Cerebral Microbleeds a Marker of Vascular Brain Disease

Saloua Akoudad

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Cerebral Microbleeds

a Marker of Vascular Brain Disease

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een marker van vasculaire hersenschade

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CHAPTER 1

General Introduction

General Introduction

With 15 million people suffering from stroke yearly and 40 million living with dementia worldwide, stroke has become the second leading cause of death and dementia a leading cause of disability and dependency.[1,2] The social and financial burden of these diseases has become enormous. The burden on public health will likely increase as demographic trends show global population aging.[3] The proverbial saying 'an ounce of prevention is worth a pound of cure' signifies the essential search for preclinical neurovascular and neurodegenerative disease markers. Identifying diseases in an early, pre-symptomatic stage may positively influence the disease course through early implementation of preventive or therapeutic strategies. To achieve this, a comprehensive understanding of disease etiology and pathophysiology is necessary. Accumulating evidence implicates vascular risk factors in the pathophysiology of above-described neurological brain diseases. In the last decades, magnetic resonance imaging has taken a central role in identifying early markers of neurovascular and neurodegenerative pathologies that greatly benefit etiologic research.

Structural imaging markers of vascular brain pathology include lacunes, white matter lesions, and cerebral microbleeds.[4] The prevailing pathologies underlying these lesions are arteriosclerosis and cerebral amyloid angiopathy. Although lacunes and white matter lesions are typically implicated in arteriosclerosis, microbleeds in certain brain regions may additionally represent amyloid pathology. The theory that vascular and neurodegenerative pathophysiological mechanisms may interact to cause overt neurological disease is not new, though still poorly understood. Cerebral microbleeds may help explain the link between cerebrovascular and neurodegenerative pathology.

Until recently, microbleed research focused mainly on standardizing their detection on neuroimaging,[5-7] determining their prevalence and incidence,[8,9] and identifying possible risk factors.[10-15] Microbleeds are recognized as hypointense, small, well-demarcated dot-like lesions on susceptibility weighted MR images.[5] These radiographically defined lesions are highly prevalent in patients with overt cerebrovascular disease although microbleed prevalence in the general population may be as high as 38% in those aged 80 years and over.[8,13] The development of new microbleeds is particularly associated with presence of baseline microbleeds, and other markers of vascular brain disease.[16] A distinct distribution of risk factors in people with lobar and non-lobar microbleeds has led to the understanding that microbleeds in various brain regions reflect different types of vasculopathy, i.e., lobar microbleeds represent underlying amyloid angiopathy whereas deep or infratentorial microbleeds represent

underlying arteriosclerosis. Histopathological studies confirmed the presence of arteriosclerotic changes to small vessels as well as β-amyloid depositions.[17,18] Despite our growing understanding of microbleed pathophysiology, the increased detection of microbleeds due to the widespread use of MRI in routine practice has led clinicians to wonder about their potential clinical implications. Concerns include the co-existence of ischemic and hemorrhagic brain lesions. In addition, it remains debatable whether it is safe to administer antithrombotic agents to people with microbleeds, due to the potential increased risk of adverse (symptomatic) bleeding events. Also, no study of sufficient sample size reported on temporal associations of microbleeds with stroke, cognitive deterioration, and dementia in the general population.

The aim of this thesis is to expand our current knowledge on microbleeds as a marker of vascular and neurodegenerative brain disease. To this end microbleeds were correlated with other established markers of small vessel disease –cerebral or systemic- to investigate whether microbleeds represent active and diffuse vasculopathy. Furthermore, I aimed to determine whether microbleeds yield clinical relevance by investigating their relation to development of overt neurological diseases such as stroke and dementia. All studies were conducted within the Rotterdam Study, a large population-based cohort that uses neuroimaging techniques to investigate occurrence, determinants, and consequences of chronic diseases in an aging population.[19,20]

Chapter 2 comprises studies on the etiology and pathophysiology of cerebral microbleeds. This chapter is dedicated to correlations of microbleeds with other imaging markers of cerebrovascular disease, with renal vascular markers, and finally with lipid genetics. In ***Chapter 2.1***, I discuss how microbleeds relate to progression of ischemic vascular brain lesions, namely white matter lesions and lacunes. Since white matter lesions and lacunes are known to represent diffuse brain damage despite their focal appearance on MRI, we used diffusion tensor imaging to study whether microbleed presence also associates with diffuse loss of microstructural brain white matter (***Chapter 2.2).*** As microbleeds in specific brain regions have different risk factor profiles it is suggested that microbleeds in strictly lobar regions may distinctively represent underlying cerebral amyloid angiopathy. It is known that cerebral amyloid angiopathy has a predilection for posterior brain lobes. We used functional MRI in ***Chapter 2.3*** to investigate whether cerebrovascular reactivity to visual stimulation was impaired in a sample of persons with strictly lobar microbleeds. The link between cerebral small vessel disease and systemic small vessel disease is further discussed in ***Chapter 2.4*** by studying multiple markers of renal dysfunction and markers of cerebral small vessel disease, including microbleeds. Genetic risk scores of lipids

as a determinant of increased silent and symptomatic bleeding risk are discussed in ***Chapter 2.5***.

The effects of oral anticoagulant and selective serotonin reuptake inhibitor drug use on the prevalence and incidence of microbleeds are discussed in ***Chapter 3.1*** through ***Chapter 3.3***.

Chapter 4 focuses on the clinical implications of cerebral microbleeds in the general population. Microbleeds as a determinant of mortality (***Chapter 4.1***), ischemic and hemorrhagic stroke (***Chapter 4.2***), and cognitive impairment and dementia (***Chapter 4.3***) are discussed. ***Chapter 4.4*** describes MRI-correlates, including microbleeds, in mild cognitive impairment.

I conclude the scientific content of this thesis in ***Chapter 5*** with a general discussion of the main findings in context of current knowledge, methodology, and directions for future research.

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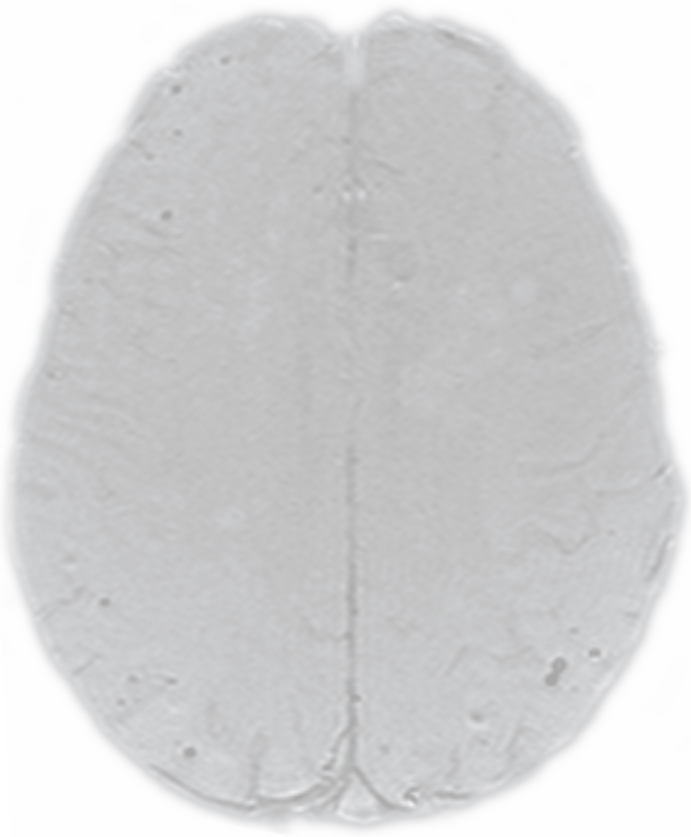
CHAPTER 2

Correlates of Cerebral Microbleeds



2.1

Cerebral microbleeds and progression of ischemic vascular lesions

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Abstract

Background: Despite their different appearance on imaging, hemorrhagic and ischemic vascular lesions frequently co-occur in the brain and are hypothesized to progress concurrently. Although silent hemorrhagic and ischemic vascular brain lesions are highly prevalent in the general population, the concomitant progression of these lesions has only been studied to a limited extent in this population. We therefore aimed to investigate whether pre-existing and incident cerebral microbleeds are related to the progression of ischemic lesions in the general population.

Methods: In the prospective population-based Rotterdam Scan Study, 803 individuals aged ≥60 years underwent magnetic resonance imaging at baseline and after an average interval of 3.4 years. Trained research physicians visually rated the presence of microbleeds and lacunes, and white matter lesions (WMLs) were automatically segmented at both time points. Logistic regression was used to investigate the association of microbleeds with incident lacunes, and linear regression was used to investigate the relation between microbleeds and progression of WML volume. All analyses were adjusted for age, sex and the time interval between baseline and follow-up scanning. The analyses were repeated after additional adjustments for cardiovascular risk factors: blood pressures; total and high-density lipoprotein cholesterol; smoking; diabetes mellitus; lipid lowering, anti- hypertensive and antiplatelet medications, and *apolipoprotein E* ε4. The analyses involving WMLs were also adjusted for intracranial volume.

Results: We found that pre-existing microbleeds in any location of the brain were related to a higher incidence of lacunes (odds ratio [OR] adjusted for age, sex and scan interval: 4.67; 95% confidence interval [CI]: 1.84–11.85). Pre-existing microbleeds were not related to progression of WML volume (mean difference in WML volume increase: –0.03; 95% CI: –0.15 to 0.09). Additional adjustments for cardiovascular risk factors did not change the results considerably. Incident microbleeds in any location of the brain were associated with a higher incidence of lacunes (OR: 9.18; 95% CI: 3.61–23.35), whereas only incident microbleeds located in cortico-subcortical regions were related to progression of WML volume (mean difference in WML volume increase: 0.41; 95% CI: 0.21–0.62). Again, adjustments for cardiovascular risk factors did not change the results significantly.

Conclusions: Our findings suggest that in the general population, microbleeds serve as a predictor of ischemic brain lesions and may represent an imaging marker of active vasculopathy. These results support the hypothesis of a common underlying pathway in the development of ischemic and hemorrhagic brain lesions.

Introduction

Vascular brain pathology is highly prevalent in aging populations and may clinically manifest as stroke or dementia. Before the onset of clinical disease, vascular pathology accumulates over the course of several years, during which it can be visualized by magnetic resonance imaging (MRI) as lacunes of presumed vascular origin (henceforth lacunes),[1] white matter lesions (WMLs) and cerebral microbleeds.[2]

Based on pathologic features, lacunes and WMLs are presumed to indicate an ischemic vascular pathology in the brain, whereas microbleeds are thought to represent a bleeding-prone state. Despite their different appearance on imaging, lacunes, WMLs and microbleeds very often co-occur in the brain.[3-5] Moreover, the many shared risk factors for lacunes, WMLs and microbleeds suggest that these lesions are at least partly affected by common pathological mechanisms.[4-9] Therefore, in the progression of vascular brain pathology, one would expect that one common pathological pathway ultimately diverges into two distinct pathways leading to the different phenotypes, i.e., ischemic and hemorrhagic events (Figure). However, evidence in support of this 'diverging pathway' hypothesis is scarce.[3,10] Hemorrhagic and ischemic brain lesions and their progression are often studied as separate entities and typically in clinical populations, even though both types of lesions are also highly prevalent in the general population.[11]

We have previously shown that, in a population-based setting, pre-existing microbleeds increase the risk of developing new microbleeds.[12] In addition, we showed that pre-existing ischemic lesions, i.e., lacunes and WMLs, were related to both the presence and incidence of microbleeds.[12-14] Thus far, no study in the general population has explored the opposite association. Evidence for microbleeds predicting and simultaneously progressing with silent ischemic vascular lesions would strengthen the argument of a shared etiological pathway. We therefore investigated, in the population-based Rotterdam Scan Study, whether pre-existing microbleeds and incident microbleeds were related to incident lacunes and progression of WML volume.

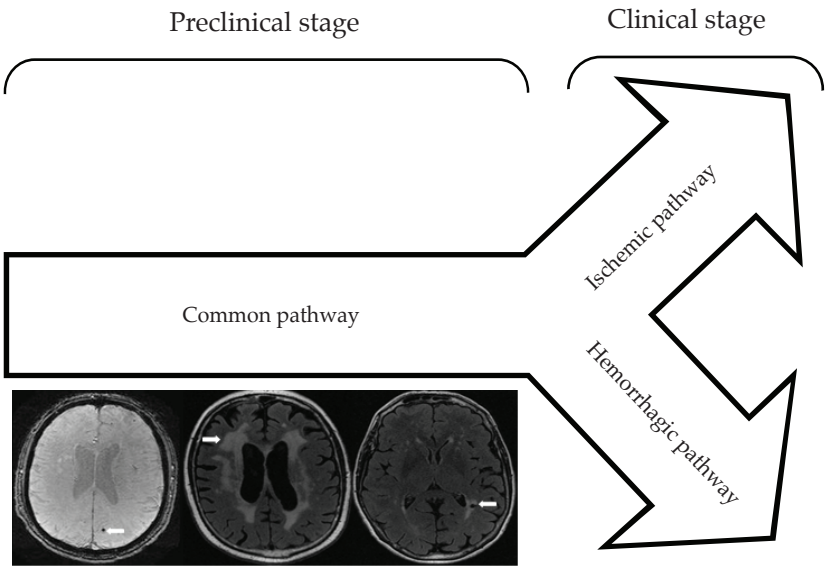

Figure. *Accumulation of ischemic and hemorrhagic vascular pathology.*
Hypothetical scheme of accumulating vascular brain pathology. In a first stage, subclinical ischemic and hemorrhagic vascular pathology may occur concurrently due to common risk factors or pathophysiological mechanisms (images from left to right: microbleed on T2-weighted sequence; WMLs on FLAIR; lacune of presumed vascular origin on FLAIR). In a later stage, a common pathway may diverge into distinct final pathways in which ischemic or hemorrhagic specific risk factors cause one overall clinical phenotype of vascular brain disease.*

Methods

Participants

The study was conducted as part of the population-based Rotterdam Scan Study,[15] a neuroimaging investigation embedded in the larger prospective Rotterdam Study.[16] Between 2005 and 2006, 1,375 non-demented study participants were randomly invited to undergo baseline brain MRI scanning. The institutional review board approved the study. A total of 146 participants were ineligible to undergo MRI (typically due to MRI contraindications). Of the 1,229 eligible participants at baseline, 1,062 (86.4%) underwent complete MRI scanning after informed consent was obtained. Subsequently, the participants were invited to undergo a repeat brain MRI between 2008 and 2010, i.e., 3–4 years after baseline. Of the 1,062 participants with baseline MRI examinations, 982 were eligible to participate in the follow-up scanning and 848 (86.4%) gave their written informed consent. Physical inabilities prohibited the completion of scanning in 14 individuals. Of the 834 complete scans, 3 had to be excluded because of artifacts, leaving a total of 831 scans for analysis.

