first step, MBs of 20 patients were assessed and counted on both sequences blinded to clinical data of interest, by 3 observers with different levels of experience with MR imaging [reader 1, J.D.C.G. [2 years experience]; reader 2 M.P.W. [9 years experience]; reader 3, F.B. [22 years experience]] for inter-observer reliability purposes. All raters were blinded for any clinical and paraclinical information. First, all GRE images were randomly presented and analyzed. Secondly, SWI images were rated blinded for MB scores on GRE. After more than two months, a MB recount of those 20 scans was performed by reader 1 (J.D.C.G.), to assess intra-observer reliability. Subsequently, the remaining scans were rated by a single rater (J.D.C.G.) in the same fashion.

The inter-rater agreement concerning the detection of MBs was excellent on both modalities, which is reflected by weighted Cohen’s kappas for the three observers of at least 0.82 for MBs on GRE and 0.87 on SWI. Intra-rater agreement was also excellent with weighted Cohen’s kappa of 0.83 for GRE and 0.86 for SWI.
**Demographical, clinical, laboratory and radiological associations**

We assessed the univariate associations of MB prevalence for both sequences with patient, clinical, laboratory and radiological characteristics (table 1). On GRE, age, sex and diagnosis were not associated with MB prevalence. Regarding medical history, only diabetes mellitus was found to be less frequent in patients with MBs (p=0.04). MB presence on GRE was not associated with statins, anticoagulant, platelet inhibitor or alcohol use, smoking status, MMSE or APOE status. Regarding other MRI characteristics, only lacunar infarctions were more frequent in patients with MBs present than patients without MBs on GRE (p=0.002).

Using SWI, age was associated with presence of MBs (p<0.001). In agreement with GRE, diagnosis and sex were not associated with MB prevalence. Medical histories were similar for patients with and without MBs, except for hypercholesterolemia, which was more frequent among patients with MBs on SWI. In contrast to GRE, there was no difference in prevalence of diabetes according to presence of MBs detected on SWI. Groups did not differ regarding statin or anticoagulant use, antiplatelet use however, was more frequent in the group with MBs (p<0.05). Similar to GRE, MMSE and APOE status were not associated with MB presence. In agreement with GRE, lacunar infarcts were more frequent in patients with MBs (p<0.001), but there were no associations with WMH or large vessel infarcts. Afterwards, we restricted analyses of MB prevalence to the 49 AD patients only. In contrast to the total population, age was significantly higher in AD patients with MBs on both sequences. Furthermore, no differences in clinical or laboratory variables in AD patients with and without MBs were found on any sequence. Similar to the total population, of the MRI characteristics, presence of lacunes was associated with MB prevalence on both sequences.

Eighty-one patients had no MBs on both sequences. Of the 60 patients that presented at least one MB on either sequence, 28 patients, almost half, had MBs on both sequences. Another large subgroup of 28 patients had MBs on SWI not detected by GRE, i.e. the patients identified by the higher sensitivity of SWI. Only 4 patients were MB positive on GRE only. Analyses of these subgroups compared to patients without MBs and to patients with MBs on both sequences can be found online.

In a subsequent analysis, we used negative binomial regression to study relationships between number of MBs and the abovementioned parameters (table 2). On GRE, the following univariate associations were found. Higher MB numbers were associated with high age (p<0.004), sex (p=0.02), absence of diabetes mellitus (p<0.001), absence of hypercholesterolemia (p=0.03), moderate to severe WMH (p=0.02) and presence of lacunar infarctions (p=0.01). When we entered age and sex as covariates in the negative binomial regression model, only age and sex remained independently associated with MB number using GRE.

On SWI, higher MB numbers were also univariately associated with higher age (p=0.001), sex (p=0.01), moderate to severe WMH (p=0.02), presence of lacunar infarctions (p=0.004) and, in addition to GRE, current smoking (p=0.02). After adjustment for age and sex, comparable to GRE, only age and sex remained independently associated with MB number using SWI.

When we restricted these analyses to AD patients, the model could not be used due to sample size.

**Discussion**

We confirmed that SWI is more sensitive for the detection of MBs in terms of overall prevalence and number, compared to conventional GRE. The assessment of MBs was accurate and reproducible, with excellent inter-rater and intra-rater agreements. Using SWI, prevalence of MBs almost doubled and MB numbers per scan were higher, without any anatomical preference. As a result, probable CAA was seemingly more frequently detected using SWI. However, this gain in detection using SWI did not result in substantially improved relationships of MBs with clinical characteristics, vascular risk factors or other MRI expressions of small vessel disease (i.e. WMH and lacunar infarctions), suggesting that even with the conventional, less sensitive, way of visualizing MBs, most (patients with) clinically relevant MBs are captured.
In addition, since a growing number of studies suggest that the amount of MBs may be more clinically relevant than mere presence of MBs, we investigated influence of MB counts on both sequences. We found that by looking at MB count instead of presence only, male sex became additionally associated with MBs. The small vessel disease marker WMH became strongly related to MBs, after accounting for MB number. This effect of MB number is in line with the notion that MBs may serve as a marker for small vessel disease severity. In addition, we found that GRE and SWI were largely in agreement on these significant associations with MB number, underlining their validity. Discrepancies were hypercholesterolemia which was more prevalent in patients with MBs on SWI, but had a negative association with MB number on GRE, without adjustment. In the absence of a gold standard these differences are hard to interpret; hypercholesterolemia is a known vascular risk factor, although low cholesterol levels are associated with MBs as well. Unfortunately, cholesterol levels were not assessed in our study. Nevertheless, lipid lowering medication use could not explain these observations in our population, since this was not different between groups. Another surprising finding was that current smoking seemed to protect against MBs on SWI, a trend also found on GRE in our population. Although counterintuitive, we think this could still be a valid observation, since the negative association was observed on both sequences and moreover has been found in patients with cerebrovascular disease in a systematic review of 11 large studies.

Only 8 patients had higher MB counts using GRE. Upon close examination of these MBs using both sequences, these MBs were either misclassified on GRE, for example a vessel, visible only using SWI, identified as MB (figure 2), or not identified on SWI caused by various technical reasons. This indicates that both GRE and SWI may have some limitations regarding true MB assessment. The higher number of MBs on SWI did not result in an improvement of the clinical and radiological associations compared to the associations found using GRE. One may argue that the additional lesions detected by SWI, probably smaller lesions, may be more frequently artefacts. However, this is unlikely and not supported by a recent pathological-radiological correlation study, relating all MBs on SWI to various pathological findings. Furthermore, although the significance of MB size remains controversial, it may be argued that larger MBs, readily detectable on GRE, serve as small vessel disease marker, whereas perhaps smaller lesions, visible on SWI only, do only to a lesser extent. In addition, MB number may be considered the tip of the iceberg for underlying vascular pathology. Possibly, SWI merely exposes a slightly larger tip of the iceberg already visualized on GRE. Alternatively, power issues, due to relatively low prevalence of MBs, modest differences in MB counts, and heterogeneity of our current population may have hampered differences in clinical and radiological associations, despite our advanced statistical approach. In the literature dealing with MBs, considerable attention has been paid to the variability in prevalence and number of MBs depending on the used MR sequence. Despite the observed difference in prevalence and number of MBs, associations with most characteristics were comparable. Therefore, our results provide support for the notion that in terms of clinical associations, the relevance of MBs is much more robust than previously thought.

Among the strengths of the current study are the direct comparison performed by a single rater of the two sequences on the same 1.5T Siemens MR system, in the same patient at the same time. Therefore, true pathology underlying MB lesions may be considered constant. Furthermore, our associations of MBs with clinical and MRI characteristics were performed within the same patients using the same measurements. We used negative binomial regression, because it has been demonstrated to be especially suitable for lesion count data, and to provide a statistically more powerful parametric approach and it also allows for adjustment of covariates. Limitations of the current study are that our sample was heterogeneous, with relatively small subgroups, resulting in limited power, limited generalizability and may thus leave our findings as largely hypothesis-generating. In addition, we did not use a validated MB rating scale. Our definitions of definite MBs and MB mimics however, are equal to the MARS scale and our agreements were also excellent. Apart from minor anatomical scoring differences, the most notable difference is that we did not assess possible MBs in our current study. Nevertheless, the authors of the MARS scale proposed that for reliability purposes only definite MBs may be used, since possible MBs come with moderate reliability, possible reflected by our excellent agreement.
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Furthermore, that due to the small difference in slice thickness between the sequences, this study was not able to assess whether the benefit in detection is exclusively due to the use of added phase information. Another limitation is that due to difference in coverage, we could not consider infratentorial MBs, which may have altered the relations we have described. For example, MBs in the brainstem are common in patients with chronic hypertension. MBs in the cerebellum however, have been associated with both CAA and hypertension. Therefore, we are not able to predict to what extent our associations of only supratentorial MBs with risk factors are biased. The size of this bias however, may be rather small since in a previous study in AD patients with multiple MBs, infratentorial MBs only accounted for 2% of the total MB count. Finally, several adverse outcomes associated with baseline MBs, for example large intracerebral hemorrhages and subsequent cognitive impairment, and mortality, could not be evaluated due to the cross-sectional design of our current study.

The benefit of SWI over GRE may differ depending on the used magnetic field strength since susceptibility is proportional to the square of the magnetic field strength. Therefore, the expected gain in improved MB detection at higher magnetic field strengths in combination with SWI and follow-up of the abovementioned adverse possible clinical implications should be evaluated in further studies. Notably, the current definitions of possible and probable CAA may need to be re-established using these more sensitive techniques.

In conclusion, using SWI higher MB numbers were detected in more patients, irrespective of MB location. On both sequences, assessing MB number contributed to clinical and radiological associations. On SWI, the associations found on GRE were corroborated; however the higher MB numbers found on SWI compared to GRE, did not improve these associations. Therefore, previous clinical MB studies using GRE may still be valid. Although SWI may present a promising role in clinical practice, by possibly offering earlier detection of patients with bleeding-prone small vessel disease, the exact clinical relevance of improved MB detection needs to be determined.

Acknowledgements
We thank Ton Schweigmann for his technical assistance. We thank Siemens Medical Systems (Erlangen, Germany) for kindly providing a work-in-progress version of their SWI protocol for research purposes.

Table 1. Characteristics of patients without and with MBs for both sequences

<table>
<thead>
<tr>
<th>Variables</th>
<th>No MBs on GRE (n=109)</th>
<th>MBs on GRE (n=32)</th>
<th>No MBs on SWI (n=85)</th>
<th>MBs on SWI (n=56)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Demographics</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>62±9</td>
<td>65 ±10</td>
<td>60±8</td>
<td>65±9**</td>
</tr>
<tr>
<td>Male sex</td>
<td>61(56%)</td>
<td>20(63%)</td>
<td>47(55%)</td>
<td>34(61%)</td>
</tr>
<tr>
<td>Diagnosis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SMC</td>
<td>16(85%)</td>
<td>3(15%)</td>
<td>14(70%)</td>
<td>6(30%)</td>
</tr>
<tr>
<td>MCI</td>
<td>11(69%)</td>
<td>5(16%)</td>
<td>9(54%)</td>
<td>7(44%)</td>
</tr>
<tr>
<td>AD</td>
<td>39(80%)</td>
<td>10(20%)</td>
<td>30(61%)</td>
<td>19(39%)</td>
</tr>
<tr>
<td>Other dementia</td>
<td>17(74%)</td>
<td>1(6%)</td>
<td>11(21%)</td>
<td>7(39%)</td>
</tr>
<tr>
<td>Other disease</td>
<td>25(66%)</td>
<td>13(34%)</td>
<td>21(45%)</td>
<td>17(45%)</td>
</tr>
<tr>
<td>Clinical</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypertension</td>
<td>22(20%)</td>
<td>3(9%)</td>
<td>16(19%)</td>
<td>9(16%)</td>
</tr>
<tr>
<td>Diabetes Mellitus</td>
<td>13(12%)</td>
<td>0(0%)*</td>
<td>10(12%)</td>
<td>3(5%)</td>
</tr>
<tr>
<td>Hypercholesterolemia</td>
<td>14(13%)</td>
<td>4(13%)</td>
<td>7(8%)</td>
<td>11(20%)*</td>
</tr>
<tr>
<td>Current smoking</td>
<td>22(22%)</td>
<td>5(18%)</td>
<td>17(22%)</td>
<td>10(20%)</td>
</tr>
<tr>
<td>Alcohol &gt;2/day</td>
<td>11(11%)</td>
<td>4(14%)</td>
<td>8(10%)</td>
<td>7(14%)</td>
</tr>
<tr>
<td>Statin use</td>
<td>7(11%)</td>
<td>8(9%)</td>
<td>7(8%)</td>
<td>8(14%)</td>
</tr>
<tr>
<td>Antiplatelet use</td>
<td>15(14%)</td>
<td>5(16%)</td>
<td>8(9%)</td>
<td>12(21%)*</td>
</tr>
<tr>
<td>Anticoagulant use</td>
<td>12(2%)</td>
<td>2(3%)</td>
<td>11(1%)</td>
<td>2(4%)</td>
</tr>
<tr>
<td>Systolic tension</td>
<td>139±17</td>
<td>143±14</td>
<td>138±8</td>
<td>143±14</td>
</tr>
<tr>
<td>Diastolic tension</td>
<td>85±9</td>
<td>86±8</td>
<td>85±9</td>
<td>86±10</td>
</tr>
<tr>
<td>MMSE</td>
<td>25±5</td>
<td>25±4</td>
<td>25±5</td>
<td>25±4</td>
</tr>
</tbody>
</table>

Furthermore, that due to the small difference in slice thickness between the sequences, this study was not able to assess whether the benefit in detection is exclusively due to the use of added phase information. Another limitation is that due to difference in coverage, we could not consider infratentorial MBs, which may have altered the relations we have described. For example, MBs in the brainstem are common in patients with chronic hypertension. MBs in the cerebellum, however, have been associated with both CAA and hypertension. Therefore, we are not able to predict to what extent our associations of only supratentorial MBs with risk factors are biased. The size of this bias however, may be rather small since in a previous study in AD patients with multiple MBs, infratentorial MBs only accounted for 2% of the total MB count. Finally, several adverse outcomes associated with baseline MBs, for example large intracerebral hemorrhages and subsequent cognitive impairment, and mortality, could not be evaluated due to the cross-sectional design of our current study.

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Table 2. Associations between MB number and baseline characteristics

<table>
<thead>
<tr>
<th>Variables</th>
<th>MBs on GRE</th>
<th>MBs on SWI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Unadjusted</td>
<td>Regression coefficient adjusted for age and sex</td>
</tr>
<tr>
<td>Age</td>
<td>1.06 (1.00-1.12)**</td>
<td>1.06 (1.00-1.12)**</td>
</tr>
<tr>
<td>Male sex</td>
<td>3.17 (1.19-8.45)*</td>
<td>3.06 (1.16-9.04)*</td>
</tr>
<tr>
<td>Diagnosis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SMC</td>
<td>Ref.</td>
<td>0.97 (0.20-4.80)</td>
</tr>
<tr>
<td>MCI</td>
<td>2.72 (0.54-13.74)</td>
<td>2.06 (0.45-9.41)</td>
</tr>
<tr>
<td>AD</td>
<td>0.12 (0.012-1.27)</td>
<td>0.14 (0.012-1.48)</td>
</tr>
<tr>
<td>Other dementia</td>
<td>1.05 (0.25-4.44)</td>
<td>1.26 (0.34-7.1)</td>
</tr>
<tr>
<td>Other disease</td>
<td>0.43 (0.086-2.12)</td>
<td>0.56 (0.13-2.47)</td>
</tr>
<tr>
<td>Hypertension</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ref.</td>
<td>0.52 (0.11-2.40)</td>
<td>0.48 (0.12-1.84)</td>
</tr>
<tr>
<td>Diabetes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ref.</td>
<td>0.30 (0.10-0.88)*</td>
<td>0.43 (0.10-1.93)</td>
</tr>
<tr>
<td>Alcohol &gt;2/ day</td>
<td>0.37 (0.12-1.10)</td>
<td>0.77 (0.17-3.47)</td>
</tr>
<tr>
<td>Smoking</td>
<td>0.39 (0.12-1.24)</td>
<td>0.50 (0.16-1.53)</td>
</tr>
<tr>
<td>Systolic tension</td>
<td>0.97 (0.94-1.01)</td>
<td>0.98 (0.95-1.02)</td>
</tr>
<tr>
<td>Hypercholesterolemia</td>
<td>0.98 (0.91-1.04)</td>
<td>0.98 (0.93-1.08)</td>
</tr>
<tr>
<td>MMSE</td>
<td>0.92 (0.82-1.02)</td>
<td>0.92 (0.83-1.03)</td>
</tr>
<tr>
<td>APOE e2</td>
<td>0.44 (0.12-1.72)</td>
<td>0.73 (0.18-2.94)</td>
</tr>
<tr>
<td>APOE e4</td>
<td>0.45 (0.33-5.44)</td>
<td>1.52 (0.43-5.40)</td>
</tr>
<tr>
<td>Double e4</td>
<td>0.91 (0.17-4.82)</td>
<td>1.74 (0.32-9.38)</td>
</tr>
<tr>
<td>WMH score &gt;1</td>
<td>4.13 (1.23-13.9)*</td>
<td>2.77 (0.79-9.74)</td>
</tr>
<tr>
<td>Presence of lacunes</td>
<td>4.05 (1.38-11.86)**</td>
<td>2.28 (0.71-7.36)</td>
</tr>
</tbody>
</table>

Data are represented as negative binomial regression coefficients and 95% confidence interval. *p<0.05, **p<0.01. MBs = microbleeds, SMC = subjective memory complaints, MCI = mild cognitive impairment, AD = Alzheimer disease, Hypercholesterolemia, MMSE = mini mental state examination. WMH = white matter hyperintensities on the Fazekas visual rating scale.
Microbleeds in Dementia: Connecting the dots

Literature


Microbleeds in Dementia: Connecting the dots

Microbleeds are associated with lacunar stroke defined clinically and radiologically, independently of white matter lesions. Stroke 2006;37:2633-6.


Won SS, Hwa LB, Kim EJ, Chin J, Sun CY, Yoon U, Na DL. Clinical


35 Landis JR, Koch GG. The measurement of observer agreement for categorical data. Biometrics 1977;33:159-74.


Microbleeds in Dementia: Connecting the dots

Abstract

Background and Purpose: Ultra-high field strength MRI has been found very sensitive in detecting cerebral microbleeds (MBs), suggesting that at high enough field strength, almost all Alzheimer disease (AD) patients have MBs. We aimed to compare MB imaging at 7T with 3T imaging in a relatively young AD patient sample.

Methods: 15 probable AD patients without substantial cerebrovascular comorbidity were scanned using routine 3T and subsequently 7T MRI with dual echo $T_2^*$-weighted gradient echo. Presence and number of MBs were rated. We used Wilcoxon signed rank tests to compare MB numbers. For each field strength, we determined non-parametric correlations of MB number with demographic, clinical and radiologic characteristics.

Results: Twelve patients (age 63±6, 67% male) had images of acceptable quality at both field strengths. MB presence was 33% at 3T and 42% at 7T ($p=0.10$) and MB numbers were not significantly higher at 7T ($p=0.16$). At 7T, higher age ($r=0.70$) and APOE ε4 allele number ($r=0.74$) were associated with number of MBs. By contrast at 3T, age was not associated with MB number, while the correlation with APOE ε4 allele dose was borderline significant.

Conclusion: In a sample with young AD patients without substantial cerebrovascular comorbidity, 7T dual echo detected non-significantly more MBs compared with 3T. Moreover, the majority of patients showed no MBs at 7T. Nevertheless, improved associations of MBs with age and APOE ε4 at 7T, suggest clinical relevance by indentifying relevant subgroups.

Abbreviation key

MBs = cerebral microbleeds
AD = Alzheimer disease
APOE = Apolipoprotein E
CAA = cerebral amyloid angiopathy
MMSE = mini mental state examination

4.2 MICROBLEEDS IN ALZHEIMER DISEASE USING 7T IMAGING; APPROACHING THE TRUE NUMBER?

Under revision

Authors: Jeroen DC Goos MD$^{1,2}$, Sanneke van Rooden MSc$^3$, Maarten J Versluis MSc$^3$, Jeroen van der Grond PhD$^4$, Niels D Prins MD PhD$^{1,2}$, Philip Scheltens MD PhD$^{1,2}$, Mark A van Buchem MD PhD$^4$, Frederik Barkhof MD PhD$^{1,4}$ and Wiesje M van der Flier PhD$^{1,2,5}$

Institutional affiliations: From the $^1$Alzheimer Center and $^2$Department of Neurology, VU University Medical Center, Amsterdam the Netherlands. $^3$Department of Radiology, Leiden University Medical Center, Leiden, the Netherlands. $^4$Department of Radiology, VU University Medical Center, Amsterdam the Netherlands. $^5$Department of Epidemiology and Biostatistics, VU University Medical Center, Amsterdam the Netherlands.

‘De beelden liegen niet!’

Ron Brandsteder
**Introduction**

Detection of cerebral microbleeds (MBs) is highly imaging technique dependent. Field strength for example, has great impact on MB detection.\(^1\)\(^,\)\(^2\) Ultra-high field strengths are expected to detect more MBs with greater anatomical detail, which was recently supported by studies using 7T MRI.\(^3\)\(^,\)\(^4\) In a post-mortem setting, MBs and even smaller microscopic lesions at 7T, have been neuropathologically confirmed as hemosiderin deposits in brains of Alzheimer disease (AD) patients.\(^5\)

MBs have been linked to cerebral amyloid angiopathy (CAA) in AD.\(^6\) Associations between especially lobar MBs and APOE ε4 carriearship have provided support for this hypothesis.\(^7\)\(^,\)\(^8\) Alternatively, one may reason that MBs on MRI represent nonspecific age-related cerebral iron deposition. Using enhanced MRI sequences for MB detection, MBs were indeed also frequent in an asymptomatic population and MB prevalence strongly increased with age.\(^7\)\(^,\)\(^8\)

If MBs reflect nonspecific iron deposition, one may expect that, at high enough field strengths, MB prevalence in elderly individuals and especially those with AD, may eventually approach 100%. This notion is supported by a recent report, describing a very high MB prevalence in AD patients at 7T.\(^7\) Alternatively, MBs may reflect a more specific substrate, likely CAA, in both elderly and AD populations, identifying relevant subgroups.

We aimed to investigate the difference in MB presence and number as assessed at 3T and 7T field strengths, in a fairly young sporadic AD patient population, with little cerebrovascular comorbidity. Additionally, we compared associations between MBs and demographic, clinical and MRI characteristics at both field strengths.

**Methods**

**Patients**

15 probable AD patients were scanned at 3T and subsequently at 7T (median 8 months, range 6-15). From two memory clinics (VU University Medical Center and Leiden University Medical Center) we included patients that were recently diagnosed with probable AD and were willing and able to undergo scanning at 7T (be able to lie still, have MMSE scores of ≥20, no contra-indications for 7T MRI and be mobile). One patient had a scan of unacceptable quality at 3T and two other patients at 7T, resulting in 12 patients for the direct comparison. Patients received a standardized dementia work-up, including general medical and neurological examination, neuropsychological and laboratory testing and 3T MRI. All patients fulfilled the NIA-AA criteria for probable AD.\(^10\) Systolic and diastolic blood pressure were measured manually using a sphygmomanometer, with the patient in sitting position, or when absent the average of two measurements in supine position. Presence of hypertension, hypercholesteremia or diabetes mellitus was determined based on medical history and/or medication use. Current smoking status was recorded. The local ethics committee had approved the study protocol and all participants gave written informed consent.

**Imaging and analysis**

Routine work-up included 3T MRI on a whole-body 3T MR system (Signa HDxt, General Electric, Milwaukee, WI, USA) using an 8-channel head coil. The protocol included the following pulse sequences: (1) used for MB rating: axial 2-dimensional T\(_2\)*-weighted gradient-echo, with an echo-planar read-out (matrix: 256x480, field of view [FOV] 2519 cm\(^2\), slice thickness 3mm, TR/TE=5300ms/25ms, 2 excitations); (2) sagittal 3-D fluid-attenuated inversion-recovery (FLAIR) (3) axial 2-dimensional proton-density/ T\(_2\)-weighted fast spin echo; (4) oblique reconstructions of a 3-D fast spoiled gradient recalled echo-based sequence (FSPGR), parameters described elsewhere.\(^11\) On the 3T FLAIR sequence, WMH were visually assessed according to the modified Fazekas rating scale.\(^12\) The scale ranges from 0-3 [none, punctuate, early confluent and confluent]. Presence of large vessel and lacunar infarcts was assessed. Large
vessel infarcts were rated as present or absent, based on hyperintensity of the lesion on both FLAIR and T2-weighted sequences. Lacunar infarcts were defined as well-demarcated lesions from 3-15 mm, with a cerebrospinal fluid-like signal on all sequences. In addition, 7T MR imaging was performed on a whole-body human 7T MR imaging system (Achieva; Philips Healthcare, Best, the Netherlands), with a 16-channel head coil (Novo Medical, Wilmington, MA, USA). For MB detection, we performed a dual-echo T2*-weighted sequence with an acquired resolution of 0.35x0.4x0.6mm³, reconstructed to 0.35x0.35x0.3mm³. TR/first TE/second TE=20/2.5/15ms, with an acquired matrix of 508x399 with 167 slices, further details published by Conijn et al. In order to reduce slice number and enhance MB hypointensity, minimum intensity projections (MinIPs) were reconstructed of the first and the second echo, for transversal slabs (thickness 3mm, 2mm overlap, 150 slices). The dual echo approach leads to improved MB detection by enhancing the smaller MBs by using a long TE of the second echo, while the first echo enables MB detection in regions with high susceptibility effects.