Assessment of MRI Markers of Cerebrovascular Disease

Both baseline and follow-up scanning were performed on a 1.5-Tesla MRI scanner (GE Healthcare, Milwaukee, Wis., USA) [15]. The presence of MRI markers of cerebrovascular disease was rated by 1 of 5 trained research physicians. The raters were blinded to the clinical data. The presence, number and location of microbleeds was rated on three-dimensional T2*-weighted gradient-recalled echo MRI at both time points. Baseline or follow-up scans that showed ≥1 microbleeds were rated blinded for time point in a side-by-side comparison.[12] The participants were categorized into groups of individuals who had microbleeds in lobar brain regions only and individuals who had microbleeds in deep or infratentorial regions (regardless of the presence of lobar microbleeds). The presence of lacunes and cortical infarcts was rated on fluid-attenuated inversion recovery (FLAIR), proton density-weighted and T1-weighted sequences.[13] Participants with ≥1 infarcts at any of the two time points were included in a side-by-side comparison to assess the final number of infarcts on each scan. Lacunes were defined as focal lesions ≥3 and <15 mm in size with the same signal intensity as cerebrospinal fluid on all sequences and a hyperintense rim on FLAIR (when located supratentorially). Infarcts showing the involvement of gray matter were classified as cortical infarcts. Quantitative measures of WML volume (in milliliters) and intracranial volume (in milliliters) were obtained at both time points, using validated automated post-processing steps that included conventional k-nearest-neighbor brain tissue classification extended with WML segmentation.[17,18]

Assessment of Cardiovascular Risk Factors

All participants were examined by trained personnel.[16,19] We used standardized interviews as well as laboratory and physical examinations around the time of baseline MRI scanning to assess cardiovascular risk factors. Blood pressure measurements were averaged over 2 readings, measured on the right arm with a random- zero sphygmomanometer. Serum total and high-density lipoprotein cholesterol were determined using an automated enzymatic procedure.[20] Smoking habits were defined as 'ever' or 'never' smoking. Participants were considered diabetic if their fasting glucose levels were ≥7.0 mmol/L or if they used any glucose-lowering medication. The use of lipid-lowering and blood pressure-lowering medications was assessed by interviews during home visits. Information on antiplatelet medication use (i.e., aspirin or carbasalate calcium preparations) was obtained from automated pharmacy records and categorized as 'ever' versus 'never' used before MRI. *Apolipoprotein E* (*APOE*) genotyping was done on coded genomic DNA samples,[21] with allele frequencies being in Hardy-Weinberg equilibrium.

Data Analysis

We investigated the microbleed status dichotomously and by location (strictly lobar versus no microbleeds and deep or infratentorial versus no microbleeds), as described previously.[13] Additionally, we investigated categories of microbleed count, namely, single versus no microbleeds and multiple versus no microbleeds. Logistic regression models adjusted for age, sex and scan interval (in years) were used to investigate the association of pre-existing and incident microbleeds with incident lacunes. WML progression (in milliliters per year) was assessed by calculating the differences in lesion load volume (in milliliters) on baseline and follow-up scans and subsequently dividing this number by the time interval between the two scans (in years).

We applied linear regression models to investigate the association of pre-existing and incident microbleeds with progression of WML volume. All analyses of WMLs were adjusted for age, sex, scan interval and intracranial volume. The analyses were repeated after adjustment for cardiovascular risk factors, adjustment for *APOE* ε4 and, finally, after stratification by *APOE* ε4 carriership. Individuals with cortical infarcts at baseline or follow-up scanning were excluded from all analyses (n=28) because tissue loss and the gliosis surrounding cortical infarctions may influence image registration to an extent that WML segmentation measures become unreliable. For the analyses of WMLs, an additional 27 scans had to be excluded due to segmentation errors in baseline or follow-up scans. The analyses were performed using the statistical software package SPSS version 21.0 (SPSS Inc., Chicago, Ill., USA), using an α-value of 0.05.

Results

The characteristics of the study population are shown in Table 1. The mean age was 68.3 years, and 410 participants (51.1%) were women. Microbleeds were present in 195 participants (24.3%) at baseline, and 78 (9.7%) developed ≥1 new microbleeds during follow-up. Incident microbleeds were located strictly in lobar regions in 49 participants (6.1%) and in deep or infratentorial brain regions in 29 (3.6%). At baseline, 59 participants (7.3%) had lacunes, and 20 (2.5%) developed ≥1 new lacunes. The median baseline volume of WMLs was 3.7 mL, and a median increase of 0.18 mL/year was seen during a mean scanning interval of 3.4 years.

Table 2 shows the association between cerebral microbleeds and incident lacunes. Pre-existing microbleeds, at any location in the brain, were related to incident lacunes. Although deep or infratentorial microbleeds were more strongly associated with incident lacunes after adjustment for cardiovascular risk factors

Table 1. *Characteristics of the study population*

	N= 803
Age, years	68.3 (6.2)
Women	410 (51.1)
Pre-existing microbleeds	195 (24.3)
Strictly lobar	129 (16.1)
Deep or infratentorial	66 (8.2)
Incident microbleeds	78 (9.7)
Strictly lobar	49 (6.1)
Deep or infratentorial	29 (3.6)
Baseline white matter lesion volume, mL	3.7 (2.2; 7.1)
White matter lesions volume progression*	0.18 (0.03; 0.53)
Pre-existing lacunes	59 (7.3)
Incident lacunes	20 (2.5)
Scan interval, years	3.4 (0.2)
Intracranial volume, mL	1119.1 (115.6)
Systolic blood pressure, mmHg	143.7 (18.0)
Diastolic blood pressure, mmHg	80.7 (10.3)
Total cholesterol, mmol/L	5.7 (1.0)
High-density lipoprotein cholesterol, mmol/L	1.4 (0.4)
Smoking	564 (70.2)
Diabetes mellitus	64 (8.0)
Lipid lowering medication use	170 (21.2)
Antihypertensive medication use	282 (35.1)
Antiplatelet medication use	207 (25.8)
Apolipoprotein E ε4 carriership	198 (24.7)

*Data are presented as mean (standard deviation) for continuous variables, number (%) for categorical variables. *White matter lesions are presented as median (interquartile range). The following variables had missing data: systolic and diastolic blood pressure n=3, total and high-density lipoprotein cholesterol n=6, smoking n=8, diabetes mellitus n=11, lipid lowering and antihypertensive medication n=8, apolipoprotein E genotype n=4.*

(OR for incident lacunes: 6.10; 95% CI: 1.61–23.14), strictly lobar microbleeds were most strongly related to incident lacunes after adjusting for *APOE* ε4 carriership (OR: 4.79; 95% CI: 1.46–15.67). Also, incident microbleeds at any location in the brain were strongly associated with incident lacunes (OR: 9.18; 95% CI: 3.61–23.35). Adjusting for baseline WML volume did not alter these results (data not shown). Both single and multiple pre-existing or incident microbleeds increased the risk of incident lacunes, and although the numbers were small, the associations were strongest for multiple incident microbleeds (Supplementary Table 1).

Table 2. *Microbleeds and incident lacunes*

		Incident lacunes		
	n/N	**Model 1**	**Model 2**	**Model 3**
No pre-existing microbleeds	8/608	Reference	Reference	Reference
Pre-existing microbleeds (all)	12/195	4.67 (1.84; 11.85)	4.25 (1.56; 11.60)	4.67 (1.59; 13.74)
Pre-existing strictly lobar	6/129	3.56 (1.18; 10.74)	3.27 (0.97; 11.00)	4.79 (1.46; 15.67)
Pre-existing deep or infratentorial	6/66	6.60 (2.17; 20.09)	6.10 (1.61; 23.14)	4.16 (0.96; 17.93)
No incident microbleeds	10/725	Reference	Reference	Reference
Incident microbleeds (all)	10/78	9.18 (3.61; 23.35)	6.28 (2.26; 17.47)	8.41 (2.82; 25.10)
Incident strictly lobar	4/49	5.60 (1.63; 19.30)	4.19 (1.03; 17.07)	7.75 (2.07; 28.98)
Incident deep or infratentorial	6/29	17.09 (5.60; 52.20)	10.43 (2.80; 38.77)	9.25 (2.17; 39.38)

Model 1: adjusted for age, sex, and scan interval.
Model 2: as model 1, additionally adjusted for blood pressures, total and HDL cholesterol, smoking, diabetes mellitus, lipid lowering medication, antihypertensive medication, and antiplatelet medication.
Model 3: as model 1, additionally adjusted for apolipoprotein E ε4 genotype.
Values represent odds ratios for incident lacunes in participants with pre-existing and incident microbleeds compared to no microbleeds.
n= number of participants with incident lacunes; N= total number of participants.

The association between cerebral microbleeds and WML volume progression is shown in Table 3. Pre-existing microbleeds were not related to WML volume progression. However, the progression of strictly lobar microbleeds was strongly associated with the progression of WML volume (mean difference in WML volume increase: 0.41; 95% CI: 0.21– 0.62). This was true for individuals with a single incident microbleed, but even more so for those with multiple strictly lobar incident microbleeds (Supplementary Table 2). Adjusting for baseline lacunes did not alter these results (data not shown). Although statistical significance was not reached, it appeared that there was more WML volume progression in individuals with strictly lobar incident microbleeds who carried the *APOE* ε4 allele than in non-carriers (*P* interaction: 0.14; Supplementary Table 3).

Table 3. *Microbleeds and progression of WML volume*

	Difference in annual progression of WML volume		
	Model 1	**Model 2**	**Model 3**
No pre-existing microbleeds	Reference	Reference	Reference
Pre-existing microbleeds (all)	-0.03 (-0.15; 0.09)	-0.05 (-0.17; 0.08)	-0.01 (-0.14; 0.11)
Pre-existing strictly lobar	-0.04 (-0.18; 0.10)	-0.06 (-0.20; 0.08)	-0.07 (-0.21; 0.08)
Pre-existing deep or infratentorial	0.01 (-0.19; 0.18)	-0.01 (-0.21; 0.18)	0.09 (-0.11; 0.28)
No incident microbleeds	Reference	Reference	Reference
Incident microbleeds (all)	0.23 (0.05; 0.40)	0.19 (0.01; 0.37)	0.22 (0.04; 0.41)
Incident strictly lobar	0.41 (0.21; 0.62)	0.39 (0.18; 0.61)	0.33 (0.10; 0.55)
Incident deep or infratentorial	-0.10 (-0.37; 0.17)	-0.17 (-0.45; 0.11)	0.02 (-0.28; 0.32)

Model 1: adjusted for age, sex, scan interval, and intracranial volume.
Model 2: as model 1, additionally adjusted for blood pressures, total and HDL cholesterol, smoking, diabetes mellitus, lipid lowering medication, antihypertensive medication, and antiplatelet medication.
Model 3: as model 1, additionally adjusted for apolipoprotein E ε4 genotype.
Values represent differences in annual white matter lesion (WML) volume progression in participants with pre-existing and incident microbleeds compared to no microbleeds.

Discussion

In our longitudinal population-based study, we found that both pre-existing and incident microbleeds were related to incident lacunes and progression of WML volume. Strengths of our study are its population-based setting and the large number of participants with a follow-up scan. Also, we performed an identical MRI protocol on the same MRI scanner at both time points without software or hardware alterations to optimize comparability between scans over time. A possible limitation of the study is that selective dropout may have influenced our results as the participants who underwent follow-up MRI scanning were younger and healthier than those who refused or were ineligible to undergo a second MRI scanning. However, if present, selective dropout would have underestimated the true strength of the association between microbleeds and ischemic vascular lesions in our study because the individuals who would have dropped out would have been more likely to have a worse cardiovascular risk profile. As for the analysis stratified by *APOE* ε4, the sample size was rather small, and these results should be interpreted with caution.

We found that incident microbleeds, which pathologically correspond to hemorrhagic lesions,[22-24] related to an increased risk of progression of ischemic vascular lesions. This was particularly true for individuals with multiple incident

microbleeds. Unfortunately, our study setting did not allow us to identify the chronological order of lesion occurrence. However, the co-occurrence of incident microbleeds, incident lacunes and progression of WML volume does suggest that the accumulation of these vascular pathologies follows a common pathway, which may depend on shared pathophysiological mechanisms. Person-specific modifiable risk factors (e.g., oral anticoagulant drug use) or non-modifiable risk factors (e.g., genetic variations) may then tilt individuals toward a final pathway of either major symptomatic hemorrhagic events or major symptomatic ischemic events.

We have previously shown that in preclinical asymptomatic individuals, pre-existing ischemic vascular lesions were related to the presence and incidence of microbleeds.[12,13] Taken together with our current findings, this strengthens the suggestion of a common pathogenic pathway with a divergence for ischemic and hemorrhagic lesions. One point of note is that the progression of hemorrhagic and ischemic vascular brain lesions did not solely depend on shared cardiovascular risk factors, as adjusting for these factors did not change our findings. Although the underlying mechanism explaining this diverging pathway remains unclear, we speculate that advanced cerebral amyloid angiopathy (CAA) pathology or hypertensive arteriopathy may give rise to both incident microbleeds and incident ischemic lesions. Additionally, in small vessel disease, impaired vessel tone and impaired autoregulation may lead to hypoperfusion after vessels rupture.[1,25,26] Finally, pro-inflammatory pathways may be triggered in the vasculopathy, advocating the synchronic progression of both hemorrhagic and ischemic lesions.[27] Interestingly, there are also observations from pathology suggesting that microbleeds themselves have an ischemic origin.[28] Microbleeds may not exclusively reflect extravasation of erythrocytes but may partly signify the inability of the aging brain to store ferritin iron released from ischemic damaged brain cells.[28]

Consistent evidence from observational and pathology studies implies that CAA is the prevailing pathology underlying microbleeds confined to cortico-subcortical (i.e., lobar) regions of the brain, whereas microbleeds located in deeper or infratentorial regions of the brain are more suggestive of hypertensive arteriopathy.[13,23,29] These observations have led to the assumption that depending on the location of microbleeds in the brain, and thus on their underlying pathology, microbleeds may differentially relate to ischemic small vessel lesions, i.e., lacunes or WMLs.