We defined MBs as small (no minimum size and up to 10mm), round well defined hypointense lesions. MBs were regionally counted. We did not consider lesions in regions of cerebral infarction, or sulci probably representing flow voids from vessels and lesions in the globus pallidus, supposedly representing iron or calcium deposits. Neither choroid plexus and pineal calcifications, nor lesions suggestive of partial-volume effects were taken into account. Due to limited coverage of the cerebellum and brainstem in most scans, infratentorial MBs were not considered. On 7T, MBs were counted by the same rater on the MinIPs viewed in a dimly lit room, with two parallel screens to systematically assess both echo images side-by-side, one brain quadrant at a time, averaging well over 30 minutes per scan. Rating was performed blinded for clinical information and 3T imaging results, using the modified MARS visual rating scale.

In doing so, rating at 7T dual echo slightly differed from assessment at 3T, as MBs had to be substantially larger on the second echo image at side-by-side comparison. As part of the training, 10 scans of elderly controls who underwent the same 7T MRI protocol were rated (mean age 75±3 [range 70-80], 64% male, MMSE 29±1, observed MB prevalence 80%). Additionally, a subset of AD scans was scored in a consensus meeting with an experienced MB rater at 7T. Several months after the first rating, a second rating of all scans was performed in random order to establish an intra-rater reliability. The reliability for definite MBs was excellent, reflected by a weighted Cohen’s kappa of 0.93. When lesions not fulfilling the modified MARS criteria for a definite MB (possible MBs) were included in the analysis, the kappa dropped to 0.69, rendering only substantial agreement. Therefore, possible lesions were disregarded in further analyses, as has been recommended before.

APOE ε4 genotyping
DNA was isolated from 10mL of ethylenediaminetetra-acetic acid/blood and was available for 12 patients (80%). Apolipoprotein E (APOE) genotype was determined with the light cycler APOE mutation detection method (Roche diagnostics GmbH, Mannheim, Germany). We categorized patients according to APOE ε4 status into noncarriers, heterozygous carriers and homozygous carriers.

Statistics
Statistical analyses were performed using SPSS 18.0 for mac (SPSS, Chicago, IL). To compare MB number directly between field strengths, Wilcoxon signed rank tests were used. Student’s t-tests were performed for continuous variables to investigate group differences. Group differences for categorical variables were assessed with χ² tests. Correlations of MB number with patient characteristics, including APOE ε4 dose, blood pressure measurements and MRI variables were assessed with non-parametric Spearman correlations. A p value <0.05 was considered significant.

Results
Table 1 presents the demographic, clinical and radiologic characteristics of our total sample. Patients had a mean age of 64±6 years (range 51-78) and 10 patients were male (67%). Four patients had hypertension, one had diabetes mellitus, one had hypercholesterolemia and one patient smoked cigarettes. Three patients had moderate WMH. No lacunar or large vessel infarcts were observed.
At 3T, we counted 5 MBs in 4 out of 14 patients compared to 10 MBs in 5 out of 13 patients at 7T. Eight out of 10 (80% of total) MBs at 7T were lobar, compared to 3 out of 5 (60%) at 3T (example in figure 1). When we compared the patients who had both scans available, four patients had one or more MBs visible at 3T (33%) and 5 patients (one additional patient) had one or more MBs at 7T (42%, p=0.10) illustrated by figure 2. MB numbers did not significantly differ either with a median of 0 (0-2) at 3T compared to 0 (range 0-3) at 7T (p=0.16). At 7T, equal or higher MB numbers were visible in all patients, except for one lesion, which was counted as a MB at 3T, but appeared to be a vessel at 7T. Most lesions only visible at 7T were very small; in the (sub)millimetre range. At 7T, patients with MBs were older (68±3 versus 60±6, p<0.05) than patients without MBs and had more APOE ε4 alleles (p<0.05), no other differences were found. Regarding MB presence at 3T, we found no group differences. Subsequently, we assessed Spearman correlations of MB number with demographic, clinical and radiologic characteristics. At 7T, we found MB number correlated with age [Spearman’s Rho 0.70, p<0.05] and number of APOE ε4 alleles [Spearman’s Rho 0.74, p<0.05], but no other correlations were found. At 3T, only an association with APOE dose [Spearman’s Rho 0.62, p<0.05] was found, but not with age, or any other variable. When restricting analyses to patients with acceptable 3T and 7T scans, results did not essentially change for the 7T correlations. At 3T however, the correlation of MB number and APOE ε4 alleles lost significance [Spearman’s Rho 0.60, p<0.10].

Visually, we observed greater anatomical and lesion detail, with enhanced contrast at 7T, allowing detection of submillimetre lesions on both echos. Figure 3 shows an example of atypical hypointense linearly appearing lesions at 3T and illustrates the hypertense aspect of this region on the FLAIR images. At 7T, individual tiny (<1mm) hypointense foci could be appreciated. These lesions were not considered as MBs however, as a capillary telangiectasia or an ischemic region with secondary hemorrhage could not be ruled out. No visible continuous vessel structures at the MinIP of the second (venous), or MaxIP of the first echo (arterial) were found. Therefore, larger vascular malformations were unlikely, which illustrates a potential advantage of dual echo imaging in detection of MB mimics.

Discussion

In this radiologic comparison of MBs as assessed at different field strengths in a sample of relatively young sporadic AD patients, we found that imaging at 7T revealed a non-significantly higher number of MBs than at 3T. Scans obtained at ultra-high field strength did not reveal MBs in (nearly) every AD patient, suggesting that there is a ceiling to the ”true number” of MBs. Moreover, our results seemingly provide support for the notion that MBs reflect a specific substrate, such as severe CAA. Stronger associations with age and especially APOE ε4 also point in this direction.

MB prevalence at 7T in our study was lower than reported elsewhere. Two studies assessed different populations (vascular dementia and atherosclerotic disease), likely with more severe vasculopathies. One 7T study in patients with AD and mild cognitive impairment however, also reported a clearly higher prevalence (78%). As imaging aspects were similar, our prevalence probably reflects the lower age of our population with little vascular risk factors or concomitant vascular disease. The observed correlation between MB number and age at 7T, as well as the high prevalence of MBs in our older controls, who were rated as part of the training procedure, support this. Furthermore, in a small familial AD sample, younger than ours, 3T MB prevalence was lower (25%) than ours (33%), but 0% in controls. These findings support the notion that MB prevalence is not only related to age-related brain changes, but may also be specific to AD. Neuropathological evidence of CAA is observed in almost every AD patient to some extent, but MBs as observed on MRI during life are not. Probably only severe CAA, which is present in approximately 25% of autopsied AD patients, leads to MBs. In AD, this prevalence of severe CAA closely resembles the MB prevalence of roughly one out of four, detected with conventional MRI. This suggests that MB detection at 7T may approximate the ”true” CAA related MB load.

We observed several submillimetre lesions at MRI in vivo, likely reflecting hemosiderin deposits of 0.2 mm or smaller, as former study has reported that post-mortem 7T MRI enabled detection of microscopic hemosiderin deposits in AD brain slices, appearing...
2.5-5 times larger on MRI than at neuropathological examination. This finding corresponds to another recent neuropathological study, which found (pericapillary hemosiderin deposits that ranged from 0.05-0.2 mm). Whether these microscopic hemosiderin deposits should even be called MBs is still debated. Nevertheless, these observations may suggest (larger) capillary related hemosiderin foci may be detected using 7T MRI. In AD, this would be of special importance, as (pericapillary CAA is currently not detectable during life, but is pathologically associated with AD.

Among strengths of the current study are the sample of relatively young AD patients, with little vascular risk factors and hardly any signs of ischemic small vessel disease. This maximized the likelihood that the observed MBs indeed reflected a specific AD related substrate, such as advanced CAA. An additional strength was the use of a dual echo sequence at 7T and a validated rating scale by an experienced rater. A single rater however, may also be a limitation, since modest inter-rater agreements at 7T have been reported, with poor agreements for possible MBs. Therefore, only definite MBs were taken into account, similar to a previous report. A major limitation is our sample size. Therefore, we had to deal with little power, possibly explaining the lack of a significant difference between 3T and 7T in prevalence of MBs. Nonetheless, the gain in MB sensitivity at ultra-high field strength was unexpectedly low, suggesting that the “true” bleeding load in a relatively pure and young AD sample may actually be smaller than suggested by previous reports. At the same time, these findings confirm the relevance of MBs, which are not omnipresent, nonspecific findings, but may rather identify an additional disease process in a subgroup of AD patients.

### Table 1. Patient, vascular risk and MRI characteristics of the total sample

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>n=15</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>64±6</td>
</tr>
<tr>
<td>Sex (male)</td>
<td>10(67%)</td>
</tr>
<tr>
<td>MMSE</td>
<td>23±3</td>
</tr>
<tr>
<td>APOE ε4 status&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Noncarriers</td>
<td>3(25%)</td>
</tr>
<tr>
<td>Heterozygous carriers</td>
<td>4(33%)</td>
</tr>
<tr>
<td>Homozygous carriers</td>
<td>5(42%)</td>
</tr>
<tr>
<td>Hypertension</td>
<td>4(27%)</td>
</tr>
<tr>
<td>hypercholesterolemia</td>
<td>1(7%)</td>
</tr>
<tr>
<td>Diabetes Mellitus</td>
<td>1(7%)</td>
</tr>
<tr>
<td>Current smoking&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1(8%)</td>
</tr>
<tr>
<td>Systolic BP, mmHg&lt;sup&gt;c&lt;/sup&gt;</td>
<td>142±24</td>
</tr>
<tr>
<td>Diastolic BP, mmHg&lt;sup&gt;c&lt;/sup&gt;</td>
<td>84±11</td>
</tr>
<tr>
<td>Moderate/severe WMH</td>
<td>3(21%)</td>
</tr>
<tr>
<td>Lacunar infarct</td>
<td>0(0%)</td>
</tr>
<tr>
<td>Large vessel infarct</td>
<td>0(0%)</td>
</tr>
<tr>
<td>GCA</td>
<td>0.9±0.8</td>
</tr>
<tr>
<td>MTA mean</td>
<td>1.2±0.8</td>
</tr>
<tr>
<td>Total MBs at 3T&lt;sup&gt;d&lt;/sup&gt;</td>
<td>5</td>
</tr>
<tr>
<td>Total MBs at 7T&lt;sup&gt;e&lt;/sup&gt;</td>
<td>10</td>
</tr>
<tr>
<td>Total Lobar MBs at 3T&lt;sup&gt;d&lt;/sup&gt;</td>
<td>3</td>
</tr>
<tr>
<td>Lobar MBs at 7T&lt;sup&gt;e&lt;/sup&gt;</td>
<td>8</td>
</tr>
</tbody>
</table>

Data are represented as number of patients with variable present (%), or mean±SD.

<sup>a</sup> n=12, <sup>b</sup> n=13, <sup>c</sup> n=12, <sup>d</sup> n=14, <sup>e</sup> n=13. Abbreviations: MMSE: Mini mental state examination, APOE: Apolipoprotein E; BP: blood pressure; MBs: cerebral microbleeds; Moderate/severe WMH: white matter hyperintensities with a Fazekas visual rating scale score >1; GCA: global cortical atrophy; MTA mean: medial temporal lobe atrophy average score of left and right.
Figure 1  
3T T<sub>2</sub>*-weighted gradient-echo echo-planar read-out (A), from a 65 year old male Alzheimer disease patient, homozygous for APOE ε4, showing a microbleed, in the right occipital cortex (lobar). The lesion is also clearly visible at 7T on the minimum intensity projections of the first echo (B) and is larger at the second (C) echo.

Figure 2  
Histogram of number of patients (Y-axis) and MB number per field strength (X-axis). White bars represent 3T data and black bars represent 7T data.
Figure 3 3T T₂*-weighted gradient-echo echo-planar read-out (A), from a 67 year old female Alzheimer disease patient, homozygous for APOE ε4, showing an atypical small hypointense region, with a linear aspect in the right frontal lobe. Hyperintensity of the same region on 3T FLAIR images (B). Magnification of the region with 3T T₂*-weighted sequence (C), first (D) and second T₂*-weighted echo images (E) at 7T showing multiple submillimetre satellite lesions around a subcortical hypointense focus in the right frontal lobe, suggesting small hemosiderin deposits, as they were markedly hypointense on the first and appeared larger at the second echo (so called “blooming”). Distance between the small scale bars=1mm. These lesions are not counted as MBs, because a vascular malformation, likely a capillary telangiectasia, or secondary hemorrhaging from due to ischemia cannot be ruled out. In total, this patient shows 3 definite lobar MBs at 7T. A definite MB close to the cortico-subcortical junction of the left frontal operculum at the first echo (F), which appears larger at the second echo (G) at 7T, but is not visible at the 3T T₂*-weighted gradient-echo echo-planar read-out (H).