In the current study, we found that both pre-existing lobar and deep or infratentorial microbleeds increased the risk of incident lacunes. Also, the

development of lobar microbleeds concurred with a higher progression of WML volume. In previous work, we showed that a higher WML load increased the risk of both deep or infratentorial and lobar microbleeds,[12] and that pre-existing microbleeds increased the risk of new microbleeds.[12] Taken together, these findings suggest that the pathology underlying microbleeds – be it hypertensive arteriopathy, amyloid angiopathy or a combination of both – may thus contribute to the progression of both ischemic and hemorrhagic lesions.

In CAA, vascular β-amyloid accumulates and might eventually lead to destruction of the vessel lumen, which leads to the development of lobar microbleeds.[30] However, vascular amyloid deposition may also cause stenosis, occlusion, loss of the contractile components of the vessel wall as well as impaired reactivity to physiologic stimulation.[3] The abnormal relaxation and constriction of small vessels may in turn contribute to repetitive hypoperfusion and, possibly, ischemic events.[31] Indeed, in patients with CAA-related hemorrhages, a progression of ischemic brain lesions on MRI has increasingly been recognized.[9,27,32,33] Given our previous and new findings,[12-14] we might consider that in the general population, possible CAA-related microhemorrhages and ischemic lesions develop more steadily throughout the 'common pathway'. We did not find an association between deep or infratentorial microbleeds and WML volume progression. As individuals with deep or infratentorial microbleeds have higher WML volumes compared with those without microbleeds in these locations,[12-14] a possible 'ceiling effect' may be created in this group. This group nonetheless develops more lacunes, another likely ischemic manifestation.

In conclusion, we found support for a common underlying pathway in the development of ischemic and hemorrhagic vascular brain lesions. Furthermore, our findings suggest that cerebral microbleeds may represent an imaging marker of active vasculopathy, which serves as a predictor of ischemic brain lesions.

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Supplementary Table 1. *Categories of microbleed count and incident lacunes*

	n/N	Incident lacunes
No pre-existing microbleeds	8/608	Reference
Single pre-existing microbleeds (all)	6/123	3.98 (1.33; 11.90)
Multiple pre-existing microbleeds (all)	6/72	5.70 (1.79; 18.13)
Single pre-existing strictly lobar	5/93	4.42 (1.39; 14.02)
Multiple pre-existing strictly lobar	1/36	1.53 (0.17; 13.71)
Single pre-existing deep or infratentorial	1/30	2.46 (0.29; 20.86)
Multiple pre-existing deep or infratentorial	5/36	10.31 (3.06; 34.73)
No incident microbleeds	10/725	Reference
Single incident microbleed (all)	3/43	4.65 (1.19; 18.09)
Multiple incident microbleed (all)	7/35	16.18 (5.60; 46.78)
Single incident strictly lobar	2/34	4.00 (0.82; 19.51)
Multiple incident strictly lobar	2/15	9.72 (1.79; 52.72)
Single incident deep or infratentorial	1/9	7.40 (0.80; 68.40)
Multiple incident deep or infratentorial	5/20	23.67 (6.98; 80.25)

Values are adjusted for age, sex, and scan interval.
Values represent odds ratios for incident lacunes in participants with pre-existing and incident microbleeds compared to no microbleeds.
Abbreviations: n=number of participants with incident lacunes; N=total number of participants.

Supplementary Table 2. *Categories of microbleed count and progression of WML volume*

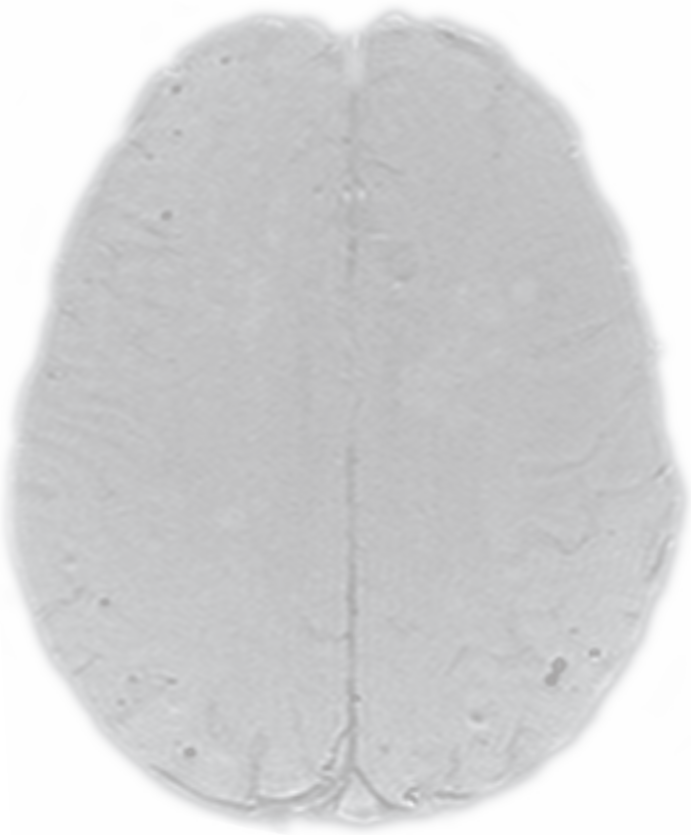
	Difference in annual progression of WML volume
No pre-existing microbleeds	Reference
Single pre-existing microbleeds (all)	-0.09 (-0.22; 0.05)
Multiple pre-existing microbleeds (all)	0.07 (-0.12; 0.26)
Single pre-existing strictly lobar	-0.15 (-0.30; 0.00)
Multiple pre-existing strictly lobar	0.24 (-0.00; 0.49)
Single pre-existing deep or infratentorial	0.10 (-0.16; 0.36)
Multiple pre-existing deep or infratentorial	-0.10 (-0.34; 0.15)
No incident microbleeds	Reference
Single incident microbleeds (all)	0.20 (-0.02; 0.41)
Multiple incident microbleeds (all)	0.26 (0.01; 0.51)
Single incident strictly lobar	0.30 (0.06; 0.54)
Multiple incident strictly lobar	0.65 (0.30; 1.01)
Single incident deep or infratentorial	-0.17 (-0.61; 0.27)
Multiple incident deep or infratentorial	-0.06 (-0.39; 0.27)

Values are adjusted for age, sex, scan interval, and intracranial volume.
Values represent differences in annual white matter lesion volume progression in participants with pre-existing and incident microbleeds compared to no microbleeds.
Abbreviations: WML=white matter lesion.

Supplementary Table 3. *Microbleeds and progression of WML by APOE ε4 status*

	Differences in annual progression of WML volume	
	Carriers (N=188)	Non-carriers (N=454)
Pre-existing microbleeds (all)	0.10 (-0.12; 0.32)	-0.12 (-0.25; 0. 0004)
Pre-existing strictly lobar	0.10 (-0.14; 0.34)	-0.12 (-0.27; 0.02)
Pre-existing deep or infratentorial	0.14 (-0.20; 0.47)	-0.14 (-0.34; 0.06)
Incident microbleeds (all)	0.28 (-0.04; 0.61)	-0.03 (-0.22; 0.16)
Incident strictly lobar	0.34 (-0.02; 0.71)	0.05 (-0.19; 0.28)
Incident deep or infratentorial	0.09 (-0.56; 0.73)	-0.15 (-0.43; 0.14)

Adjusted for age, sex, scan interval, and intracranial volume.
Values represent differences in annual white matter lesion volume progression in APOE ε4 carriers and non-carriers with pre-existing and incident microbleeds compared to no microbleeds.
Abbreviations: WML= white matter lesion; APOE= apolipoprotein E.



2.2

Cerebral microbleeds and white matter microstructural integrity

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Abstract

Background: Cerebral microbleeds are highly frequent in the general population and are increasingly recognized as a manifestation of cerebral small vessel disease (CSVD). Although microbleeds present on imaging as focal lesions, it remains unclear whether the underlying CSVD is also restricted to that focal area or actually affects the brain more diffusely. We investigated whether the presence, number, and location of microbleeds in the general population is related to loss of microstructural integrity of brain white matter, as measured by diffusion tensor imaging (DTI).

Methods: In the prospective, population-based Rotterdam Scan Study, a total of 4,493 participants underwent brain MRI to determine microbleed status. With diffusion tensor imaging, global fractional anisotropy (FA) and mean diffusivity (MD) were measured in normal-appearing white matter. Multiple linear regression models, adjusted for age, sex, cardiovascular risk factors, white matter lesions, and infarcts, were applied to investigate the independent association between microbleeds and organization of brain white matter. Analyses were repeated after stratification by *APOE* ε4 carriership.

Results: Presence of microbleeds was related to a lower mean FA and higher mean MD, in a dose-dependent manner, and was already apparent for a single microbleed (standardized FA: -0.13, 95% confidence interval -0.21 to -0.05; MD: 0.12, 95% confidence interval 0.05 to 0.19). For lobar microbleeds, alterations in diffusion tensor imaging measurements were solely driven by *APOE* ε4 carriers.

Conclusions: Presence of microbleeds relates to poorer microstructural integrity of brain white matter, even after adjusting for cardiovascular risk and other markers of cerebral small vessel disease. Our data suggest that microbleeds reflect diffuse brain pathology, even when only a single microbleed is present.

Introduction

As the elderly population is growing, cerebral small vessel disease (CSVD) and its phenotypes are expected to put a larger psychosocial and economic burden on society.[1] CSVD is, despite its diffuse character, typically recognized by focal lesions such as white matter lesions (WMLs) and lacunes on conventional MRI.

Recent advances in noninvasive brain imaging techniques have expanded the possibilities to study brain changes that cannot be visually appreciated on conventional MRI. Diffusion tensor imaging (DTI) is an MRI technique that allows for quantification of subtle pathology in the architecture of brain white matter.[2] Indeed, with DTI, it has been shown that people with WMLs or lacunes on brain MRI have diffuse loss of white matter integrity not seen on conventional MRI.[3]

In the past decade, cerebral microbleeds have emerged as a novel marker of CSVD. Microbleeds also appear as well-demarcated focal lesions on MRI.[4-6] It may be hypothesized that these microbleeds, like WMLs and lacunes, mark more widespread brain damage. Although there is some pathologic evidence linking microbleeds to surrounding white matter damage,[7-10] large *in vivo* studies on this topic are lacking. DTI can provide us with new insights regarding global white matter structural deterioration in the presence of these focal microbleeds.

We investigated whether the presence of microbleeds is related to diffuse loss of microstructural integrity of brain white matter in a large sample of middle-aged and elderly people from the general population.

Methods

Participants

This study was conducted within the Rotterdam Scan Study, an ongoing prospective population-based imaging study designed to investigate preclinical brain changes in the elderly. The Rotterdam Scan Study is performed in the context of the population-based Rotterdam Study. Its rationale and study design for both studies have been described extensively elsewhere.[11,12] We have previously published the prevalence of microbleeds in 3,979 Rotterdam Study participants who completed brain MRI scanning between 2005 and 2008.[13] Since then, additional participants were scanned, and until 2012, a total of 5,990 participants were invited to undergo brain MRI. Of 5,445 eligible participants (all without dementia and MRI contraindications), 4,843 (88.9%) gave written informed consent. After excluding participants with the inability to complete MRI (n=71),

scans of inadequate quality (n=125), and subjects with cortical brain infarcts (n=154), data on 4,493 participants were available for analyses.

Brain MRI and Assessments of MRI Markers

We performed a multisequence MRI protocol on a 1.5-Tesla scanner (GE Healthcare, Milwaukee, WI) in all participants.[11] All scans were reviewed by 1 of 5 trained research physicians, who recorded the presence, number, and location of microbleeds and infarcts using a protocol that was initiated at the beginning of the study in 2005 with good inter- and intraobserver agreement.[14] Raters were blinded to clinical data, including *APOE* genotyping. Cerebral microbleeds were rated as focal areas of signal loss on 3-dimensional T2* gradient recalled echo–weighted MRI. Presence of microbleeds was categorized by location based on presumed differences in underlying etiology: deep or infratentorial microbleeds are thought to represent hypertensive arteriopathy whereas strictly lobar microbleeds point toward cerebral amyloid angiopathy (CAA).[14] The presence of lacunes and cortical infarcts was rated on fluid-attenuated inversion recovery (FLAIR), proton density–weighted, and T1-weighted sequences. Lacunes of presumed vascular origin[15] were defined as focal lesions of ≥3 mm and <15 mm in size with the same signal intensity as CSF on all sequences and a hyperintense rim on the FLAIR (when located supratentorially). Infarcts larger than 15 mm were identified as subcortical infarcts, irrespective of their location in the brain (subcortical or striatocapsular).[14] Infarcts showing involvement of gray matter were classified as cortical infarcts. Brain tissue was segmented into gray matter, white matter, CSF, and background using a fully automated approach incorporating a multiatlas strategy with 6 manually labeled atlases for learning specific tissue intensities. WMLs were automatically segmented based on the FLAIR image using a post-processing step.[16] This enabled identification of the normal-appearing white matter as white matter unaffected by WMLs. This segmentation approach was specifically developed for MRI data acquired in the Rotterdam Scan Study. The automated detection of WMLs was achieved with high similarity index (0.72) when compared with gold-standard manual segmentations, which is comparable to the interobserver variability for manual segmentation (similarity index 0.75).[16]

Processing of DTI Data

Loss of white matter microstructural integrity is accompanied by changes in DTI-derived measurements. DTI measurements most frequently used are fractional anisotropy (FA) and mean diffusivity (MD). Higher values of FA and lower values of MD generally indicate better microstructural integrity. Moreover,

directional diffusivity measurements (axial and radial diffusivity) are thought to provide more subtle insight into underlying pathophysiologic processes, as myelin damage has been associated with increased radial diffusivity and axonal damage with increased axial diffusivity.[17]

Diffusion data were preprocessed using a standardized pipeline.[18] In summary, to compensate for subject motion and eddy currents, all acquired volumes were co-registered using affine registrations performed with Elastix.[19] Data were resampled at an isotropic resolution of 1 mm^3, and diffusion tensors were fitted using a Levenberg-Marquardt nonlinear least-squares algorithm available in ExploreDTI.[20] The diffusion data were then co-registered to the T1-weighted image using affine registration. The tensor image, resampled in the T1-weighted space, was used to obtain FA, MD, and directional diffusivity images. These diffusion metrics were then combined with the tissue segmentation results to obtain global measurements in the normal-appearing white matter of the brain, which were used in the analyses. All FA images were visually inspected, and 61 subjects were excluded because of scanning artifacts or excessive motion.