Literature
11. Goos JDC, Teunissen CE, Veerhuis R et al. Microbleeds relate to

‘No blood... no blood at all. Why hadn’t I thought of that? No blood. What a beautiful idea!’

Dexter Morgan

MICROINFARCTS ASSESSED BY POST-MORTEM QUANTITATIVE T1 RELAXATION TIME AT 1.5 TESLA IN ALZHEIMER AND NON-ALZHEIMER DISEASE CASES

Submitted

Authors: Jeroen DC Goos MD¹, Jeroen Hoozemans PhD¹, Alida A Gouw MD PhD¹, Dirk L Knol PhD¹, Adriaan Versteeg³, Hugo Vrenken PhD¹, Anneemieke JM Rozemuller MD PhD³, Frederik Barkhof MD PhD¹, Philip Scheltens MD PhD¹, Wiesje M van der Flier PhD¹ and Jeroen JG Geurts PhD⁷.

Institutional affiliations: From the ¹Alzheimer Center and ²Department of Neurology, ³Department of Pathology, ⁴Department of Epidemiology and Biostatistics, ⁵Department of Radiology, ⁶Department of Physics and Medical Technology ⁷Anatomy & Neurosciences, Section of Clinical Neuroscience.

VU University Medical Center, Amsterdam, the Netherlands.
Abstract

Background and Purpose: Although of likely clinical relevance, cerebral microinfarcts (MIs) are currently not detectable with conventional imaging. To investigate whether neuropathologically defined MIs in post-mortem tissue of Alzheimer disease (AD) and non-AD cases can be measured by quantitative T1 relaxation time mapping MRI (T1-RTM).

Methods: forty-seven formalin fixed coronal brain slices from 11 AD and 7 non-AD subjects with white matter hyperintensities (WMH) were scanned at 1.5T using qualitative dual-echo T2-weighted spin-echo and T1-RTM based on flip-angle array. We counted MIs on brain tissue slices stained with immunohistochemistry. The grey matter, normal appearing white matter (NAWM) and WMH were semi-automatically outlined on T2-weighted scans and subsequently copied to corresponding T1-maps. Linear mixed models were used to assess the relation between MIs and T1-RTM values and WMH size.

Results: twenty-nine percent of non-AD and 64% of AD cases (p=0.15) showed MIs. MI numbers and distribution per slice (cortical versus deep white matter) were comparable. A larger effect of MI presence on higher T1-RTM values of the WMH was found in the non-AD than in the AD cases. Presence and number of MIs were also associated with larger WMH size in non-AD cases (p=0.05).

Conclusion: MIs are related to severe small vessel disease and changes may be detected with T1-RTM at conventional field strength. This may now open up additional possibilities for in vivo detection of these lesions and their associated microvascular damage.

Introduction

MIs are focal lesions attributed to ischemia, which are found only on microscopic examination after autopsy.1 While MIs are not detected with conventional in vivo neuroimaging, they are a common finding at autopsy.2-4 Moreover, post-mortem tissue studies have suggested that MIs are independently associated with dementia and cognitive deficits and may even be the most important cause of vascular dementia, stressing the importance of MI detection during life.1, 5-8

Several recent studies, using ultra high-field MRI or diffusion weighted imaging, have detected small (acute) ischemic lesions in vivo, but these studies lacked pathological evaluation to confirm that these lesions are indeed MIs.4 Post-mortem MRI may provide the necessary link between this neuropathologically defined entity and MRI observations. A recent study using 7T post-mortem high-resolution MRI, was able to detect neuropathologically confirmed cortical MIs in a single case with Cerebral Autosomal-Dominant Arteriopathy With Subcortical Infarcts and Leukoencephalopathy.9 In a previous study, we have shown that post-mortem MRI at 1.5T with T1-RTM enables the detection of subtle pathological changes in NAWM and differentiates between areas of WMH in AD cases and elderly non-AD cases, both with signs of cerebral small vessel disease.10 Moreover, T1-RTM values correlated with severity of pathological damage to myelin, axons and extent of microglial activation, all processes involved in microinfarction.11 We therefore hypothesized that presence of MIs at autopsy might be measurable by T1-RTM at 1.5T, which might then serve as a valuable tool for in vivo detection of this subtle but important type of pathology. To investigate the relation between MIs and T1-RTM changes and WMH size, we neuropathologically assessed presence and number of MIs in AD and non-AD cases, both with MRI apparent signs of chronic cerebral small vessel disease, i.e. WMH. To determine associations between these histopathologically defined MIs and imaging, post-mortem T1-RT maps and WMH sizes of all the imaged slices were calculated.
Methods

Cases
We selected 11 brain specimens of patients over 70 years of age with a clinical diagnosis of AD from the Netherlands Brain Bank (NBB). Brain specimens of nine non-AD cases, all over 70 years and non-demented, were selected from the VU University Hospital, who died during hospital admission. Subjects were included if extensive periventricular WMH and/or (beginning) confluent areas of deep WMH were found on post-mortem T2-weighted MRI. Specimens were excluded if subjects underwent resuscitation, or presented clinically with large vessel infarcts, or neurological disease other than AD. Additionally, history of hypertension, or known histories of coronary, peripheral, or cerebral atherosclerotic disease during life were recorded. The institutional ethics review board approved the study protocol, and all patients, or their next of kin, had approved the use of their brain tissue and review of their medical history for research.

Pathological assessment
General pathological assessment was performed according to the NBB dementia protocol. The clinical diagnosis of AD was pathologically confirmed using the Braak criteria and Consortium to Establish a Registry for Alzheimer’s disease (CERAD) criteria, whereas non-AD cases all had a Braak stage score for neurofibrillary tangles <2. Subjects were excluded when either pathological evidence of large vessel infarction or other types of dementia were present. Furthermore, in a subset of 13 subjects an overall degree of vascular amyloid deposition, indicating cerebral amyloid angiopathy (CAA), was noted (none/mild versus moderate/severe), using Congo red staining.

After rapid autopsy (all within 24 hours) and at least 4 weeks of fixation in 10% formalin, one hemisphere was cut into 10mm thick coronal brain slices using a 10mm deep cutting panel. One to four slices per case were used for MRI scanning, including pre-frontal slice(s) (first slice cranial of frontal horns of the lateral ventricle), frontal slice(s) (at the level of the hippocampus, caudate and lenticular nuclei) and parietal slice(s) (at the level of the cella media).

After scanning, slices were paraffin-embedded and subsequently cut in half to reveal the center of the imaged plane on which we defined the regions of interest (ROIs), capturing the grey matter (GM), NAWM and extensive WMH. This way, the brain slices could be matched to the MR images as report before. Serial large 8-μm thick sections were then cut, mounted onto glass slides and stained with haematoxylin and eosin, Luxol Fast Blue/Cresyl Violet and Bodian silver. Immunohistochemical stainings were performed, including HLA-DR primary antibodies (clone 3, mouse antibody, dilution 1:50) for microglial activation, also described in more detail elsewhere.

Assessment of MIs
In agreement with previously published guidelines, we defined MIs as microscopic foci of tissue rarefaction, with increased microglial immunoreactivity. We excluded lesions that were larger than 5mm, or strictly perivascular and not involving the parenchyma, or when lesions consisted of only a few HLA-DR immunoreactive cells. By using this definition of MIs, we attempted to exclude acute and subacute MIs, which are not considered to contribute to dementia during life. Furthermore, not mandatory for our counting, but considered as supportive features, were signs of edema, evidence of a relationship of microglia with an adjacent vessel, or pallor on adjacent Luxol Fast Blue/Cresyl Violet stained slices. In a consensus meeting of two raters, rectangles were manually drawn around areas scored as MIs on immunohistochemically stained brain slices. In case of disagreement, a third reviewer was consulted. Additionally, the MIs were regionally classified as cortical (cortex and the cortico-subcortical junction), or deep white matter.

Post-mortem MRI protocol
Post-mortem MRI scanning was performed using a 1.5-T MRI scanner (Vision, Siemens AG, Erlangen, Germany). The brain slices were separately placed in a custom-made perspex multi-slice holder for the MRI head-coil. The MRI protocol, that was optimized for fixed brain slices, included: Qualitative dual-echo T2-weighted spin-echo (T2SE) MRI with TR/TE/NEX: 2755ms/45ms and 90ms/2; field of view: 80x128mm; matrix size 160x256; slice thickness: 3mm; acquisition time: 7 min 25 sec. Quantitative T1-RTM MRI: 3D fast low-angle shot (FLASH), TR/TE/NEX: 15ms/4ms/4;
Results

Two initial non-AD cases were excluded as they both had a Braak stage of 3, resulting in the final study sample of 11 AD patients and 7 non-AD cases. Demographic, clinical and neuropathologic characteristics are shown per subject group in Table 1. Age, sex, hypertension, or history of peripheral, coronary, or cerebral vascular disease during life did not differ between groups. Post-mortem delay, fixation times and number of available slices per subject were also comparable between groups.

We found a total of 36 MIs (Figure 1) in all cases. MIs were observed in 7 AD patients (64%) versus 2 non-AD cases (29%, p=0.15). Numbers of MIs per slice were comparable between groups. Furthermore, distribution of MIs did not differ between subjects groups: 71% of AD patients had MIs in the cortex or at the cortico-subcortical junction (cortical MIs), whereas both non-AD cases with MIs showed cortical MIs (p=0.39). Deep white matter MIs were also present in 5 AD patients and in the 2 non-AD cases (p=0.39). Larger lacunar infarctions (5-15mm) often readily visible with the naked eye, were observed on pathological assessment of the slices in 1 non-AD case (14%) versus 3 AD patients (27%, p=0.52). The occurrence of these lacunar infarctions was unrelated to presence of MIs (p=0.26). For a subset of 13 subjects (8 AD versus 5 non-AD cases) global CAA scoring was available. Moderate/severe CAA was more frequent in AD patients compared to non-AD cases, since it was observed in half of the evaluated AD cases, but absent in non-AD cases (p=0.057).

Table 2 shows the mean T1-RTM values in ms and WMH sizes in mm² per subject group, further divided by presence of MIs, estimated by mixed models based on mean values of age and adjusted for sex. In non-AD cases, a trend towards higher T1-RTM values of the WMH was observed and a significantly larger size of the WMH, when one or more MIs were present. In the AD group however, no differences between cases with or without MIs were found for any of the measurements.

To further assess the relation of MIs and T1-RTM values of the different tissue types and size of the (beginning) confluent WMH, we used linear mixed models again [Table
Microbleeds in Dementia:
Connecting the dots

vessel disease on MRI, and a strong relation between MIs and severe WMH, MIs relate
to hypertensive vasculopathy rather than CAA, which is more frequently associated
with MIs in AD. The finding that 50% of the assessed AD patients exhibited moderate/severe CAA, but moderate/severe CAA was absent in non-AD cases, might support
this notion of different pathomechanisms, which should now be investigated in a
larger sample.

We found that the presence of MIs in the non-AD group was associated with more
abnormal WMH T1-RTM values and also with larger sizes of the (beginning) confluent
WMH. Therefore, MIs probably relate to more severe forms of small vessel disease.
We have previously shown that T1-RTM values relate to axonal and myelin loss and
also reflect microglial activation. These pathological processes are known to be
associated with microinfarction, especially in the white matter, but in our previous
study MIs were not specified. Effects of microinfarction on T1-RTM values in the less
severely affected NAWM in subjects with MIs may be too small to be detected by our
methods, at the current field strength. In a post-mortem setting, 7T MRI has been
shown to provide better signal to noise ratios and a higher spatial resolution and even
to enable visualization of individual MIs. Therefore, using post-mortem T1-RTM at
higher field strengths might enable detection of more subtle MI associated changes,
for example in the NAWM or GM, eventually opening up additional possibilities for
detection of these lesions and their associated microvascular damage in vivo.

Different pathomechanisms for development of MIs and WMH have been suggested, although our results imply a close association between MIs and WMH, especially when
WMH is extensive. Whether MIs contribute to the heterogeneous tissue changes seen
as WMH on T2-weighted MRI, or that these changes precede microinfarction, cannot
be answered by our study. It has been suggested before, that MIs may contribute to
the aspect of WMH on T2-weighted MRI. Moreover, in line with our current findings,
MIs were only found in areas of confluent WMH and were not typically observed in less
severe forms of WMH, or the NAWM. Taken together, our present findings support
the notion that MIs are associated with more severe underlying microvascular
pathology, as evidenced by more severe WMH.

We found no univariate associations (model 1) between presence of MIs and mean
T1-RTM values of the NAWM, GM, WMH, or size of the (beginning) confluent WMH
in mm³. Adjusted for age, sex and subject group (model 2), MI presence showed an
interaction (p<0.05) with subject group, only for T1-RTM values of the WMH. A larger,
but non-significant, main effect of MI presence on higher T1-RTM values of the WMH
was found in the non-AD [p<0.10] than in the AD cases [p<0.10]. MI presence did not
influence T1-RTM values of the NAWM or GM. The effect of MI presence on the T1-
RTM values for these regions did not differ per subject group. Presence of MIs related
to a larger WMH size however, but only in non-AD cases [p<0.01].

Subsequently, we repeated these analyses with the number of the MIs per slice as the
independent variable (Table 3). No univariate associations were observed between
MI number and any of the imaging characteristics. After adjustment for age, sex and
subject group (model 2), still no effects of MI number on T1-RTM values of any tissue
type were found. Measuring WMH size however, MI number revealed an interaction
(p<0.05) with subject group, where higher MI numbers were associated with a larger
mean WMH size in the non-AD cases [p<0.05], but not in AD patients.

Discussion

In this comparative MRI-neuropathology study, we found that MIs were common
in brains of non-AD cases with signs of chronic cerebral small vessel disease and
seemingly even more so in AD patients. Statistically, presence of MIs had a larger
effect on T1-RTM values of the WMH in non-AD cases at 1.5T MRI. Furthermore,
presence and number of MIs related to larger confluent WMH surface areas in non-
AD cases.

Intriguingly, different effects of MIs were observed per subject group. Although we
achieved more statistical power using several slices per patient, we only included
2 non-AD cases with MIs and therefore these data probably should be interpreted
carefully. It is conceivable however, that in our 2 non-AD cases with signs of small

3). We found no univariate associations (model 1) between presence of MIs and mean
T1-RTM values of the NAWM, GM, WMH, or size of the (beginning) confluent WMH
in mm³. Adjusted for age, sex and subject group (model 2), MI presence showed an
interaction (p<0.05) with subject group, only for T1-RTM values of the WMH. A larger,
but non-significant, main effect of MI presence on higher T1-RTM values of the WMH
was found in the non-AD [p<0.10] than in the AD cases [p<0.10]. MI presence did not
influence T1-RTM values of the NAWM or GM. The effect of MI presence on the T1-
RTM values for these regions did not differ per subject group. Presence of MIs related
to a larger WMH size however, but only in non-AD cases [p<0.01].

Subsequently, we repeated these analyses with the number of the MIs per slice as the
independent variable (Table 3). No univariate associations were observed between
MI number and any of the imaging characteristics. After adjustment for age, sex and
subject group (model 2), still no effects of MI number on T1-RTM values of any tissue
type were found. Measuring WMH size however, MI number revealed an interaction
(p<0.05) with subject group, where higher MI numbers were associated with a larger
mean WMH size in the non-AD cases [p<0.05], but not in AD patients.

Discussion

In this comparative MRI-neuropathology study, we found that MIs were common
in brains of non-AD cases with signs of chronic cerebral small vessel disease and
seemingly even more so in AD patients. Statistically, presence of MIs had a larger
effect on T1-RTM values of the WMH in non-AD cases at 1.5T MRI. Furthermore,
presence and number of MIs related to larger confluent WMH surface areas in non-
AD cases.

Intriguingly, different effects of MIs were observed per subject group. Although we
achieved more statistical power using several slices per patient, we only included
2 non-AD cases with MIs and therefore these data probably should be interpreted
carefully. It is conceivable however, that in our 2 non-AD cases with signs of small

vessel disease on MRI, and a strong relation between MIs and severe WMH, MIs relate
to hypertensive vasculopathy rather than CAA, which is more frequently associated
with MIs in AD. The finding that 50% of the assessed AD patients exhibited moderate/severe CAA, but moderate/severe CAA was absent in non-AD cases, might support
this notion of different pathomechanisms, which should now be investigated in a
larger sample.

We found that the presence of MIs in the non-AD group was associated with more
abnormal WMH T1-RTM values and also with larger sizes of the (beginning) confluent
WMH. Therefore, MIs probably relate to more severe forms of small vessel disease.
We have previously shown that T1-RTM values relate to axonal and myelin loss and
also reflect microglial activation. These pathological processes are known to be
associated with microinfarction, especially in the white matter, but in our previous
study MIs were not specified. Effects of microinfarction on T1-RTM values in the less
severely affected NAWM in subjects with MIs may be too small to be detected by our
methods, at the current field strength. In a post-mortem setting, 7T MRI has been
shown to provide better signal to noise ratios and a higher spatial resolution and even
to enable visualization of individual MIs. Therefore, using post-mortem T1-RTM at
higher field strengths might enable detection of more subtle MI associated changes,
for example in the NAWM or GM, eventually opening up additional possibilities for
detection of these lesions and their associated microvascular damage in vivo.

Different pathomechanisms for development of MIs and WMH have been suggested, although our results imply a close association between MIs and WMH, especially when
WMH is extensive. Whether MIs contribute to the heterogeneous tissue changes seen
as WMH on T2-weighted MRI, or that these changes precede microinfarction, cannot
be answered by our study. It has been suggested before, that MIs may contribute to
the aspect of WMH on T2-weighted MRI. Moreover, in line with our current findings,
MIs were only found in areas of confluent WMH and were not typically observed in less
severe forms of WMH, or the NAWM. Taken together, our present findings support
the notion that MIs are associated with more severe underlying microvascular
pathology, as evidenced by more severe WMH.
Among strengths of the current study are, comparison of pathologically defined MIs using published criteria, on clinically and pathologically well-defined large hemispheric and mostly multiple slices per subject with post-mortem imaging. Furthermore, we used an MRI field strength that is currently common in most clinical settings. In doing so, we chose a pragmatic rather than an idealistic approach. In order to improve our detection, we used T1-RTM methods, which have been shown to be more sensitive to subtle tissue changes, such as microglial activation. Unfortunately, consensus guidelines to pathologically define and assess MIs are currently still lacking. We opted to use a recently published scoring method, offering a clear and practical definition of MIs using immunohistochemistry. However it has been suggested, that the increase in sensitivity by immunohistochemical staining may come at the cost of a decrease in specificity and that this method may detect (sub) acute MIs that may have occurred around time of death and are therefore unlikely to contribute to cognitive problems during life. As mentioned, a major limitation is the small sample size, especially with respect to the number of non-AD cases with MIs. The inherent insufficient power may explain lack of difference regarding MI prevalence between groups, while presence of one or more MIs in AD patients was twice as high as in non-AD cases. However, the observed prevalence for the 2 groups was comparable with two recent reports. Concerning the imaging results, one may argue that a 4mm MRI slice may contain many more MIs than could be observed on the 8-μm pathology slices alone. Therefore, the observed MI numbers on histopathological assessment present an underestimation of the true number of MIs in the imaged slices. Nevertheless, the observed MIs may serve as a marker to estimate true MI burden, and this underestimation is constant between cases. Using this marker we were able to find a relation between MI number and WMH size, at least in the non-AD cases.

To conclude, our findings suggest that MIs in the brain are associated with more severe forms of small vessel disease, mostly in non-AD, and that they might be indirectly detectable with T1-RTM at conventional field strengths. These findings may now open up new possibilities for in vivo detection at higher field strengths of these currently obscure, but clinically relevant lesions and their associated microvascular damage in the brain.

Acknowledgements
JDCG is supported by a grant from Stichting Dioraphte (VSM 09 01 2000).

Table 1: Demographic, clinical and neuropathologic characteristics per subject group

<table>
<thead>
<tr>
<th>Variables</th>
<th>Non-AD group (n=7)</th>
<th>AD group (n=11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>78±10</td>
<td>83±10</td>
</tr>
<tr>
<td>Sex (% male)</td>
<td>3 (43%)</td>
<td>3 (28%)</td>
</tr>
<tr>
<td>Hypertension*</td>
<td>1 (17%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Any vascular disease*</td>
<td>5 (83%)</td>
<td>5 (50%)</td>
</tr>
<tr>
<td>Neuropathology</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Post-mortem delay (h:min)</td>
<td>&lt;24 hours</td>
<td>6.09 ± 3.05</td>
</tr>
<tr>
<td>Fixation times (months)</td>
<td>2.6±1.2</td>
<td>2.3±0.9</td>
</tr>
<tr>
<td>Number of slices</td>
<td>2.3±1</td>
<td>2.9±1</td>
</tr>
<tr>
<td>Microinfarction</td>
<td>2 (29%)</td>
<td>7 (64%)</td>
</tr>
<tr>
<td>MIs per slice</td>
<td>0.62±1.1</td>
<td>0.72±0.89</td>
</tr>
<tr>
<td>Cortical MIs</td>
<td>2 (100%)</td>
<td>5 (71%)</td>
</tr>
<tr>
<td>Deep WM MIs</td>
<td>2 (100%)</td>
<td>5 (71%)</td>
</tr>
<tr>
<td>Lacunar infarction</td>
<td>1 (14%)</td>
<td>3 (27%)</td>
</tr>
<tr>
<td>Moderate/severe CAA*</td>
<td>0 (0%)</td>
<td>4 (55%)+</td>
</tr>
</tbody>
</table>

Data are represented as number of cases with the variable present (%), or mean±sd. Non-AD=non-demented cases without significant Alzheimer’s pathology, AD=Alzheimer disease cases, MIs=Microinfarcts, Cortical MIs=Microinfarcts in cortex and/or grey/white junction, Deep WM MIs=Microinfarcts in the deep white matter, CAA=cerebral amyloid angiopathy, any vascular disease=history of peripheral, coronary or cerebral atherosclerotic disease during life. *Availability of the data: hypertension and any vascular disease 16/18 (89%) and of CAA 13/18 (72%). ++p=0.037.
Table 2: estimated mean T1 relaxation times and size of the white matter hyperintensities per subject group with and without MIs.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Non-AD group</th>
<th>AD group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No MIs</td>
<td>MIs</td>
</tr>
<tr>
<td>T1-RTM (ms)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NAWM</td>
<td>353.9(23)</td>
<td>326.1(30)</td>
</tr>
<tr>
<td>GM</td>
<td>459.5(29)</td>
<td>440.0(37)</td>
</tr>
<tr>
<td>WMH</td>
<td>388.0(27)</td>
<td>490.4(43)+</td>
</tr>
<tr>
<td>WMH size (mm²)</td>
<td>75.5(27)</td>
<td>226.3(37)**</td>
</tr>
</tbody>
</table>

Values represent the estimated mean T1 relaxation time in milliseconds, or mean size of the white matter hyperintensities in square millimeters and their standard errors, estimated by mixed models based on mean age and adjusted for sex. T1-RTM = T1 relaxation time mapping values, No MIs = no microinfarcts observed, MIs = presence of at least one microinfarct, AD = Alzheimer disease, NAWM = normal appearing white matter, GM = grey matter, WMH = white matter hyperintensities.

+p<0.1, **p<0.01, both compared with non-AD cases without MIs.

Table 3: unadjusted and adjusted effects of MI presence and MI number on T1 relaxation times and WMH size

<table>
<thead>
<tr>
<th>Variables</th>
<th>MI presence</th>
<th>MI per slice</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Model 1</td>
<td>Model 2</td>
</tr>
<tr>
<td>T1 values</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NAWM</td>
<td>-15.9(30)</td>
<td>-27.8(28)</td>
</tr>
<tr>
<td>GM</td>
<td>-18.9(35)</td>
<td>-19.6(35)</td>
</tr>
<tr>
<td>WMH</td>
<td>1.1(38)</td>
<td>102.5(50)+</td>
</tr>
<tr>
<td>WMH size</td>
<td>40.1(29)</td>
<td>150.8(45)+</td>
</tr>
</tbody>
</table>

Values represent regression coefficients and their standard errors, estimated with linear mixed models. The dependent variables were T1 relaxation time mapping values for the different tissue types in milliseconds and WMH size in mm². When a significant interaction with subject group was observed, the regression coefficients and their standard errors are separately presented per group. MIs = microinfarcts, AD = Alzheimer disease, NAWM = normal appearing white matter, GM = grey matter, WMH = white matter hyperintensities. Model 1 = unadjusted, Model 2 = adjusted for age, sex, and subject group and interaction between MI presence (or MI number per slice) and group when necessary. += <0.1, *= <0.05, **= <0.01.

Figure 1 Immunohistochemical staining for HLA-DR of the parietal cortex in a 71-year-old female non-AD case, showing demarcated local increased presence of microglia (reddish brown) in close relation to a vessel [A]. Same lesion at higher magnification, with seemingly diminished perivascular cell density (blue/purple), suggesting tissue rarefaction and/or edema [B].
Microbleeds in Dementia: Connecting the dots

‘We know accurately only when we know little, with knowledge doubt increases.’

Johann Wolfgang von Goethe

General Discussion

Objective

In this thesis we aimed to gain novel insights into the radiological construct of MBs in dementia in relation to: I clinical relevance, II underlying vasculopathies and associated pathologies and III novel imaging techniques.