Assessment of Cardiovascular Risk Factors

Cardiovascular risk factors were assessed during study visits by interview and laboratory and physical examinations at regular visits of study participants to the research center.[12] Risk factors corrected for in our analyses included systolic and diastolic blood pressure (measured twice with a random-zero sphygmomanometer), total cholesterol (determined by using an automated enzymatic procedure, Hitachi analyzer, Roche Diagnostics), diabetes mellitus (fasting blood glucose of ≥7.0 mmol/L, and/or the use of any glucose-lowering medication), smoking (ever versus never smoked), and lipid-lowering and antihypertensive medication. Medication use was assessed during home visits. *APOE* genotyping was performed on coded genomic DNA samples.[21] Distribution of *APOE* genotype and allele frequencies in this population was in Hardy-Weinberg equilibrium.

History of Cardiovascular Disease

A history of symptomatic stroke and coronary heart disease was assessed by self-report, and by continuous monitoring of medical records through automatic linkage of general practitioners' files with the study database, as described previously.[22]

Data Analysis

WML volume was natural log-transformed because of its skewed distribution. Diffusion measures were averaged inside the normal-appearing brain white matter for each subject. Subject-specific mean FA, MD, and axial and radial diffusivity were standardized to Z-scores. Microbleed status was investigated dichotomously (none versus one or more microbleeds), categorically for microbleed count (none [reference category] versus 1, 2–4, and ≥5 microbleeds), and by location (none versus strictly lobar microbleeds, and none versus deep or infratentorial microbleeds [with or without the presence of lobar microbleeds]). We used linear regression to evaluate the association between microbleed status and integrity of brain white matter and evaluated 4 models. In the first model, analyses were age- and sex-adjusted. Model 2 was additionally adjusted for cardiovascular risk (systolic and diastolic blood pressure, total cholesterol, smoking, diabetes, and lipid-lowering and antihypertensive medication). We adjusted model 3 for age, sex, intracranial volume, and for other markers of CSVD, namely, lacunes, white matter volume, and WML volume. A final fourth model was adjusted for both cardiovascular risk and other markers of CSVD. We also examined R^2 in the regression models to evaluate the proportion of total variance in FA and diffusivity measurements that was attributed to microbleeds after having corrected for the other variables in models 2, 3, and 4. We additionally investigated whether adjustments for age-squared would give a better adjustment for confounding by age.

Quantitative risk factors such as blood pressures and total cholesterol were modeled continuously per SD increase. Analyses were repeated after stratification by *APOE* ε4 carriership, and formal interaction tests were applied to determine significant differences in subgroups. Finally, apart from cortical infarcts, we additionally excluded persons with lacunes, subcortical infarcts, and a history of symptomatic stroke in sensitivity analyses. All analyses were performed using statistical software package SPSS 20.0 (IBM Corp., Armonk, NY), and an α-value of 0.05. Additionally, we adjusted for multiple testing using Bonferroni correction (*P* value threshold of 0.0028) based on 18 independent tests.

Standard Protocol Approvals, Registrations, and Patient Consents

The institutional review board approved the study. We obtained consent for examinations from all participants. The study conforms to the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) statement guidelines.

Results

Table 1 provides characteristics of our study population. Mean age was 63.9 years (range, 45.7–100.1 years) and 2,493 of the participants were female. A total of 875 participants had at least one microbleed of whom 588 had strictly lobar and 287 deep or infratentorial microbleeds (with or without presence of lobar microbleeds). Mean brain normal-appearing white matter volume was 389.7 mL with a mean FA of 0.34, mean MD of 0.7 $x10^{-3}$ mm^2/s, mean axial diffusivity of 1.0 $x10^{-3}$ mm^2/s, and mean radial diffusivity of 0.6 $x10^{-3}$ mm^2/s.

The association between the presence of one or more microbleeds and diffusion measurements of brain normal-appearing white matter is shown in Table 2. When adjusted for age and sex, there was a significantly lower mean FA and higher mean MD in brain white matter, reflecting loss of integrity of brain white matter in subjects with microbleeds compared with those who did not have any microbleeds. Additional adjustments for cardiovascular risk did not change the results meaningfully (model 2). The association also remained unchanged after adjustment for other markers of CSVD (WML volume and presence of lacunes, model 3), and after adjusting for a full model of cardiovascular risk and imaging markers of CSVD (model 4). Both axial and radial diffusivity were higher in subjects with microbleeds compared with those who did not have microbleeds. A maximum of 1.1% of the variance in DTI measurements was explained by microbleeds in these models. All associations described above survived multiple comparison correction. When analyzing microbleed count, we found that the presence of a single microbleed was associated with a significantly lower mean FA and higher MD. These associations became more prominent with increasing microbleed count (Figure).

Table 1. *Baseline characteristics of the study population*

Characteristics	No microbleeds (n=3618)	Microbleeds (n=875)
Age, yrs	62.4 (10.7)	69.9 (10.8)
Female	2027 (56.0)	466 (53.3)
Normal appearing white-matter volume, mL	391.2 (47.9)	383.9 (48.5)
Fractional anisotropy in normal appearing white-matter	0.34 (0.02)	0.33 (0.02)
Mean diffusivity in normal appearing white-matter, $x10^{-3}$ mm^2/s	0.73 (0.02)	0.75 (0.03)
Axial diffusivity in normal appearing white-matter, $x10^{-3}$ mm^2/s	1.01 (0.03)	1.03 (0.03)
Radial diffusivity in normal appearing white-matter, $x10^{-3}$ mm^2/s	0.59 (0.04)	0.61 (0.03)
Intracranial volume, mL	1125.3 (118.6)	1128.7 (121.3)
White matter lesion volume, mL*	2.5 (3.4)	4.9 (9.3)
Lacunes	195 (5.4)	141 (16.1)
History of symptomatic stroke	39 (1.1)	32 (3.7)
History of coronary heart disease	67 (1.9)	81 (9.3)
Apolipoprotein E ε4 carriers	940 (31.9)	251 (34.9)
Systolic blood pressure, mmHg	136.9 (20.8)	144.3 (21.8)
Diastolic blood pressure, mmHg	82.4 (10.8)	82.8 (11.2)
Total cholesterol	5.6 (2.2)	5.4 (1.1)
Smoking (ever in life)	289 (8.1)	92 (10.6)
Diabetes	289 (8.1)	92 (10.6)
Lipid lowering medication	783 (21.8)	267 (30.7)
Antihypertensive medication	759 (21.1)	180 (20.7)

*Data are presented as mean (standard deviation) for continuous variables, and number (%) for categorical variables. * White matter lesion volume presented as median (interquartile range).*

Following variables had missing data: blood pressure (n=38), total cholesterol (n=67), smoking (n=14), diabetes (n=60), lipid lowering medication (n=24), antihypertensive medication (n=26), apolipoprotein E ε4 genotype (n=294).

Table 2. *Microbleeds (yes versus no) and white-matter microstructural integrity*

	Difference in adjusted Z-score							
	Fractional anisotropy	***P* value**	**Mean diffusivity**	***P* value**	**Axial diffusivity**	***P* value**	**Radial diffusivity**	***P* value**
Presence of microbleeds Model 1	-0.27 (-0.33; -0.21)	<0.001	0.25 (0.19; 0.30)	<0.001	0.17 (0.11; 0.23)	<0.001	0.26 (0.21; 0.32)	<0.001
Model 2	-0.25 (-0.32; -0.19)	<0.001	0.24 (0.19; 0.30)	<0.001	0.18 (0.11; 0.24)	<0.001	0.26 (0.20; 0.31)	<0.001
Model 3	-0.15 (-0.21; -0.10)	<0.001	0.15 (0.10; 0.20)	<0.001	0.10 (0.05; 0.16)	0.001	0.16 (0.11; 0.21)	<0.001
Model 4	-0.15 (-0.20; -0.09)	<0.001	0.15 (0.10; 0.20)	<0.001	0.11 (0.05; 0.17)	0.001	0.16 (0.10; 0.21)	<0.001

Model: adjusted for age and sex.

Model 2: as Model 1, additionally adjusted for systolic and diastolic blood pressure, total cholesterol, smoking, diabetes lipid lowering medication, and antihypertensive medication.

Model 3: adjusted for age, sex, lacunes, normal appearing white-matter volume, white matter lesion volume (log transformed), and intracranial volume.

Model 4: adjusted for age, sex, cardiovascular risk as in Model 2, and imaging markers as in Model 3.

Values represent difference in Z-score of mean fractional anisotropy and mean diffusivity parameters in normal-appearing white matter for presence of any microbleeds compared to no microbleeds.

Bonferroni-corrected threshold for significance: P value=0.0028.

Table 3 shows the association between microbleed location and integrity of brain white matter. Persons with microbleeds in either deep or infratentorial, or strictly lobar locations had lower mean FA and higher mean MD in the brain white matter compared with those who did not have microbleeds. Again, these associations survived multiple comparison correction. Associations were stronger for microbleeds situated in the deep or infratentorial regions. Also, axial and radial diffusivity were highest in subjects with deep or infratentorial microbleeds. After additional stratification by *APOE* ε4carriership, we found that only persons carrying an *APOE* ε4 allele showed a clear association between lobar microbleeds and loss of brain white matter microstructural integrity, whereas such an association was not present for non-carriers (*P* interaction FA=0.022, MD=0.002) (Table 4). Effect modification by *APOE* ε4 carriership was not present for deep or infratentorial microbleeds.

Additional exclusion of persons with a history of symptomatic stroke or those with MRI-defined lacunes and/or subcortical infarcts did not change any of the above-mentioned results.

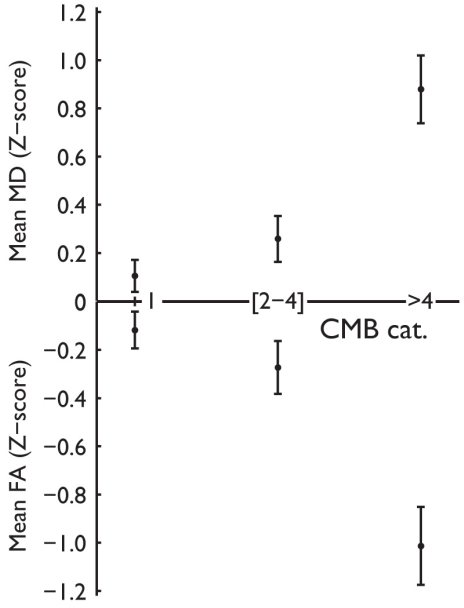

Figure. *Categories of cerebral microbleed count and white-matter microstructural integrity.*
The y-axis represents age- and sex-adjusted changes in Z-score in mean fractional anisotropy (FA) and mean diffusivity (MD) for each category of cerebral microbleed (CMB) count (x-axis), compared with a reference group without microbleeds.
Categories: 0 microbleeds, n=3618; 1 microbleed, n=530; 2–4 microbleeds, n=242; and ≥5 microbleeds, n=103.

Table 3. *Microbleeds by location and white-matter microstructural integrity*

	Difference in adjusted Z-score							
Microbleeds	**Fractional anisotropy**	***P* value**	**Mean diffusivity**	***P* value**	**Axial diffusivity**	***P* value**	**Radial diffusivity**	***P* value**
Deep / infratentorial								
Model 1	-0.49 (-0.59; -0.39)	<0.001	0.46 (0.37; 0.55)	<0.001	0.33 (0.23; 0.43)	<0.001	0.49 (0.40; 0.58)	<0.001
Model 2	-0.48 (-0.58; -0.37)	<0.001	0.46 (0.37; 0.55)	<0.001	0.34 (0.24; 0.44)	<0.001	0.48 (0.40; 0.57)	<0.001
Model 3	-0.24 (-0.34; -0.15)	<0.001	0.25 (0.16; 0.33)	<0.001	0.18 (0.08; 0.28)	<0.001	0.26 (0.18; 0.34)	<0.001
Model 4	-0.24 (-0.34; -0.15)	<0.001	0.25 (0.17; 0.34)	<0.001	0.19 (0.09; 0.29)	<0.001	0.27 (0.18; 0.35)	<0.001
Strictly lobar								
Model 1	-0.17 (-0.24; -0.10)	<0.001	0.16 (0.09; 0.22)	<0.001	0.11 (0.04; 0.18)	0.008	0.17 (0.10; 0.23)	<0.001
Model 2	-0.15 (-0.22; -0.08)	<0.001	0.15 (0.08; 0.21)	<0.001	0.11 (0.04; 0.18)	0.010	0.16 (0.09; 0.22)	<0.001
Model 3	-0.12 (-0.19; -0.05)	0.001	0.11 (0.05; 0.17)	0.001	0.08 (0.01; 0.14)	0.030	0.12 (0.06; 0.18)	<0.001
Model 4	-0.11 (-0.17; -0.04)	0.002	0.11 (0.05; 0.17)	<0.001	0.08 (0.01; 0.15)	0.028	0.11 (0.06; 0.17)	<0.001

Model 1: adjusted for age and sex.
Model 2: as Model 1, additionally adjusted for systolic and diastolic blood pressure, total cholesterol, smoking, diabetes, lipid lowering medication, and antihypertensive medication.
Model 3: adjusted for age, sex, lacunes, normal appearing white-matter volume, white matter lesion volume (log transformed), and intracranial volume.
Model 4: adjusted for age, sex, cardiovascular risk as in Model 2, and imaging markers as in Model 3.
Values represent difference in Z-score of mean fractional anisotropy and mean diffusivity parameters in normal-appearing white matter for presence of any microbleeds by their location compared to no microbleeds.
Bonferroni-corrected threshold for significance: P value=0.0028.