In this chapter, the main observations are summarized and reviewed in the context of the current knowledge. Subsequently, in order to aid interpretation, methodological issues will be addressed. Finally, the possible clinical implications of our findings are presented, together with recommendations for further research.

Summary and interpretation of main findings

Part I Clinical Relevance

Several former studies did not find any associations between MBs and cognition in AD patients,\(^1\,^2\) in contrast to non-AD populations, often with higher MB burdens.\(^3\,^4\) Possibly, most AD studies were hampered by insufficient power as they did not include enough cases with a substantial number of MBs to measurably add to the effects on cognition of advanced neurodegenerative disease. We therefore took a different approach, attempting to maximize the supposed MB associated effect. We did this...
Microbleeds in Dementia: Connecting the dots

Determinants predicting incident MBs were MBs at baseline, but also MRI signs of small vessel disease, i.e. WMH grade and lacunar infarcts, predicted incident MBs. This was more prominent for nonlobar MBs, whereas smoking related more to strictly lobar MBs. In addition APOE ε2, a risk factor associated with CAA related hemorrhage, was also associated with incident MBs.

From these studies concerning the relevance of MBs in dementia, we may conclude that MBs or the underlying pathology they represent, can contribute to cognitive deficits, when severe enough. It is debatable however whether the subtle effects on cognition of one or a few MBs are relevant when the devastating effects of advanced AD pathology are already present. This is indirectly supported by the observation that in most studied populations, for example healthy elderly or stroke survivors, a relation between MBs and cognition has been observed, but typically not when AD is diagnosed.

Part II Underlying vasculopathies and associated pathologies

The findings of two studies in part I already hinted at a possible relation between MBs and amyloid pathology. In the first study (chapter 2.1) we found that the CSF levels of AD patients with multiple MBs were lower than in AD patients without MBs. Decreased CSF levels of Aβ are generally held to reflect increased intracerebral amyloid deposition. Whether increased amyloid deposition in AD patients with MBs, as indicated by CSF abnormalities occurs mainly in plaques or in cerebral small vessels, known as CAA, is not known. Furthermore, we found that both APOE ε4 (chapter 2.1) and APOE ε2 (chapter 2.3) were related to MBs. In addition to the consistently observed predominantly lobar MB pattern in our AD patients, these genetic findings may further support a relationship of these MBs with CAA. Moreover, a study in sporadic non-demented CAA patients, with large intracerebral hemorrhages (ICH), which usually presents without significant amyloid plaques, corroborated our CSF findings, showing that CSF levels of Aβ were both decreased compared with controls and even with AD patients, suggesting intravascular amyloid deposition.

Although prevalence of MBs in AD and risk factors have been investigated, data on occurrence of new MBs from longitudinal studies in AD were lacking. Studying MB incidence is important for several reasons. First, the known risk factors were mostly derived from cross-sectional studies and longitudinal assessment may provide more evidence for any causal relationships. Second, since trials with amyloid-lowering therapy reported occurrence of incident MBs, an ample need arose for data on the natural MB incidence in AD. We studied a cohort of over 250 memory clinic patients with a mean follow-up of approximately 2 years. We found a two-year incidence of one or more new MBs of 12% (same estimate in total population and in AD patients only; chapter 2.3). Baseline and incident MBs were not associated with cognitive decline over time. Determinants predicting incident MBs were MBs at baseline, but also MRI signs of small vessel disease, i.e. WMH grade and lacunar infarcts, predicted incident MBs. This was more prominent for nonlobar MBs, whereas smoking related more to strictly lobar MBs. In addition APOE ε2, a risk factor associated with CAA related hemorrhage, was also associated with incident MBs.
levels in plasma have been found to be higher in patients with signs of small vessel disease.\textsuperscript{11} We therefore set out to study levels of both amyloid peptides in CSF and plasma, at the same time assessing blood-brain barrier (BBB) integrity as measured by albumin ratios in AD patients with and without MBs, but also in patients with vascular dementia (VaD; mostly with MBs), using patients with subjective complaints without MBs as controls. In this study (chapter 3.1) we replicated our previous observation, by showing that MBs in AD were associated with additional decreases in CSF levels in an independent sample with any number of MBs. However, no relation between MBs and Aβ40 levels was found in AD. This may be explained by a less advanced stage of CAA, known to be associated with less Aβ40 deposition relative to \textsuperscript{12} or alternatively by a different subtype of CAA in AD. In VaD subjects however, CSF levels of Aβ40 were decreased, whereas in plasma Aβ40 levels were non-significantly increased, together with signs of BBB dysfunction, which seems to suggest leakage from CSF to the circulation. Subsequently, in the PET study, we zoomed in on the BBB function using verapamil (chapter 3.3). We did not find any differences in P-glycoprotein transporter function between AD patients with and without MBs. The finding that MBs in AD are not associated with any measurable BBB changes, may thus provide validation for previous studies using amyloid imaging in relation to MBs, which neglected the possibility of BBB effects. Finally, we had the opportunity to directly link the clinical observation of high numbers of MBs on MRI during life to post-mortem evaluation in a case study. We evaluated a case with rapidly progressive dementia and multiple “stroke-like” or “seizure-like” episodes, with innumerable MBs at MRI as most prominent imaging abnormality, suggesting CAA. In the absence of major pathology related to AD, other dementias or macroscopic infarcts or hemorrhages, this clinical and radiologic diagnosis was neuropathologically confirmed and further refined as severe capillary CAA with dyshoric changes and multiple microinfarcts with and without surrounding iron depositions. Extending on the conclusions of part I, this case, again as a proof-of-principle, showed that not only cognitive deficits, but even severe dementia may arise from a vascular amyloidopathy, diagnosable during life using MB pattern on MRI, notably in the absence of other pathologies explaining the decline. Furthermore, this case provided direct neuropathological evidence that even without classic plaques, perivascular amyloid can result in measurable CSF changes. This may suggest that the extremely low CSF amyloid levels in AD patients with MBs, reported in part II, indeed partially reflect vascular deposition of amyloid.

From the multi-disciplinary evidence presented in this part, we conclude there is a robust link between (lobar) MBs and amyloid pathology in dementia. In addition, we found no measurable effects reflecting BBB dysfunction associated with MBs in AD, potentially rendering arterial sampling obsolete in these patients, although this may differ for VaD patients.

**Part III Novel techniques in microvascular imaging**

From studies in parts I&II, we have concluded that MBs in an abundant quantity may relate to cognition and are associated with amyloid pathology, especially when their location is lobar. These findings stress the importance of accurate MB detection in diagnosing, following and experimentally treating memory clinic patients.

In chapter 4.1 we found that the new SWI post-processing method detected more MBs than the conventional method at the same scanner in the same patients at the same time. No substantial differences in associations with imaging and patient characteristics were found however. Another technical development, which has been shown to be highly sensitive in detecting MBs is ultra-high field strength MRI.\textsuperscript{13} In a sample of young AD patients, 7T scanning did not reveal significantly more MBs compared with 3T, although stronger relations of MBs with age and APOE ε4 were observed, indicating clinical relevance of the additional lesions. Probably, the gain in MB detection of ultra-high field and SWI imaging, does not reflect artefacts, but rather reflects true consequences of an underlying bleeding-prone vasculopathy.\textsuperscript{14} This is further supported by findings in an elderly population, reporting risk factors of patients with MBs on an advanced technique were more similar to those of patients with MBs on conventional scanning than patients without MBs.\textsuperscript{15} The additional MBs seen with the advanced technique only, were not associated with any of the studied risk factors, however. Moreover, the total MB load at the advanced scanning technique in that study was found to predict future MBs more accurately, supporting the concept of a true underlying bleeding-prone vascular state. Findings from our work and of
Approximately half of our memory clinic population is under the age of 65. Therefore, the prevalence of MBs we report may be lower than in other studies typically describing older AD patients. Although, this likely limits the generalizability of our findings, our population offers a unique opportunity to study relatively pure AD patients, compared to older populations probably presenting with more mixed disease. Another issue that comes with any outpatient clinic, is the notion that follow-up may not include patients with extremely fast decline, severe disability or short survival, which prevents them from returning. This healthy survivor effect may have affected the longitudinal studies reported in this thesis, hereby possibly underestimating the adverse clinical effects of MBs. Another possible source of selection bias, are the exclusion criteria we inevitably used during our dementia work-up. For example, pacemakers are a frequent cause of MRI exclusion. This is unfortunate because cardiovascular disease, dementia and MBs share many risk factors. For lumbar puncture, oral anti-coagulation use presented an exclusion criterion. Again, these patients likely have vascular disease making them very interesting to study. However, the relations of MBs with CSF abnormalities we have reported may rather reflect true vasculopathy than an enhanced bleeding state by medication. In addition, the relatively infrequent diagnosis of VaD in our memory clinic and the age of our population may lead to an underestimation of the presence and role of hypertensive vasculopathy in dementia.

**Methodological considerations**

**Selection of patient population**

MBs have most consistently been found to be associated with age. As a specialized center focused on patients with early onset dementia, we attract many young patients.
a cut-off of 8 or more MBs, to define the multiple MB group. This value reflected
the top 5% of AD patients with the highest MB numbers and as a result most likely
represents the most severe underlying vasculopathy. Moreover, when we used 5 and
10 MBs as cut-off values, results were essentially comparable.

In the chapters comparing different modalities, we were not able to separate field
strength from other parameters as echo time, in-plane resolution and slice thickness,
also known to influence MB detection. Another technical limitation may be that in 2
studies we excluded infratentorial regions, due to insufficient coverage. Pathologists
consider CAA moderately extensive when vessels in the cerebellum are affected.
From chapter 2.1 we know that even in the AD patients with the most extended CAA
based on MRI, only a fraction of the MBs resides in the cerebellum. Furthermore, the
brainstem is not considered to show CAA related MBs. In contrast, MBs associated
with hypertensive vasculopathy may occur in both infratentorial regions and as such
may be underestimated in these studies.

Although we have ample experience and consistently used well-defined criteria, MB
counting is not an exact science, and thus remains subjective to a certain extent.
We have reported excellent inter- and intrarater reliabilities on different modalities,
deriving consistency of our rating. Whenever possible, we used multiple raters in
consensus to reach higher levels of ascertainment.

MBs are a radiologic construct and they may not always reflect neuropathologically
defined sites of (past) bleeding. In recent years, the number of radiologic-pathologic
correlation studies has grown, but they are still relatively limited. Additionally,
although evidence that radiologic MBs represent past bleeding has accumulated, it is
not yet conclusive. Numerous technical radiologic, but also pathologic factors and
MB mimics (such as calcium deposits) potentially contribute to this current lack of full
agreement. A study using more advanced SWI MRI techniques in AD patients concluded
that all hypointense foci on MRI were found to reflect several pathological CAA
related phenomena. These lesions were found to represent recent or past bleeding,
microaneurysms, but importantly also microinfarcts with secondary hemorrhage,
which all related to amyloid laden vessels. Findings from our own pathologic case
study, corroborated that MRI defined MBs, may be pathologically heterogeneous, as
they may also represent microinfarcts associated with hemosiderin.

Clinical implications
In this paragraph we will discuss possible implications of our work regarding
diagnosing, performing clinical trials, potentially treating and finally preventing
dementia.

MBs in diagnosing dementia
Although even at 7T, MBs were only found in a minority of AD patients, presence of
specific MB patterns, readily observable on conventional clinical scanners, may still
aid the clinician in diagnosing dementia. Studies in this thesis may suggest that a
lobar MB pattern in dementia patients may be a non-invasive marker of severe CAA.
In patients suspected of dementia, lobar MBs may therefore favor the diagnosis of
an amyloidopathy, most frequently AD, but Lewy body dementia, or even capillary or
dyshoric CAA alone, as described in this thesis, may also be considered. Conversely,
a pattern of multiple nonlobar MBs may provide further support of hypertensive
vasculopathy, supporting a suspected diagnosis of vascular dementia or mixed
pathology in AD. However, MBs are currently not included in the most widely used
criteria of VaD. Nonetheless, MBs are increasingly recognized as a marker of small
vessel disease. Moreover, as various studies have demonstrated, MBs may relate
to cognition, especially in non-AD populations. Possibly as an advantage over the
traditional small vessel disease markers, WMH and lacunar infarcts, MBs have an
additional value as their location may indicate specific etiology, i.e. hypertensive
vasculopathy or CAA.