Table 4. *Microbleeds and white-matter microstructural integrity, by APOE ε4 status*

	Difference in adjusted Z-score							
	Carriers *APOE* ε4				Non- carriers *APOE* ε4			
Microbleeds	**Fractional anisotropy**	***P* value**	**Mean diffusivity**	***P* value**	**Fractional anisotropy**	***P* value**	**Mean diffusivity**	***P* value**
Presence of any								
Model 1	-0.32 (-0.44; -0.20)	<0.001	0.31 (0.19; 0.43)	<0.001	-0.21 (-0.30; -0.13)	<0.001	0.19 (0.12; 0.26)	<0.001
Model 2	-0.30 (-0.42; 0.18)	<0.001	0.30 (0.18; 0.42)	<0.001	-0.19 (-0.28; -0.11)	<0.001	0.19 (0.11; 0.26)	<0.001
Model 3	-0.22 (-0.33; -0.11)	<0.001	0.21 (0.10; 0.32)	<0.001	-0.13 (-0.21; -0.05)	0.001	0.11 (0.04; 0.18)	0.001
Model 4	-0.20 (-0.31; -0.09)	<0.001	0.21 (0.10; 0.32)	<0.001	-0.12 (-0.20; -0.04)	0.004	0.11 (0.04; 0.18)	0.001
Deep / infratentorial								
Model 1	-0.44 (-0.63; -0.24)	<0.001	0.40 (0.22; 0.58)	<0.001	-0.46 (-0.60; -0.32)	<0.001	0.44 (0.32; 0.55)	<0.001
Model 2	-0.42 (-0.62; -0.22)	<0.001	0.40 (0.21; 0.59)	<0.001	-0.44 (-0.58; -0.30)	<0.001	0.44 (0.32; 0.55)	<0.001
Model 3	-0.20 (-0.38; -0.02)	0.027	0.19 (0.02; 0.37)	0.033	-0.26 (-0.39; -0.14)	<0.001	0.25 (0.15; 0.36)	<0.001
Model 4	-0.18 (-0.37; -0.001)	0.049	0.19 (0.01; 0.36)	0.043	-0.26 (-0.39; -0.14)	<0.001	0.26 (0.16; 0.37)	<0.001
Strictly lobar								
Model 1	-0.26 (-0.40; -0.13)	<0.001	0.28 (0.15; 0.41)	<0.001	-0.11 (-0.20; -0.01)	0.038	0.08 (0.002; 0.17)	0.044
Model 2	-0.24 (-0.38; -0.10)	0.001	0.26 (0.12; 0.39)	<0.001	-0.09 (-0.19; 0.01)	0.079	0.08 (-0.004; 0.16)	0.061
Model 3	-0.22 (-0.35; -0.10)	0.001	0.23 (0.11; 0.36)	<0.001	-0.08 (-0.17; 0.01)	0.096	0.06 (-0.02; 0.13)	0.151
Model 4	-0.21 (-0.34; -0.08)	0.001	0.22 (0.10; 0.35)	<0.001	-0.06 (-0.15; 0.03)	0.179	0.05 (-0.02; 0.13)	0.175

Model 1: adjusted for age and sex.
Model 2: as Model 1, additionally adjusted for systolic and diastolic blood pressure, total cholesterol, smoking, diabetes, lipid lowering medication, and antihypertensive medication.
Model 3: adjusted for age, sex, lacunes, normal appearing white-matter volume, white matter lesion volume (log transformed), and intracranial volume.
Model 4: adjusted for age, sex, cardiovascular risk as in Model 2, and imaging markers as in Model 3.
Values represent difference in Z-score of mean fractional anisotropy and mean diffusivity parameters in normal-appearing white matter for presence of any microbleeds, deep or infratentorial microbleeds, and strictly lobar microbleeds compared to no microbleeds.
Bonferroni-corrected threshold for significance: P value=0.0028.

Discussion

We found that the presence of microbleeds was associated with a poorer global microstructural integrity of brain white matter, even when only a single microbleed was present. Associations were present after adjusting for important cardiovascular risk factors and other MRI markers of CSVD. When investigating directional diffusivity, we observed that both axial and radial diffusivity were higher in subjects with microbleeds.

The large sample size and population-based setting increase generalizability of our results and are major strengths of our study. We used sensitive MRI sequences to detect microbleeds[23] and to measure microstructural integrity of brain white matter.[24] Also, because of availability of a wide range of other subject characteristics, we were able to investigate the association between microbleeds and white matter integrity after adjusting for important cardiovascular risk factors and other macroscopic imaging markers of CSVD, i.e., WMLs and lacunes. Some limitations of our study need to be mentioned. Because of the cross-sectional design of our study, we were not able to assess temporality of associations. Also, we should note that microbleed detection strongly depends on MRI acquisition parameters and field strength used.[23,25] Although our scan protocol was optimized for the detection of microbleeds on a 1.5-Tesla scanner,[23] we may have missed small bleeds that would have become apparent when, for example, using higher field strengths. Furthermore, registrations of DTIs are to some extent susceptible to artifacts and distortions and may therefore have small registration inaccuracies. All registration results were, however, visually inspected and no registration errors caused by distortions or susceptibility artifacts were observed. Finally, although we aimed to address all potential important confounders, residual confounding due to unmeasured confounders or measurement inaccuracy in variables may have affected our results to some extent. Specifically, residual confounding by age may have overestimated the association presented in our study.

Previously, pathology studies have suggested that the presence of microbleeds reflects a more diffuse pathologic process in the brain by showing that white matter changes surround the actual microbleeds. However, these studies were often limited by sample size and generalizability.[8-10] Moreover, it is difficult to draw conclusions on diffuse brain pathology from these studies because pathologic examinations were not performed on the entire brain. DTI-MRI allows us to study the entire cerebral white matter *in vivo*, and alterations in DTI parameters have previously been linked to both cognitive deterioration as well as neurodegenerative disease,[26-28] suggesting that clinically relevant white matter

changes can be assessed with DTI. Our DTI study in the general population now provides evidence to support the hypothesis that microbleeds are associated with subtle, but diffuse brain pathology *in vivo*, advocating the idea that presence of microbleeds on MRI may present merely a "tip of the iceberg" regarding the true extent of the underlying brain damage.

Several theories can be proposed when speculating about the underlying pathophysiologic mechanisms that link microbleeds and white matter integrity loss. First, microbleeds may reflect vascular pathology that leads to white matter integrity loss via a pathway of shared risk factors, e.g., *APOE* genotype, cardiovascular risk, or inflammatory factors. Alternatively, microbleeds may be linked to white matter integrity loss more directly through failure of the blood-brain barrier. For example, leakage of blood products due to blood-brain barrier dysfunction may result in microbleeds on one hand, and lead to subtle damage to the surrounding neuronal and white matter tissue on the other hand.[29,30]

We found that even the presence of a single microbleed related to a diffuse loss of white matter microstructure. This is particularly interesting because the presence of a single microbleed as isolated marker of CSVD remains an issue of debate.[31,32] Our results support the hypothesis that microbleed presence reflects damage to functional pathways more diffusively[33] rather than focally,[34] and thus that even a single microbleed marks relevant underlying vascular pathology. However, we should bear in mind that a single microbleed threshold should be interpreted within the context of MRI acquisition parameters and field strength used.[23,25]

Subjects with deep or infratentorial microbleeds had poorer global white matter microstructure compared with subjects with strictly lobar microbleeds. One explanation could be that hypertensive vasculopathy may affect the brain microvasculature more diffusely, whereas CAA is primarily restricted to the cortical and meningeal vessels. Another consideration is that CAA pathology is known to cluster in occipital lobes,[35] and this may not be adequately reflected in a global brain white matter diffusion measurement. Future studies could use a lobar or regional approach to account for these specific focal changes.

For persons with strictly lobar microbleeds, poorer white matter integrity was confined to *APOE* ε4 allele carriers, although results were based on a smaller study sample. White matter pathology is a frequent finding in patients with CAA,[36] and the *APOE* ε4 allele is a well-known risk factor for CAA. It may be that subjects with strictly lobar microbleeds who carry an *APOE* ε4 allele are more susceptible to diffuse white matter damage because both factors may act synergistically.

The association between microbleeds and WMLs is well established, as is the association between WMLs and poorer white matter microstructural integrity. The novelty of our study is that we found a direct association between microbleeds and white matter microstructure integrity loss. We showed that the relation between microbleeds and worse white matter microstructure remained after adjusting for burden of WMLs or presence of lacunes. A previous small study in patients with CAA did not find the same independency,[33] but differences in study population and sample size complicate comparison. Although the percentage of explained variance by microbleeds on DTI measurement in our study was only modest, the relation between microbleeds and white matter microstructural integrity provides important etiologic insights. Our finding suggests that microbleeds may reflect a more severe or unique form of vasculopathy that is not captured by other imaging markers of CSVD. This may implicate microbleeds as an important marker of risk stratification or therapy allocation in future studies.

Finally, we found that the presence of microbleeds was associated with increased axial and radial diffusivity. Axial diffusivity describes water movement parallel to, and radial diffusivity perpendicular to, the main fiber orientation. From animal studies the hypothesis arose that an increase in axial diffusivity may correlate with axonal damage,[37] while increased radial diffusivity better reflects myelin damage.[17,38,39] This would suggest that the presence of microbleeds may indicate both axonal and myelin damage. However, it is known from patient-based studies that demyelination and axonal damage often correlate and concordantly influence directional diffusivities. Also, the presence of both acute and chronic tissue damage may influence measures of axial diffusivity.[40,41] Thus, although it is tempting to extrapolate our understanding of directional diffusivity from animals to humans, interpretation of directional diffusivity measurements in observational studies is complex and warrants caution.

Overall, we found that microbleeds may reflect a more severe or unique form of underlying vasculopathy because their presence associates with diffuse microstructural integrity loss of brain white matter even after adjusting for the presence of lacunes and WMLs. This subtle white matter pathology may be more severe in *APOE* ε4 allele carriers with lobar microbleeds.

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2.3

Lobar microbleeds and cerebrovascular reactivity: the EDAN Study

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Abstract

Introduction: In patients with symptomatic cerebral amyloid angiopathy (CAA), cerebrovascular reactivity to visual stimuli is reduced. Lobar microbleeds are a diagnostic hallmark of CAA, but are also highly prevalent in asymptomatic individuals. In the latter group it remains uncertain whether lobar microbleeds reflect underlying CAA. In the general population, we investigated whether cerebrovascular reactivity is impaired in individuals with lobar microbleeds.
Methods: From the population-based Rotterdam Study, we invited 35 participants with lobar microbleeds and 15 age-matched controls (all ≥ 55 years) for functional MRI (fMRI) as part of the Early Detection of Angiopathy Network (EDAN) Study. Cerebrovascular reactivity parameters (i.e., amplitude and time to peak responses) were assessed in response to visual stimulation using fMRI. Student's t-test, linear regression, and linear mixed effects models were used to compare fMRI parameters in participants with and without microbleeds.
Results: Amplitude and time to peak responses did not differ between participants with and without microbleeds (respectively, P=0.179 and P=0.555). Participants with microbleeds, especially when located in occipital lobe, had slightly higher amplitude responses compared to participants without microbleeds. After excluding individuals with mixed microbleeds, we found no significant difference in cerebrovascular reactivity for persons with a single microbleed or multiple microbleeds compared to persons without microbleeds.
Conclusions: In the general population, lobar microbleeds are not related to impaired cerebrovascular reactivity. In asymptomatic individuals, lobar microbleeds may either reflect less advanced CAA pathology insufficient to cause functional vascular impairment, or reflect vascular pathology other than CAA.

Introduction

Cerebral amyloid angiopathy (CAA) is one of the most common forms of cerebral small vessel disease in old age, and an established cause of intracerebral hemorrhage and cognitive impairment.[1,2] One of the hallmarks of CAA in patients with an appropriate clinical history is the presence of strictly lobar microbleeds on brain MRI.[3] In the general population, strictly lobar cerebral microbleeds are also frequently observed on MRI and are potential markers of CAA.[4-7] To date, mainly indirect evidence is available to support this notion. Evidence includes the strong predilection of lobar microbleeds for posterior brain regions, along with preponderance in persons with *APOE* ε4 genotype, and correlations with ischemic cerebral small vessel lesions.[4-8]

Aside from microbleeds, accumulating evidence from clinical studies suggests that impaired cerebrovascular reactivity is also a phenotypic trait of advanced CAA.[9-11] Microbleeds and cerebrovascular reactivity may be linked via a mechanism in which vascular β-amyloid deposits cause cerebral small vessel to lose normal elements of the vessel wall and thereby stiffen. This impairs the vasodilation function of vessels, and consequently, impairs blood flow responses.

In CAA patients who present with intracerebral hemorrhage or other clinical events, impaired cerebrovascular responses have been reported, by means of Doppler ultrasound and functional magnetic resonance imaging (fMRI).[9,10,12] In the general population, the association between lobar microbleeds and cerebrovascular reactivity has not yet been studied. An inverse correlation between lobar microbleeds and cerebrovascular reactivity would strengthen the notion that lobar microbleeds reflect sufficiently advanced CAA in the general population to impair normal vessel function. We therefore performed fMRI on a subset of microbleed-positive and –negative participants in the population-based Rotterdam Study

Methods

Study population

This study was conducted as part of the Early Detection of Angiopathy Network (EDAN) Study, which aims to unravel early imaging markers of cerebral angiopathy in a clinical and population-based setting. For the present study, 41 stroke- and dementia-free persons with lobar microbleeds (cases) and 19 age-matched controls were recruited from the population-based Rotterdam Study,[13,14] to undergo fMRI with a visual stimulation task for assessment of cerebrovascular

reactivity. Eligible cases were individuals who previously underwent complete brain MRI between 2012 and 2014 with usable T2* GRE imaging data for microbleed assessment, and did not have any of the following: a definite cause of microbleeds (i.e., antecedent head trauma, excessive anticoagulation [INR>3.0], ischemic stroke, central nervous system tumors, vasculitis, vascular malformation); history of intracerebral hemorrhages; dementia; contraindication to (functional) MRI [i.e., pacemakers]; seizures; non correctable visual impairment. Participants were invited via telephone. Using pre-hoc power calculations, we invited one- third of the participants with single microbleeds, one-third with multiple microbleeds, and one-third with no microbleeds on previous brain MRI. Note that some persons may have switched categories since the number of microbleeds could have increased in the meantime. The Medical Ethics Committee of the Erasmus Medical Center approved the study, and all participants provided written informed consent. For the current study we excluded 10 participants in whom BOLD fMRI-signal could not be calculated, leaving 50 participants for analysis.

Structural brain MRI and cerebral microbleeds

Participants were scanned on a 3.0-Tesla MRI scanner (GE Healthcare, Milwaukee, WI) using an 8-channel head coil. We used a multi-sequence MRI protocol, which included 3D SPGR T1-weighted images, and a T2*-weighted susceptibility-weighted angiography (SWAN) scan. Cerebral microbleeds were visually rated and recognized as focal, small, round to ovoid areas of signal loss on SWAN (repetition time= 37 ms, echo time= 22, matrix size=320 x 224, flip angle=12, field-of-view=25.6 X 0.75, parallel imaging acceleration factor= 2, 94 slices encoded with a slice thickness of 1.6 mm zero padded to 184 slices of 0.8 mm, acquisition time 3 min 26 sec). The presence and number of microbleeds were scored by 2 trained research physicians. The intraobserver (n=60, single rater, k=0.92) and interobserver reliability (n=60, k=0.91) was excellent. In addition, the lobar distribution of microbleeds was assessed automatically.[7] In short, after microbleeds were manually labelled, automated lobe segmentation was done by nonrigid registration of 6 manually annotated lobe atlases to the participant under investigation using the Elastix software. Lobe segmentations were combined with the manually labeled microbleeds, to determine the lobar distribution of microbleeds. Infarcts and brain tissue segmentations (total brain volume, gray matter, white matter, and white matter lesions volume) were defined within the Rotterdam Study, as described extensively before.[14]

Functional brain MRI and visual stimuli task design

BOLD-weighted echo-planar imaging (EPI) volumes positioned on the occipital lobes were acquired with a voxel size of 2.5 x 2.5 x 3 mm, repetition time of 1500 ms, echo time of 38 ms, flip angle 78º. A total of 512 imaging volumes were acquired over four consecutive runs, each with an acquisition time of 3 minutes 12 seconds.

The visual stimulus was presented in 16 consecutive blocks (4 per run), each consisting of 20 seconds of a 8-Hz flashing radial black-and-white checkerboard pattern followed by 28 seconds of gray screen. These visual stimuli were projected onto a screen within the MRI scanner bore, which the participant observed through a mirror attached to the head coil. To maintain participants attention and fixation, each participant was given a fixation task to indicate by pushbutton when a dot positioned in the center of the projection screen changed from lighter to darker red, which occurred at random intervals of approximately 2 seconds throughout the stimulus presentation.

Functional MRI processing

A total of four BOLD-weighted echo-planar imaging (EPI) runs were collected for each subject, each consisting of 128 imaging volumes. Pre-processing of those volumes was done using FEAT (FMRI Expert Analysis Tool) Version 6.00, part of FSL (FMRIB's Software Library, www.fmrib.ox.ac.uk/fsl). Specifically, the raw EPIs underwent motion correction, brain extraction, spatial smoothing (at a 3.0 mm FWHM Gaussian kernel), high-pass temporal filtering, and pre-whitening. Then, FEAT was used to yield volumetric statistical parametric maps of activation strength of each subject, using a canonical hemodynamic response function (HRF) (double-gamma function). Those statistical maps were thresholded using clusters with a Z-statistic value greater than 2.3 and a corrected cluster significance threshold of $p = 0.05$ (Worsley, 2001). In addition to the statistical parametric maps, FEAT (FSL) generates time-series plots for data and model, averaged over all the significant voxels. The signals predicted by the canonical HRF model at each time-point were then averaged, over all 4 runs, and each was expressed as a percentage of the mean signal intensity. The BOLD amplitude was subsequently defined as the difference between the peak signal and the baseline signal. In order to calculate a robust Time-to-Peak, a double-gamma function was fitted to the data, predicted by FEAT, using the curve fitting tool of MATLAB. Then the Time-to-peak was defined as the time it takes the double-gamma fitted function to reach its peak.

Covariates

Blood pressure and blood pressure-lowering medication were assessed during a regular Rotterdam Study research center visit, on average 1.1 years (SD 0.5) prior to fMRI.[15]

Statistical analysis

Student's t-test and Fisher's exact test were used to compare characteristics of study participants with and without cerebral microbleeds. Functional MRI parameters (i.e., amplitude and time to peak responses) were compared in participants with and without microbleeds using independent samples T-test. Afterwards, linear mixed effects models were used to assess the association of microbleed count per lobe with fMRI parameters. For this purpose, all microbleeds in distinct cortical lobes of all participants were combined. Random effects were added to the model to account for within person clustering of microbleeds.

Finally, we excluded participants who also had non-lobar microbleeds (4 persons had both lobar and deep microbleeds), and used multiple linear regression to determine the relationship between strictly lobar microbleeds and fMRI parameters. In this analysis, we classified participants as having no microbleeds (reference), a single microbleeds, two microbleeds, and more than two microbleeds, to assess potential dose-dependency. Analyses were adjusted for age at time of fMRI and sex. In sensitivity analyses, we additionally adjusted for hypertension and cortical gray matter volume. Also, we excluded a single person with a very high number of lobar microbleeds (n=46), to assure that associations were not driven solely by this participant.

Results

The characteristics of the study population with analyzable fMRI data are provided in Table 1. Overall, the distribution of characteristics was similar for participants with and without microbleeds. Of the participants with microbleeds, n=21 had a single microbleed and n=14 had multiple microbleeds (range, 1-46). The total number of microbleeds counted per lobe for all participants combined was respectively: 13 frontal, 19 temporal, 23 parietal, and 50 occipital.

Comparison of fMRI-assessed cerebrovascular reactivity to visual stimulation in participants with microbleeds relative to those without microbleeds showed no difference in amplitude of the BOLD-signal or in time to peak response (mean amplitude 4.22 ±0.78 versus 3.91 ±0.56, P=0.179; mean time to peak 7.69 ±0.77 versus 7.82 ±0.58, P=0.555) (Figure). Secondary analysis according to presence

Table 1. *Characteristics of the study population*

	Microbleeds absent (n=15)	Microbleeds present (n=35)	*P* value
Women	7 (46.7)	20 (57.1)	0.538
Age, years	63.9 (4.3)	64.0 (5.4)	0.937
Presence of hypertension	9 (60.0)	18 (51.4)	0.758
Blood pressure-lowering medication use	4 (26.7)	13 (37.1)	0.533
Intracranial volume, mL	1134.5 (114.5)	1170.2 (140.9)	0.391
Gray matter volume, mL	66.5 (5.9)	67.9 (1.5)	0.710
White matter lesions volume, mL*	2.6 (1.6-4.3)	3.7 (1.5-6.0)	0.280
Presence of lacunes	0 (0)	3 (8.6)	0.545

*Values represent mean (standard deviation) for continuous variables and number (percentages) for categorical variables. *White matter lesion volume was measured in 49 persons and presented as median (interquartile range).*

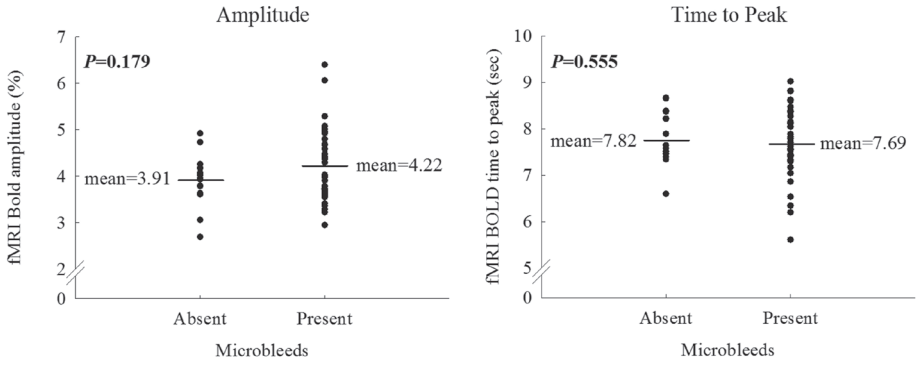

Figure. *Presence of cerebral microbleeds and cerebrovascular reactivity.*
Left graph represent BOLD fMRI amplitude responses and the right graph represent BOLD fMRI time to peak responses for persons with and without microbleeds. Horizontal bars represent the unadjusted mean response of the subgroups. fMRI= functional MRI

of microbleeds in each cortical lobe found increased amplitude of the fMRI response among persons with microbleeds in the occipital lobe (age and sex adjusted difference in amplitude: 0.69, 95% CI 0.14; 1.24) (Table 2). After excluding participants with mixed microbleeds, we found no difference in fMRI parameters of cerebrovascular reactivity in persons with a single microbleeds and multiple microbleeds relative to persons without microbleeds (Table 3).

Additional adjustments for hypertension or cortical gray matter volume did not change the results meaningfully. Also, excluding the single participant with a high microbleed count (n=46, of whom 29 occipital microbleeds) did not alter the results.

Table 2. *Lobar distribution of cerebral microbleeds and cerebrovascular reactivity*

	Mean difference in amplitude	Mean difference in time to peak
No microbleeds (n=15)	Reference	Reference
Any microbleeds (n=110)	0.41 (-0.07;0.88)	-0.12 (-0.50;0.27)
Frontal microbleeds (n=13)	0.06 (-0.59;0.71)	0.01 (-0.53;0.53)
Temporal microbleeds (n=19)	-0.03 (-0.67;0.61)	-0.29 (-0.81;0.23)
Parietal microbleeds (n=23)	0.52 (-0.08;1.12)	0.03 (-0.46;0.52)
Occipital microbleeds (n=50)	0.69 (0.14;1.24)	-0.09 (-0.54;0.36)

Values represent differences in amplitude and time to peak responses on BOLD fMRI per lobar microbleed count relative to no microbleeds. Values are adjusted for age and sex.
n= the number of microbleeds in each cortical lobe for all participants combined.

Table 3. *Categories of microbleed count and cerebrovascular reactivity*

	Mean difference in amplitude	Mean difference in time to peak
No microbleeds	Reference	Reference
Single microbleeds	0.09 (-0.39;0.57)	0.04 (-0.46; 0.53)
2 microbleeds	0.76 (0.03; 1.48)	-0.01 (-0.76; 0.73)
>2 microbleeds	0.26 (-0.46; 0.99)	-0.33 (-1.08; 0.42)

Values represent mean differences in amplitude and time to peak responses on BOLD fMRI in persons with a single microbleeds (n=21), 2 microbleeds, (n=6) and >2 microbleeds (n=8) relative to persons without microbleeds (n=15). Linear trend test across microbleed categories for amplitude P value=0.158, for time to peak P value=0.456.
Values are adjusted for age and sex. In contrast to the Figure and Table 2, we excluded persons with non-strictly lobar microbleeds (n=4) from this analysis.

Discussion

In this sample from the general population, we found no difference in cerebrovascular reactivity in response to visual stimuli in persons with cerebral microbleeds relative to those without. We hypothesized that if asymptomatic strictly lobar microbleeds were an imaging marker of advanced vascular amyloid pathology, they might associate with reduced cerebrovascular reactivity. This hypothesis was based on previous studies in patients with symptomatic CAA. In these previous studies it was shown that, compared to healthy age-matched controls, vascular reactivity in response to visual stimulation was impaired in CAA patients, reflected in lower amplitudes and delayed time to peak on BOLD-fMRI and using Doppler ultrasound in lower visual evoked mean flow velocity increase in the posterior cerebral artery.[9,10,12] Inconsistent results were however reported for the correlation of vascular reactivity parameters with number of microbleeds, with one study reporting strong correlations,[10] whereas another study did not find correlations.[9]

The presumed link between reduced cerebrovascular reactivity and severe vascular amyloid pathology may be explained by impaired vessel dilation. Beta amyloid accumulates in the wall of small and medium cerebral vessels and causes changes to or loss of smooth muscle cells of the tunica media and adventitia.[16] These changes presumably compromise the integrity of the vessel walls, and consequently predispose to bleedings. Additionally, vasodilation to appropriate stimuli may be reduced.[17-20] Cerebrovascular reactivity may also be reduced via a mechanism by which β-amyloid causes physiologic dysfunction in smooth muscle cells which impairs normal vasodilatation.[21,22] On the other hand, impaired vascular reactivity may also point towards reduced blood flow in response to reduced metabolic demand from injured neuronal tissue. A previous study however, showed no detectable changes in visual evoked potential amplitudes in CAA patients compared to healthy controls, despite the reduction of BOLD fMRI amplitude responses.[10] This finding may indeed, as suggested by the authors, point towards decoupling of vascular and neuronal responses. Thus, in CAA reduced blood flow may particularly be due to impaired vessel dilation rather than reduction of metabolic demand from neuronal inactive tissue.[10]

We did not, however, find impaired cerebrovascular reactivity in asymptomatic individuals with lobar microbleeds. This was in line with findings from a longitudinal study in CADASIL patients that did not find a relationship between cerebrovascular reactivity and microbleed progression.[23] In addition, the SMART Study failed to present an association between microbleed count and cerebrovascular dysfunction on 7-Tesla MRI, although presence of any microbleed

was associated with impaired vascular reactivity.[24]

Various explanations may be proposed for the fact that we did not find reduced cerebrovascular reactivity in our cohort whereas this was found previously in symptomatic CAA patients. First, if we assume that lobar microbleeds in community dwelling persons indeed reflect early CAA, it might be postulated that these bleeds in asymptomatic individuals reflect a stage of pathology that is insufficient to affect the cerebrovascular reactivity of small vessels. Early therapeutic intervention in this group could thus prevent or halt vascular dysfunction and positively influence the disease course of CAA. The interpretation that lobar microbleeds indeed reflects underlying CAA is supported by the finding that healthy individuals with lobar microbleeds had higher retention of the amyloid tracer Pittsburgh Compound B on positron emission tomography compared to healthy elderly individuals without lobar microbleeds.[25] Second, although an increasing body of indirect evidence suggests that strictly lobar microbleeds reflect underlying amyloid pathology in community-dwelling individuals, it is possible that many or all of these individuals do not have substantial CAA, but rather another type of vasculopathy accounting for their microbleeds. A third possibility, advocated by the suggestion of enhanced fMRI amplitude in a subset of participants, is that CAA-related vascular pathology might progress through an earlier stage of enhanced vascular reactivity before eventually demonstrating impaired reactivity. In the absence of other evidence or a well-defined vascular mechanism, this explanation remains speculative and would require further confirmation.

Small sample size is a potential limitation of our study. In addition, for 10 participants no BOLD activations were retrieved during fMRI post-processing steps. These participants were on average older and may have had more difficulties following instructions of the visual stimulation task. As such we cannot rule out effects caused by selection bias in our study. Also, the cross-sectional design of our study hampers conclusions regarding causality. Finally, we used a visual stimulation task and focused on BOLD-fMRI activation in the occipital cortex of the brain, as lobar microbleeds show a predilection for posterior brain regions. Though vascular reactivity to visual stimulation may be an accurate marker of advanced CAA burdens, it may be insensitive to less severe CAA in asymptomatic individuals with few lobar microbleeds.