MBs and bleeding risk
A very important clinical consequence of MBs, not within the scope of this thesis, but which
definitely deserves future attention, is stroke risk especially in association with use of
antithrombotic medication. Although some studies suggest MBs and use of antithrombotics
are associated with higher stroke risk, causation is currently not confirmed.
Microbleeds in Dementia:
Connecting the dots

The precise mechanism of how MBs relate to cognition remains unclear, although several hypotheses exist. They may have a direct effect on the surrounding tissue and as such disturb networks, although it is debated whether these small hemosiderin deposits are capable of causing tract degeneration, or they may also directly cause a functional disturbance of their surroundings.27 More widely propagated however, is the notion that the mechanisms are more indirect, involving narrowing of the small vessels and microischemic damage, or they may merely mark the severity of the underlying small vessel disease, which is responsible for cognitive problems.27 Nevertheless, we found evidence for a relation of multiple MBs with cognition in AD patients. Although MBs were not found to predict measurable future decline in AD, it is unlikely that incident MBs would have beneficial effects on cognition either, as for example in MCI patients MBs have been shown to predict progression to dementia.28, 29 An explanation could be that MBs, reflecting CAA, are more closely linked to the upstream phenomenon of advancing amyloid pathology,30 and therefore hardly have any relation with cognition later in the course and, similar to plaques which are closely associated with AD pathogenesis, but have a relatively low correlation with cognitive status.31 Furthermore, MBs in AD and other populations have also been associated with mortality.7, 8 For these reasons, reducing the vasculopathy causing MBs may be highly desirable as early as possible. Findings from our MB incidence study indicated that vascular risk factors and signs of related small vessel disease might cause MBs in memory clinic patients. Strikingly, past smoking was associated with less incident MBs compared to current smoking. This may suggest that cessation of smoking and possibly reduction of other modifiable risk factors, will result in a lower future total MB burden. Whether this supposed MB reduction, subsequently results in clinical relevant benefits, needs confirmation.

Future directions
Clinical relevance of MBs and associated microangiopathies
The effect of MBs on cognition, if any, may be a phenomenon most evident early in the disease process, when the relatively subtle effect of MBs has not yet been obscured by massive neurodegeneration. Therefore, it would be interesting to assess the relation

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**MBs in AD trials and treatment**

Another relevant implication of our work concerns current amyloid-lowering clinical trials. As we provided evidence that MBs are a natural occurring phenomenon in AD, with an estimated natural incidence of around 6%, not all MBs observed after vaccination may be therapy induced. These data were used in response to a cautionary letter from the Food and Drug Administration these trials, which proposed all patients with any number of baseline or incident MBs should be excluded (chapter 1.2). The recent recommendations from the Alzheimer’s Association Research Roundtable Workgroup are indeed more liberal regarding MBs, in order to include and retain more patients in trials.20 This approach may allow roughly one quarter of AD patients to additionally benefit from potential future treatment. At the same time, patients with MBs should be carefully monitored as they likely have significant pre-existent vascular amyloid loads, further complicated by the frequent presence of APOE ε4,23 an identified risk factor for vasogenic edema, MBs or superficial siderosis.22 Several mechanisms have been proposed. First, a mechanical explanation, the breakdown of neuronal plaques produces a temporary amyloid overload at the vessels, leading to congestion of the perivascular drainage pathways and increased deposition in the vessel wall, eventually causing more severe CAA with associated vessel wall fragility.24 Alternatively, direct removal of pre-existing amyloid deposits from the vessel wall may lead to increased permeability. Local inflammatory responses, for example Fc receptor/microglia mediated phagocytosis,25 may also cause the increased permeability, which leads to edema and MBs.

Current recommendations to treat patients with MBs are lacking.26 Currently, no specific treatment for CAA exists, but there is also no guideline to treat patients with MBs indicating hypertensive vasculopathy, with potentially more treatment options either. Studies are needed to assess whether strict management of vascular risk factors in AD patients with MBs will be beneficial. Furthermore, little is known about differences in response to cholinesterase inhibitors, or other symptomatic therapeutic options in AD patients with MBs specifically.
of MBs with cognition in earlier disease stages such as MCI, but possibly even also earlier in patients with subjective complaints. As MBs in MCI patients have been found to predict progression to dementia,\textsuperscript{26,27} it would be interesting to assess if MBs in even earlier stages would predict later cognitive decline. Furthermore, pattern of MBs in these early stages may identify underlying vasculopathy and may forecast progression to different types of dementia, as suggested previously.\textsuperscript{26}

The relation of MBs with amyloid in memory clinic patients should be further established. Similarly, the role of superficial siderosis in a memory clinic setting as a marker of amyloidopathy should be explored, preferably by using CSF and PET as amyloid biomarkers in large samples of memory clinic patients. If, and when, this relation is robustly confirmed, the clinician may want to reconsider more invasive and expensive CSF or PET evaluation to confirm amyloid abnormalities, in patients who already show hemorrhagic signs of CAA. Furthermore, in patients with dementia, a positive amyloid biomarker alone to diagnose AD should be interpreted with caution. T2*-weighted MRI may detect an alternative explanation for the abnormalities and CSF tau and ptau may be of additional value since they may further support the differentiation between AD and CAA without neurodegenerative characteristics.

The CAA in AD patients however, may differ from sporadic CAA. Clinically, sporadic CAA presents with ICHs rather than with dementia as opposed to AD. Furthermore, findings from our CSF study suggested biochemical differences as well, since MBs in AD were not related to CSF Aβ40 levels in contrast to sporadic CAA patients.\textsuperscript{10} Studies in this thesis however, showed that in AD lobar MB were found to be related to in CSF, which also happens to be more closely related to capillary CAA (capCAA) pathologically.\textsuperscript{32} Furthermore, capCAA has been found to be more common in AD than CAA which not involves the capillaries and was associated with AD pathology, whereas non-capCAA was not.\textsuperscript{23} Moreover, capCAA, especially with dyshoric changes, has been proposed to contribute to clinical dementia,\textsuperscript{24} this notion is possibly further corroborated by our case report, linking dementia to this specific vascular condition, notably in the absence of other explanations for the dementia. Currently, without biopsy, no in vivo diagnosis of this specific subtype can be made however. Therefore, we hypothesize that the role of capCAA is probably underestimated in AD, or at least in a subgroup. The combination of ultra high-field imaging and pathologic evaluation can potentially further explore this emerging notion. Another promising development is visualization of amyloid plaques at ultra-high field strength MRI as they contain iron.\textsuperscript{35} At high enough resolution, the interplay between MBs, (peri)vascular/-capillary amyloid and plaques may be simultaneously observed. As MRI is non-invasive and harmless, repeated scanning will possibly allow the dynamic course of amyloid deposition and removal. In addition, microinfarcts could also be included in this complex puzzle, as the relation with MBs and other small vessel disease phenomena as WMH, lacunar and microinfarcts needs further elucidation.

Including MBs in the VCI/VaD criteria may need to be considered. Although currently no strict cut-offs, due to heterogeneity in the imaging data, can easily be given, we have some suggestions for future criteria. Presence of multiple definite MBs, preferably scored using a validated rating scale, may be a first criterion. Probably, grading the number of MBs should be additionally used to assess severity or probability of the vascular cognitive impairment (VCI) or VaD. Although, the cut-offs will be arbitrary and heavily technique dependent, one could think of some sort of crude categorization. For example, 0-1 MBs=normal, 2-10 MBs=mild, 10-50 MBs=moderate and >50MBs=severe MB burden. Studying large samples with patients with these amounts of MBs, could validate if indeed severity or likelihood of VaD relates to these proposed stages. Secondly, the anatomical distribution pattern of MBs should be appreciated. A strictly lobar MB pattern and probably also superficial siderosis may indicate a probable CAA as specific cause of the VCI/VaD. Although, the cut-offs will be arbitrary and heavily technique dependent, one could think of some sort of crude categorization. For example, 0-1 MBs=normal, 2-10 MBs=mild, 10-50 MBs=moderate and >50MBs=severe MB burden. Studying large samples with patients with these amounts of MBs, could validate if indeed severity or likelihood of VaD relates to these proposed stages. Secondly, the anatomical distribution pattern of MBs should be appreciated. A strictly lobar MB pattern and probably also superficial siderosis may indicate a probable CAA as specific cause of the VCI/VaD.\textsuperscript{26} Furthermore, a mixed MB pattern may suggest possible hypertensive vasculopathy or mixed vasculopathies (hypertension and CAA), whereas strictly deep MBs may rather reflect a probable hypertensive vasculopathy underlying the clinical condition. Using MB distribution patterns could thus provide information about the specific location on the VCI/VaD spectrum, which ranges from pure hypertensive vasculopathy, via mixed vasculopathies to pure CAA. This categorization could be confirmed using amyloid biomarkers. This may be clinically relevant, as future management of these etiologically different VaD subgroups may differ considerably. Additionally, MB number and pattern might be combined with the traditional small vessel disease
markers (WMH and lacunes) to ascertain a vascular cause of the MBs, rather than being a posttraumatic phenomenon for example. Furthermore, the combination of these 3 small vessel disease markers may be used to more carefully estimate the radiologic severity of VCI/VaD due to small vessel disease.

Furthermore, since guidelines to treat patients with dementia and MBs, regarding stroke risk are currently lacking, associations of MBs and stroke incidence in memory clinic cohorts and the general population should be determined, preferably by large studies with long follow-up duration.

**Advanced imaging**

As work in this thesis on developments in MB imaging has demonstrated, both SWI and higher field strength are promising developments with respect to MB detection. Probably, a combination of these factors would be ideal and could further optimize MB detection. As feasibility of this combination may be limited, we recommend the cheaper option of upgrading to a SWI, or a similar, sequence for future studies and clinical use, as improvement in MB detection has repeatedly been reported. These modalities offer the possibility of detecting MBs even smaller than 1 millimeter, possibly including pericapillary hemosiderin deposits. If this notion will be validated, we may have a unique tool to study capCAA in vivo and have a better opportunity to study its presence and role (clinically, but also pathophysiologically) in AD. An expected challenge may be the notion that with every decline in size, possibly also the certainty level of a MB will decrease. Clinical relevance of every new MB detection method should therefore be carefully established before clinical implementation, or decision making otherwise, is being employed. Another promising tool in MB detection is semi-automated MB detection, offering practical benefits and may possibly add more objectivity and uniformity to the MB field. More insight into cognitive functioning associated with microangiopathy and MBs could come from studies assessing vasoreactivity and perfusion, using arterial spin labeling for example. Furthermore, using quantitative MRI at higher field strengths may possibly be able to find subtle microangiopathy associated tissue changes and can be valuable in our understanding of these diseases. Although these ongoing developments in imaging of microangiopathy can potentially provide a wealth of information, they may simultaneously limit the development of uniform guidelines and as such present a challenge in data harmonization.

**Clinical trials**

The current amyloid-lowering trial FDA guidelines do no longer exclude MBs, which grants us the opportunity to study the long-term effects of treatment induced MB incidence on cognition and survival. Another possibility would be to find out whether the supposedly temporary rise in vascular amyloid will indeed be removed as treatment is being prolonged, as has been suggested. Furthermore, an intriguing observation in these trials is that regions with MRI signs of increased vascular permeability showed the most amyloid clearance. Studying these mechanisms, may possibly also offer more insight into the natural processes of the complex amyloid metabolism.

In conclusion, this thesis advances the knowledge about MBs in dementia, with a focus on AD. We showed MBs or their underlying pathology may relate to the clinical presentation of AD and moreover to the supposed pathophysiology of the disease. Furthermore, by also identifying modifiable risk factors and natural occurrence of MBs, the work potentially has important clinical and therapeutic implications. With the ongoing developments in MR and molecular imaging, unraveling the complex interactions between MBs, parenchymal and vascular amyloid, hypertensive vasculopathy and ultimately their relation to cognition and other health outcomes, will become important and potentially feasible scientific goals.
Microbleeds in Dementia: Connecting the dots

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Microbleeds in Dementia:
Connecting the Dots

Opzet
Om deze inzichten te verkrijgen, worden in dit proefschrift de op MRI geobserveerde microbloedingen in verband gebracht met mate van dementie en cognitieve achteruitgang over de tijd. Het optreden van nieuwe microbloedingen wordt tevens in kaart gebracht, alsmede de risicofactoren voor het ontwikkelen van microbloedingen. In het tweede deel wordt gekeken naar microbloedingen en hun mogelijke onderliggende vaatpathologie, door middel van een combinatie van beeldvormend onderzoek (MRI en PET scans), klinisch chemische biomarkers in het hersenvocht en bloed en tot slot pathologisch hersenweefselonderzoek. Het laatste deel van het proefschrift onderzoekt de waarde van diverse nieuwe MRI technieken, wat betreft het aantonen van microvasculaire schade en de mogelijke betekenis.

Nederlandse Samenvatting

Microbloedingen bij dementie; "Connecting the Dots"

Achtergrond
De ziekte van Alzheimer (AD) is een zeer omvangrijk probleem en dit zal de komende jaren onverminderd toenemen. Een therapie is er momenteel immers niet, evenals een sluitende verklaring voor het ontstaan van de ziekte. De populairste hypothesen zijn de amyloid cascade hypothese, uitgaande van een pathologische neerslag van het amyloid eiwit in de hersenen, met het verdere ziekteproces tot gevolg. Een ander verklarend model is de vasculaire hypothese, waar de hersenvaten een belangrijke rol spelen in het ontstaan van de ziekte. Op het snijvlak van deze theorieën bevinden zich mogelijk de microbloedingen (MBs), die recent, door de introductie van nieuwe MRI technieken, als zwarte puntjes in de hersenen van dementiepatiënten zichtbaar geworden zijn.