In conclusion, in the general population, lobar microbleeds are not related to impaired cerebrovascular reactivity. In asymptomatic individuals, lobar microbleeds may either reflect CAA pathology with insufficient properties to cause functional vascular impairment or reflect pathology other than CAA.

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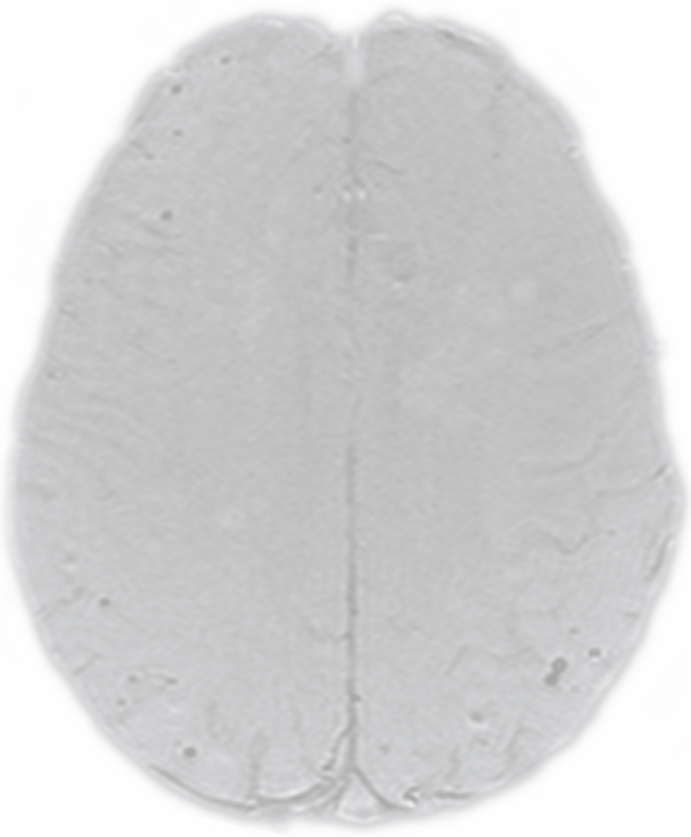

2.4

KIDNEY FUNCTION AND CEREBRAL SMALL VESSEL DISEASE

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Abstract

Background: Anatomic and hemodynamic similarities between renal and cerebral vessels suggest a tight link between kidney disease and brain disease. Although several distinct markers are used to identify subclinical kidney and brain disease, a comprehensive assessment of how these markers link damage at both end organs is lacking.

Methods: In 2,526 participants of the population-based Rotterdam Study, we measured urinary albumin-to-creatinine ratio, and estimated glomerular filtration rate (eGFR) based on serum creatinine and cystatin C. All participants underwent brain MRI. We assessed presence of cerebral small vessel disease by calculating white matter lesion (WML) volumes and rating the presence of lacunes and cerebral microbleeds. We used multivariable linear and logistic regression to investigate the association between kidney function and cerebral small vessel disease.

Results: Worse kidney function was consistently associated with a larger WML volume (mean difference per SD increase in albumin-to-creatinine ratio: 0.09, 95% CI 0.05;0.12; per SD decrease in creatinine-based eGFR: -0.04, 95% CI -0.08;-0.01, and per SD decrease in cystatin C-based eGFR: -0.09, 95% CI -0.13;-0.05). Persons with higher albumin-to-creatinine ratio or lower cystatin C-based eGFR levels had a higher prevalence of lacunes (odds ratio per SD increase in albumin-to-creatinine ratio: 1.24, 95% CI 1.07;1.43). Only participants in the highest quartile of albumin-to-creatinine ratio had a higher frequency of microbleeds compared to the lowest quartile.

Conclusions: Worse kidney function is associated with cerebral small vessel disease. Of all measures of kidney function, in particular albumin-to-creatinine ratio is related to cerebral small vessel disease.

Introduction

Small blood vessels in the kidney and the brain are closely linked because of anatomical and hemodynamic similarities.[1,2] These resemblances highlight the likelihood of a shared pathogenesis of renal and cerebrovascular disease. Since small vessel disease is considered a systemic disorder, pathology in one end organ may provide information on coexistent or future damage in another end organ. Indeed, worse kidney function has been related to cerebral small vessel disease,[3-6] which may appear as white matter lesions (WML), lacunes, and cerebral microbleeds on magnetic resonance imaging (MRI).

While the association between kidney function and cerebral small vessel disease has been studied before, there have been inconsistent reports. The inconsistencies can partly be explained by differences in population characteristics, morbidities, and ethnicities. Also, studies have typically correlated only a single marker of kidney dysfunction to markers of cerebral small vessel disease. Kidney function measures (i.e., albuminuria, estimated glomerular filtration rate (eGFR) based on creatinine or cystatin C) reflect damage to glomerular vessels of numerous kind, and may associate differently with the ischemic and hemorrhagic subtypes of cerebral small vessel disease. Thus far, no study has examined how the various serum and urinary markers of kidney function associate with the entire spectrum of subclinical cerebral small vessel lesions within one single population. Such a study would add to current literature because it allows for direct comparison of kidney markers without being limited by heterogeneity of study populations.

In a middle-aged and elderly population, we therefore investigated whether measures of kidney function – i.e., urinary albumin-to-creatinine ratio and eGFR based on serum creatinine or cystatin C - were associated with cerebral small vessel disease on MRI.

Methods

Study Population

The Rotterdam study is an ongoing prospective population-based cohort designed to investigate chronic diseases in the middle-aged and elderly population.[7] The cohort originated in 1990 and comprised 7,983 participants aged 55 years and older. In 2000 and 2006 the cohort was expanded and now counts 14,926 participants aged ≥45 years.[7] Brain MRI was implemented from 2005 onwards,[8] and a subset of the cohort had urine and serum samples collected around the time of MRI. Urine and blood samples were collected in 3,181 participants, and all three kidney function measures were obtained in 2,596 participants. The

Rotterdam Study has been approved by the medical ethics committee according to the Population Study Act Rotterdam Study, executed by the Ministry of Health, Welfare and Sports of the Netherlands. A written informed consent was obtained from all participants. Participants with eGFR <15 or > 200 mL/min/1.73m^2 were excluded from all analyses (n=3). Additionally, participants who showed cortical infarcts on MRI (n=67) were excluded because we solely aimed to study cerebral small vessel pathology and because gliosis around cortical infarcts may cause automated WML segmentation to become unreliable. This resulted in 2,526 participants who were included in our analyses. In the analyses involving WML, we additionally excluded 34 participants because the automated WML volume tissue segmentation was rendered unreliable.

Kidney Function Measures

For the assessment of albuminuria, participants were asked to collect (overnight) timed urine samples. Urinary albumin and creatinine were determined by a turbidimetric method and measured by a Hitachi MODULAR P analyzer (Roche/Hitachi Diagnostics, Mannheim, Germany).[9] Albumin-to-creatinine ratio was calculated by dividing albumin (grams) by creatinine (mol). Because albumin-to-creatinine ratio was not normally distributed we used natural log-transformed values and added 1 gram/mol to the non-transformed values to account for zero values of albuminuria (Ln[albumin-to-creatinine ratio +1.0 gram/mol]). Although serum creatinine is generally used to estimate GFR, cystatin C has been proposed to be a more stable marker of kidney function in an elderly population.[10] We therefore calculated both measures in our study. Serum creatinine was measured using an enzymatic assay method.[11] Creatinine was calibrated by aligning mean values of serum creatinine from our cohort with those of the Third National Health and Nutrition Examination Survey (NHANES III) for men and women separately in age categories of <50, 50-59, 60-69, ≥70. Cystatin C was measured with a particle-enhanced immunonephelometric assay using a Roche/Hitachi cobas c 501 analyzer which calculates the analyte concentration of each sample automatically in mg/L.[12] Estimated GFR was calculated based on either serum creatinine or serum cystatin C using the chronic kidney disease epidemiology collaboration (CKD-EPI) formula.[13] In accordance, chronic kidney disease (CKD) was defined as eGFR <60 mL/min/1.73 m^2.

Brain MRI and Markers of Cerebral Small Vessel Disease

Participants were scanned on a 1.5-Tesla MRI scanner.[8] We performed a T1-weighted, proton-density weighted, fluid-attenuated inversion recovery (FLAIR), and T2*-weighted gradient-recalled echo sequence (3D T2* GRE), as described in

detail before.[8] Automated tissue segmentation, including conventional k-nearest-neighbor brain tissue classifier extended with WML segmentation.[14] was used to segment brains into gray matter volume, white matter volume, WML volume, and cerebrospinal fluid. All scans were rated by 1 of 5 trained research-physicians to determine presence and location of infarcts (lacunes and cortical infarcts) and microbleeds. Lacunes were rated on FLAIR, proton-density-weighted and T1-weighted sequences, and were defined as focal lesions ≥3mm and <15mm in size, with the same signal intensity as cerebrospinal fluid on all sequences and a hyperintense rim on the FLAIR (when located supratentorially).[15] Infarcts showing involvement of cortical gray matter were classified as cortical infarcts. Microbleeds were visually rated as small, focal, round to ovoid areas of signal loss on 3D T2* GRE images.[15]

Assessment of Cardiovascular Risk Factors

Cardiovascular risk factors were assessed by interview, laboratory and physical examinations during the same visits in which kidney function measures were examined. Body mass index (BMI) was calculated by dividing the weight (in kilograms) by the height squared (in meters). Systolic and diastolic blood pressures were measured twice with a random-zero sphygmomanometer in sitting position at the right arm. Total and high-density lipoprotein (HDL) cholesterol (mmol/L) was determined using an automated enzymatic procedure (Hitachi analyser, Roche Diagnostics). Diabetes mellitus was defined as fasting blood glucose of ≥7.0 mmol/L, and/or the use of any glucose lowering medication. During home interviews, participants were asked about their smoking status (ever versus never smoking), and whether they used antihypertensive and/or lipid-lowering medication.

Statistical Analysis

Urinary albumin-to-creatinine ratio (g/mol) was modeled continuously per standard deviation (SD) increase. For serum creatinine (mg/dL) and serum cystatin C (mg/L) we calculated the eGFR (mL/min/1.73m^2), which was also modeled continuously per SD increase. Additionally, participants were dichotomized as having CKD yes or no (eGFR<60 mL/min/1.73m^2 used as cut off for CKD).[16] WML volume was natural log transformed because of its skewed distribution and investigated continuously. Lacunes and microbleeds were investigated dichotomously (present versus absent). In accordance with their presumed etiological background, cerebral microbleeds were categorized by location into strictly lobar versus deep or infratentorial (with or without the presence of lobar microbleeds).[15] We used multivariable linear regression to

obtain mean differences in WML volumes for every SD increase in kidney function measures and for CKD presence. Logistic regression was used to calculate odds ratios to study the relation of kidney function with lacunes and microbleeds. All models were adjusted for age and sex (model I), and additionally for cardiovascular risk factors that were considered confounders (model II). As potential confounders we took into account: BMI, systolic and diastolic blood pressure, total and HDL cholesterol, diabetes mellitus, smoking, antihypertensive and lipid-lowering medication. All analyses involving WML volume were also adjusted for intracranial volume. In sensitivity analyses we also adjusted for C-reactive protein and a history of clinical cardiovascular disease (stroke or coronary heart disease) because these factors may confound the association between kidney function and cerebral small vessel disease. Additionally, we adjusted the kidney function measurements for each other to estimate the independent association of every kidney function marker on cerebral small vessel disease. Finally, we computed quartiles of kidney function measures and compared the highest to the lowest quartiles, and studied the linear trends of these measurements in relation to markers of cerebral small vessel disease.

All analyses were done using R version 2.15.0 and IBM SPSS Statistics for Windows version 21.0, using an α-value of 0.05.

Results

Characteristics of the study population are presented in Table 1. Of the 2,526 participants, 55.6% were women and mean age was 56.9 years (SD 6.3). Median albumin-to-creatinine-ratio was 0.4 (interquartile range 0.2 to 0.7) g/mol. Mean estimated GFR was 86 mL/min/1.73m^2, measured either with serum creatinine or cystatin C. Based on serum creatinine levels, there were 64 CKD cases (eGFR <60 mL/min/1.73m^2) versus 128 CKD cases based on serum cystatin C levels. Median WML volume was 2.0 mL. In total, 3.6% of the participants had one or more lacunes, and 12.6% had one or more microbleeds.

In Table 2, we show the association between kidney function and markers of cerebral small vessel disease. Participants with worse kidney function - i.e., higher urinary albumin-to-creatinine ratio, lower creatinine or lower cystatin C-based eGFR- had more WML volume (age and sex adjusted mean difference for WML volume per SD increase in albumin-to-creatinine ratio 0.09, 95% confidence interval [CI] 0.05;0.12). Participants with a higher urinary albumin-to-creatinine ratio and higher serum cystatin C-based eGFR also had a higher prevalence of lacunes (odds ratio for lacunes per SD increase in albumin-to-creatinine ratio 1.24, 95% CI 1.07;1.43). No association was found between

continuous measures of kidney function and cerebral microbleeds. Similar associations were found when investigating the relation between CKD and imaging markers of cerebral small vessel disease (Supplementary Table 1). Participants with CKD had larger volumes of WML and more lacunes compared to those without CKD. No association was found for microbleeds. Additional adjustments for potential cardiovascular confounders did not alter any of the above-mentioned results. Also, adjusting for C-reactive protein, a history of clinical cardiovascular disease or adjusting kidney function measurements for each other did not change the interpretation of our results (data not shown).

Table 1. *Characteristics of the study population*

Characteristics	N=2526
Age, years	56.9 (6.3)
Female	1406 (55.6)
Albumin-to-creatinine ratio in urine, g/mol	0.4 (0.2-0.7)
Serum creatinine, mg/dL	0.9 (0.2)
Creatinine-based eGFR, mL/min/1.73m^2	86.2 (12.9)
Serum cystatin C, mg/dL	0.9 (0.2)
Cystatin C-based eGFR, mL/min/1.73m^2	86.4 (15.6)
White matter lesion volume, mL	2.0 (1.3-3.5)
Lacunes	92 (3.6)
Microbleeds	318 (12.6)
Strictly lobar microbleeds	233 (9.2)
Deep or infratentorial microbleeds	85 (3.4)
Body mass index, kg/m^2	27.5 (4.3)
Systolic blood pressure, mmHg	132.2 (18.7)
Diastolic blood pressure, mmHg	82.4 (10.8)
Total cholesterol, mmol/L	5.9 (1.05)
High-density lipoprotein, mmol/L	1.4 (0.4)
Diabetes mellitus	182 (7.2)
Smoking	1752 (69.3)
Antihypertensive medication	636 (25.2)
Lipid-lowering medication	530 (21.0)

Data presented as mean (standard deviation) for continuous variables and number (percentages) for categorical variables. Albumin-to-creatinine ratio and white matter lesion volume presented as median (interquartile range).