Doel
Het vergaren van nieuwe inzichten betreffende de microbloedingen bij dementie, opgesplitst in 3 hoofdthema’s: I klinische betekenis, II onderliggende pathologie en geassocieerde pathologie, III nieuwe beeldvormende technieken.

Resultaten per hoofdstuk

Deel I geassocieerde klinische factoren in een geheugenpolikliniekpopulatie
In hoofdstuk 2.1 beschrijven we een aanmerkelijk verschil in globale cognitie, gemeten met de MMSE schaal, tussen AD patiënten zonder MBs en patiënten met multipale MBs, met een verder vergelijkbare leeftijds- en geslachtsverdeling. De bevindingen in dit “proof-of-principle” stuk ondersteunden de hypothese dat MBs geassocieerd zijn met een verminderd cognitief functioneren. Het is daarom denkbaar dat AD patiënten met MBs een agressiever ziektebeloop, met een snellere cognitieve achteruitgang en kortere overleven kennen dan patiënten zonder MBs. Na het bestuderen van een grote groep AD patiënten, bleek echter dat er geen relatie bestond tussen de MBs op tijdstip 0 en de mate van cognitieve achteruitgang (hoofdstuk 2.2). Het voorkomen van MBs in AD patiënten was herhaaldelijk onderzocht, maar het optreden van nieuwe MBs (incidentie) was nog niet onderzocht. Hierdoor bestudeerden we een cohort van 254 patiënten van onze geheugenpolikliniek met een gemiddeld follow-up van circa 2 jaar en we vonden dat 12% van alle mensen in deze periode 1 of meer MBs ontwikkelde, het percentage voor AD patiënten kwam hiermee overeen (hoofdstuk 2.3). De belangrijkste voorspeller voor nieuwe MBs op de tweede scan, was de aanwezigheid van MBs reeds bij aanvang, maar ook andere tekenen van schade.
van de kleine hersenvaten op MRI en risicofactoren op hart- en vaatziekte hadden voorspellende waarde. APOE ε2, tevens een genetische risicofactor voor grote hersenbloedingen veroorzaakt door cerebrale amyloid angiopathie (CAA), was een andere factor die samenhang met incidentie van MBs.

**Deel II onderliggende vaataandoeningen en geassocieerde pathologie**

In de studie naar cognitie bij AD patiënten met multipele MBs in hoofdstuk 2.1, observeerden we reeds dat waarden in het hersenvocht, een afspiegeling van amyloid eiwit in de hersenen, een verhoogde amyloidneerslag in de hersenen suggereerden. Of deze toegenomen neerslag optreedt tussen de hersencellen in zogenaamde plaques, of in de vaatwand, bekend als CAA, was echter niet duidelijk. Daarom onderzochten we de waarden van dit amyloid in twee vormen in zowel bloed als hersenvocht, terwijl we ook de integriteit van de bloed-hersenbarrière bepaalden middels een zogenaamde albumineratio. We vergeleken deze uitkomsten tussen AD patiënten met en zonder MBs, patiënten met vasculaire dementie (VaD) en personen zonder dementie en vrij van MBs. In dit hoofdstuk (3.1) vonden we opnieuw een relatie tussen MBs en waarden in het hersenvocht van patiënten met dementie. In AD patiënten vonden we echter geen relatie tussen MBs en de andere variant van het amyloid eiwit [Aβ40] in het hersenvocht, of beide varianten [Aβ42 en Aβ40] in het bloed, ook was de bloed-hersenbarrière niet meetbaar aangetast. In VaD patiënten echter, vonden we dat MBs samenhangen met verlaagde Aβ40 waarden in hersenvocht, maar tegelijkertijd een verhoging hiervan in het bloed lieten zien, met aanwijzingen voor een verminderde bloed-hersenbarrière functie, wat mogelijk lekkage van deze eiwitten naar het bloed suggereert. In ons Verapamil PET scanonderzoek [hoofdstuk 3.2], hebben we kunnen kijken naar een ander aspect van de bloed-hersenbarrière, namelijk de PgP-functie. Dit eiwit heeft als functie, afbraakproducten uit de hersenen te transporteren. Hoewel eerdere studies een verminderde functie vonden bij patiënten met amyloid in hun hersenventrikel, een belangrijke oorzaak van MBs, hadden onze AD patiënten met en zonder MBs echter vergelijkbare transportfuncties. In hoofdstuk 3.3 hadden we de mogelijkheid om van een patiënt met klinische een bijzondere vorm van dementie en op de MRI scan talloze MBs, na overlijden de hersenen pathologisch te onderzoeken. Deze casus vormde het pathologische bewijs, dat amyloid in de kleine vaten en haarvaten, zonder andere grove met dementie geassocieerde pathologische afwijkingen, niet slechts kunnen bijdragen aan cognitieve klachten, zoals in deel I reeds gesuggereerd, maar zelfs tot volledige dementie kan leiden. Bovendien toonde de casus aan dat zelfs in afwezigheid van amyloidophopingen tussen de hersencellen (plaques), slechts het vaatgebonden amyloid de in deel II beschreven hersenvochtafwijkingen kan geven.

**Deel III Nieuwe beeldvormende technieken voor microvasculaire schade**

In hoofdstuk 4.1 beschreven we dat de nieuwe MRI techniek, susceptibiliteitsgewogen beeldvorming (SWI), meer MBs in beeld bracht dan de conventionele methode. De verbeterde detectie leidde echter niet tot substantiële verschillen in klinische of radiologische associaties, die doorgaans met MBs worden gevonden. Naast deze instellingsverbeteringen, bestaan er tegenwoordig MRI scanners met magneten van ultrahoge veldsterkte, die bijzonder gevoelig zijn voor het detecteren van MBs. In een groep jonge AD patiënten vonden we dat er niet significant veel meer MBs gevonden werden dan op een minder sterke magneet en dus zelfs met deze techniek slechts een minderheid van de patiënten MBs hadden, maar dat er een verbeterde relatie was tussen microbloedingen enerzijds en leeftijd en een genetische risicofactor voor CAA (APOE ε4) anderzijds (hoofdstuk 4.2). Een andere uiting van microvasculaire schade die recentelijk veel aandacht krijgt, zijn de microinfarcten, daar zij sterk lijken samen te hangen met dementie, maar tijdens het leven niet zichtbaar zijn met de conventionele beeldvorming van de hersenen. Op een standaard scanner hebben we door middel van geavanceerde kwantitatieve scanmethoden neuropathologisch gedefinieerde microinfarcten zichtbaar proberen te maken in hersenplakken van overleden AD patiënten en patiënten zonder dementie, maar met vasculaire schade. In deze radiologisch-pathologische correlatiestudie vonden we geen effect van microinfarcten op weefseleigenschappen gemeten met kwantitatieve methoden in AD hersenplakken. In de gevallen zonder dementie, maar met tekenen van schade aan de kleine hersenvaten, bleken de microinfarcten geassocieerd met het aspect en de grootte van zichtbare afwijkingen in de witte stof, wat een relatie suggereert tussen microinfarcten en een verder gevorderde aandoening van de kleine hersenvaten. In hoofdstuk 5 worden deze bevindingen samengevat en geïnterpreteerd in het

**Microbleeds in Dementia: Connecting the dots**

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Conclusie
Dit proefschrift heeft nieuwe kennis opgeleverd over de rol van MBs binnen dementie en AD in het bijzonder. Uit het eerste deel van dit proefschrift, kan geconcludeerd worden, dat de klinische betekenis (in het kader van cognitief functioneren) van MBs of de onderliggende vaatafwijkingen is, dat zij mogelijk bijdragen aan een verminderd cognitief functioneren van AD patiënten, maar waarschijnlijk vooral bij relatief ernstigere gevallen van deze vaatschade. De onderliggende vaatafwijking van MBs in het kader van AD, is waarschijnlijk overwegend CAA, zoals de resultaten van het werk uit dit deel van het proefschrift indirect hebben aangetoond door middel van de sterke samenhang met het in hersenvocht gemeten amyloid en genetische risicofactoren. Ook hebben we laten zien dat beïnvloedbare risicofactoren nieuwe MBs kunnen voorspellen, dus mogelijk een aangrijpingspunt voor behandelingen kan opleveren. Hoewel de klinische interpretatie steeds goed onderzocht dient te worden, impliceren de resultaten aangaande de nieuwe detectiemethoden, dat verschillende technieken veelbelovend zijn in het vroegtijdig opsporen van potentieel relevante schade van de kleine hersenvaten. In de toekomst zal het bestuderen van de belangrijke doch complexe interacties tussen MBs, amyloid in het hersenweefsel en de vaten en hypertensieve vaatschade uiteindelijk in relatie tot het functioneren van de patiënt, mogelijk worden.

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‘I never did a day’s work in my life- it was all fun.’

Thomas Edison
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28. A. Schuitemaker: Inflammation in Alzheimer’s Disease: in vivo quantification (27-01-12)
29. K. Joling: Depression and anxiety in family caregivers of persons with dementia (2-4-12)
30. W. De Haan: In a network state of mind (02-11-12) (Cum Laude)
31. D. Van Assema: Blood-brain barrier P-glycoprotein function in aging and Alzheimer’s disease (07-12-12)
32. J. D.C. Goos: Microbleeds in Dementia: connecting the dots (6-2-13)
List of publications


Goos, J.D.C.; van Rooden, S.A.; Versluis, M.J.; van der Grond, J.; Prins, N.D.; Scheltens, P.; van Buchem, M.A.; Barkhof, F.; van der Flier, W.M. Microbleeds in Alzheimer disease using 7T imaging; approaching the true number? Under revision


Goos, J.D.C.; van Assema, D.M.E.; Yaqub, M.; van der Flier, W.M.; Scheltens, P.; Barkhof, F.; Lammertsma, A.A.; Boellaard, R.; van Berckel B.N.M. Lobar Microbleeds in Alzheimer Disease a relation with regional amyloid load? Microbleeds “hot or not”? In preparation

Goos, J.D.C.; Zonneveld, H.I.; Wattjes, M.P.; Lemstra, A.W.; Scheltens, P.; van der Flier, W.M. The prevalence of cortical superficial siderosis in a memory clinic population. In preparation

van Rooden, S.A.; Goos, J.D.C.; van Opstal, M.J.; Versluis, M.J.; Webb, A.G.; Blauw, G.J.; van der Flier, W.M.; Scheltens, P.; Barkhof, F.; van Buchem, M.A.; van der Grond, J. Large numbers of small cortical hyperintense lesions in Alzheimer’s disease at high-field 7T MRI, indication for microinfarcts? In preparation

Goos, J.D.C. Morgen begin ik; the novel. In preparation
Oniipa, Namibia, has taught Jeroen that the highly criticized Dutch healthcare system proved not to be so bad after all. Experience in clinical neurology was acquired in Delft, where dr. Niekus fuelled his enthusiasm for cognitive neurology in particular. In 2008, an ideal PhD research position with prof. Philip Scheltens became available at the Alzheimer center of the VU University Medical Center in his beloved Amsterdam.

During his PhD, his star was exponentially rising, conveniently benefiting from the amazing hotness of the topic of his thesis and from the renowned Alzheimer center he was happily serving. To his own surprise, he is now often referred to as Mr. Microbleed by his colleagues and across the border as dr. Goose.

At the peak of his recently acquired fame, however, his international scientific career came to a screeching halt as in the beginning of 2013 his (PhD) student’s life was finally over, and he started a residency at the neurology department of the VU University Medical Center, as a wonderful next step in his already beautiful career.

About the author

Jeroen Dirk Cornelis Goos was born in Dordrecht on February 13th in 1979.

Shortly thereafter, his family moved to Monnickendam, but the majority of his elementary school years were spent in Zwijndrecht, where he also went to high school at the Walburg College.

After receiving his VWO diploma in 1997, Jeroen moved to Amsterdam to study medicine at the University of Amsterdam (UvA). The next year, as he joined the Amsterdamsch Studenten Corps, the shy small town adolescent slowly started to blossom as he discovered the city and intensely enjoyed his student life. In this particular period his passion for the pen manifested. The following years were spent foolishly gathering material for his long awaited debut novel: “Morgen begin ik”, unfortunately with it’s working title unchanged until this very day. Nevertheless, his writing aspirations took hold, albeit in non-fiction form, after a science internship at the Texas Heart Institute in Houston Texas.

Just before starting his rotations, Jeroen developed his skills as a monk while electronically labelling the complete farmacotherapeutisch and diagnostisch kompas with ICD10 codes, which became available online in 2005. During his rotations, neurology turned out to be his preferred medical specialty. In order to get some genuine life experience, an internship at the surgery ward of a regional hospital in
‘Don’t cry because it’s over. Smile because it happened.’

Dr. Seuss