The following variables had missing data: body mass index (n=2), blood pressures (n=6), high-density lipoprotein cholesterol (n=1), diabetes mellitus (n=20), smoking (n=5), antihypertensive and lipid-lowering medication (n=21).

White matter lesion volumes were measured in n=2,492 participants (excluding 34 scans in which tissue segmentation measures could not be obtained reliably).

Abbreviation: eGFR= estimated glomerular filtration rate

Table 2. *Kidney function and cerebral small vessel disease*

	Mean difference (95% CI)	Odds ratios (95% CI)		
	White matter lesions	Lacunes	Strictly lobar microbleeds	Deep or infratentorial microbleeds
Model I				
Albumin-to-creatinine ratio	0.09 (0.05;0.12)	1.24 (1.07;1.43)	0.99 (0.87;1.14)	1.14 (0.96;1.35)
Creatinine-based eGFR	-0.04 (-0.08;-0.01)	1.08 (0.86;1.35)	1.06 (0.92;1.23)	0.98 (0.77;1.23)
Cystatin C-based eGFR	-0.09 (-0.13;-0.05)	0.70 (0.56;0.89)	0.94 (0.81;1.10)	0.82 (0.64;1.05)
Model II				
Albumin-to-creatinine ratio	0.07 (0.04;0.11)	1.19 (1.02;1.39)	0.96 (0.84;1.11)	1.11 (0.93;1.33)
Creatinine-based eGFR	-0.04 (-0.08;-0.004)	1.07 (0.85;1.34)	1.07 (0.92;1.25)	0.96 (0.77;1.21)
Cystatin C-based eGFR	-0.08 (-0.12;-0.04)	0.74 (0.58;0.95)	0.96 (0.82;1.13)	0.83 (0.65;1.07)

White matter lesions: values represent mean differences (95% CI) in white matter lesion volume per standard deviation increase in measures of kidney function.
Lacunes: values represent odds ratios (95% CI) for lacunes per standard deviation increase in measures of kidney function.
Microbleeds: values represent odds ratios (95% CI) for microbleeds per standard deviation increase in measures of kidney function.
Model I: adjusted for age and sex.
Model II: as Model I, additionally adjusted for body mass index, systolic and diastolic blood pressure, total and high-density lipoprotein cholesterol, diabetes mellitus, smoking, antihypertensive and lipid-lowering medication. Analyses involving white matter lesions were additionally adjusted for intracranial volume. Analyses in Model II were performed as a complete case analysis.
Participants with scans in which tissue segmentation measures could not be obtained reliably were excluded from the analyses of white matter lesion volume (n=34).
Abbreviations: CI= confidence interval, eGFR= estimated glomerular filtration rate.

Figure shows the association of kidney function measures in quartiles with WML volume, lacunes, and microbleeds (in any location). Participants in the highest quartile of albumin-to-creatinine ratio had more cerebral small vessel disease compared to those in the lowest quartiles (*P* values for linear trend tests were <0.05 in relation to all markers of cerebral small vessel disease). Also, those in the lowest quartile of cystatin C-based eGFR had more WML volume and lacunes compared to those in the highest quartile (*P* values for linear trend test were <0.05 in relation to WML and lacunes).

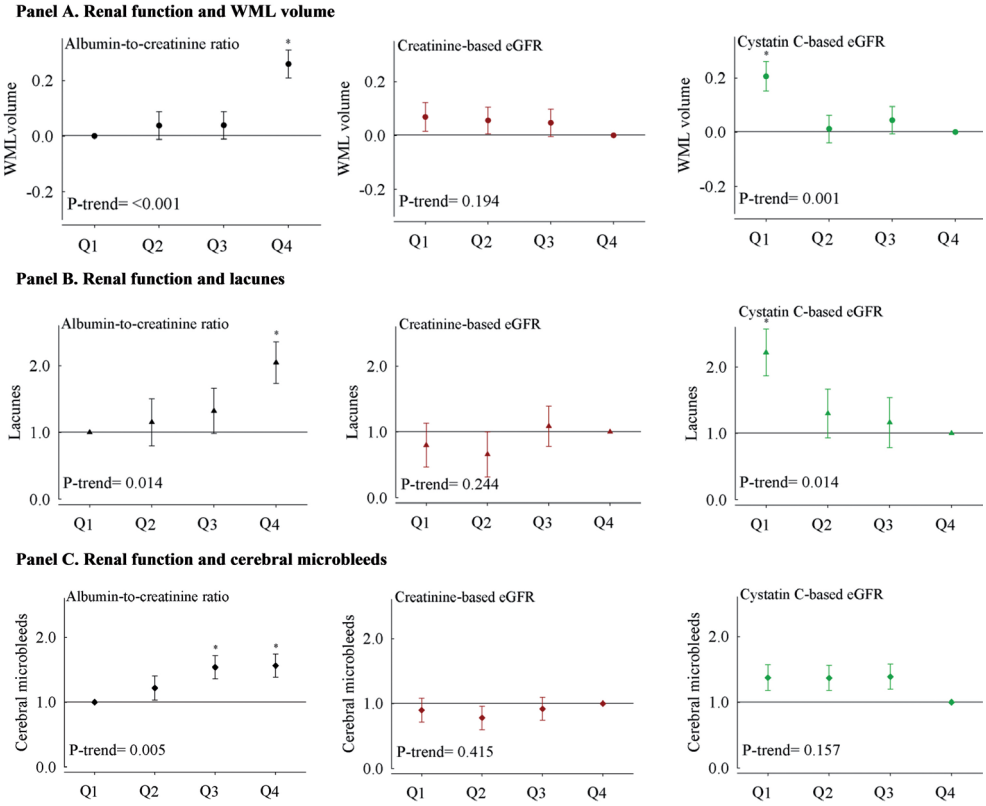

Figure. *Quartiles of kidney function measures and markers of cerebral small vessel disease.*
On the x-axis of all panels: quartiles of standardized kidney function measures. For urinary albumin-to-creatinine ratio the lowest quartile was used as reference category. For serum creatinine-based eGFR and serum cystatin C-based eGFR the highest quartile was used as reference category.
Panel A: circles represent the age, sex, and intracranial volume adjusted mean differences in WML volume.
Panel B: triangles represent age and sex adjusted odds ratios for lacunes.
Panel C: diamonds represent age and sex adjusted odds ratios for cerebral microbleeds.
*Bars represent the standard error. * Represents a P value <0.05 when a quartile was compared to the reference category. P values for linear trends are presented per kidney function measure.*
The range of albumin-to-creatinine ratio for each quartile was: Q1=0.00-0.28;Q2= 0.21-0.44; Q3=0.32-0.74; Q4=0.61-148.43, range of creatinine-based eGFR for each quartile was: Q1=17.11-82.93; †Q2=67.09-92.27; Q3=73.46-102.51; Q4=83.93-180.09, range of cystatin C-based eGFR for each quartile was: Q1=20.62-78.33; Q2=72.16-88.98; Q3=81.46-101.29; Q4=91.12-138.20.
†For lacunes and cerebral microbleeds, range of creatinine-based eGFR for Q2= 65.00-92.27.
Abbreviations: WML= white matter lesion volume, eGFR= estimated glomerular filtration rate, Q1 through Q4= quartiles 1 through 4.

Discussion

In this population-based study we investigated the association between various markers of kidney function and cerebral small vessel disease. Of all renal markers, albumin-to-creatinine ratio showed strongest associations with cerebral small vessel disease presence. Higher albumin-to-creatinine ratio, and lower eGFR based on creatinine and cystatin C were all associated with larger WML volumes. A higher albumin-to-creatinine ratio and decreased eGFR based on cystatin C were related to presence of lacunes, whereas only a higher albumin-to creatinine ratio was associated with presence of microbleeds.

Strengths of our study are the population-based character, with a large number of participants, and the extensive phenotyping within the Rotterdam Study that enabled us to investigate both urine and serum markers of kidney function in relation to several imaging markers of cerebral small vessel disease. We also acknowledge several limitations of our study. First, our study was performed cross-sectionally and we cannot draw conclusions regarding causality. Second, markers of kidney function were only measured once and this may have biased our results towards the null. Third, although we adjusted for important and evident potential cardiovascular risk factors in the association between kidney disease and cerebral small vessel disease we cannot rule out residual confounding because of unmeasured confounders.

Several hypotheses have been proposed to explain the link between renal and cerebral small vessel disease. First, small vessels in kidney and brain are both exposed to high blood flow volumes during the entire cardiac cycle.[1] It is very likely that kidney disease and cerebral small vessel disease are both signs of systemic small vessel disease affecting different end organs with anatomical and hemodynamic similarities. Shared risk factors, in particular high arterial blood pressure, may cause concurrent vascular damage to afferent arterioles in the kidney and the brain as these vessels branch off large arteries that maintain high vessel tone to ensure perfusion of kidney and brain tissue.[2] Second, endothelial dysfunction, regardless of the cause, leads to leakage of proteins into interstitial space in both kidney and brain.[17] Various markers have been suggested to cause endothelial dysfunction, including nitric oxide. Kidney dysfunction is known to induce nitric oxide deficiency due to disturbances in L-arginine metabolism or an increase in endogenous nitric oxide synthase inhibitors,[18] and may cause problems in maintaining microcirculation and blood brain barrier function.[18] Third, inflammatory processes, including the direct or indirect effects of lipoprotein phospholipase A2, myeloperoxidase and/or C-reactive protein, have been shown to affect both renal and brain vessels, and are also known for their role in endothelial dysfunction.[19-22]

Among the kidney function measures we investigated, we found that albuminuria was strongest associated with small vessel disease in the brain. This association has been described in the past, both in the general population and in patients with hypertension or acute stroke.[4,17,23-26] Although the role of albuminuria in cerebrovascular disease is not well understood, endothelium dysfunction throughout the entire body is the most appealing explanation.[27,28] Since albuminuria associates with different phenotypes of cerebral small vessel disease - both ischemic and hemorrhagic - it may suggest that endothelial dysfunction acts as a stressor and activates pathways that induce both thrombi and small vessel fragility. However, one could also argue that WML, lacunes, and microbleeds reflect markers of a single pathologic continuum. Either way, microalbuminuria may serve as an important surrogate marker for the presence of cerebral small vessel disease.

We found that a worse kidney function measured with either creatinine or cystatin C was associated with WML. This is in agreement with previous studies in the general population.[3,29-31] Although some recent studies suggest otherwise,[32-34] cystatin C is generally thought to be a more stable measurement for kidney function in the elderly than creatinine,[10] because cystatin C is hypothetically less affected by aging, loss of muscle mass and gender. We indeed found that, compared to creatinine, GFR based on cystatin C correlated better with the presence of lacunes. This confirms previous findings from the Cardiovascular Health Study.[6] In contrast to previous studies in stroke patients,[5,35-37] we did not find an association between creatinine or cystatin C and microbleeds. Potential explanations for these observations are first, a lack of statistical power in our study. Second, differences in ethnicity of study populations yield different cardiovascular risk. Third, during the acute phase of stroke, levels of creatinine and cystatin C may be affected and consequently this could have influenced the results of the studies performed in acute stroke patients. Fourth, the pathology underlying microbleeds is thought to differ according to their location in the brain.[15] The majority of microbleeds in our study were located in lobar regions and are thought to reflect cerebral amyloid angiopathy; an angiopathy typically known not to affect organs outside the brain.

In conclusion, we found that of all kidney function measures, a higher albumin-to-creatinine ratio was strongest associated with the presence of imaging markers of cerebral small vessel disease. Our study emphasizes that albumin-to-creatinine ratio is a particularly helpful marker to identify presence of or risk for subclinical cerebral small vessel disease in people with generalized small vessel disease.

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Supplementary Table 1. *Chronic kidney disease and cerebral small vessel disease*

	Mean difference (95% CI)		Odds ratios (95% CI)					
	N	White matter lesions	n/N	Lacunes	n/N	Strictly lobar microbleeds	n/N	Deep or infratentorial microbleeds
No CKD	2430	Reference	86/2462	Reference	224/2381	Reference	81/2238	Reference
Creatinine-based CKD	62	0.37 (0.14;0.59)	6/64	1.33 (0.51;3.47)	9/60	0.86 (0.39;1.88)	4/55	1.13 (0.37;3.47)
No CKD	2370	Reference	77/2398	Reference	217/2323	Reference	75/2181	Reference
Cystatin C-based CKD	122	0.49 (0.32;0.65)	15/128	2.27 (1.13;4.56)	16/118	0.73 (0.40;1.36)	10/112	1.58 (0.71;3.52)

White matter lesions: values represent mean differences (95% CI) in white matter lesion volume in people with CKD compared to those without CKD.
Lacunes: values represent odds ratios (95% CI) for lacunes in people with CKD compared to those without CKD.
Microbleeds: values represent odds ratios (95% CI) for microbleeds in people with CKD compared to those without CKD.
Analyses were adjusted for age and sex. Analyses involving white matter lesions were additionally adjusted for intracranial volume.
Participants with scans in which tissue segmentation measures could not be obtained reliably were excluded from the analyses of white matter lesion volume (n=34).
Abbreviations: CI= confidence interval, CKD= chronic kidney disease, n= number of participants with lacunes or microbleeds per exposure category, N= total population within the exposure category.

2.5

GENETIC LOCI FOR SERUM LIPID FRACTIONS AND INTRACEREBRAL HEMORRHAGE

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Abstract

Background: Serum total cholesterol and its fractions are inversely associated with intracerebral hemorrhages (ICH) and their potential subclinical precursor, cerebral microbleeds. To ascertain whether there is a genetic basis for this inverse association, we studied established genetic loci for serum total, LDL, and HDL cholesterol, and triglycerides in their association with ICH and microbleeds.

Methods: Data on 161 genetic variants for serum lipids was collected in 9,011 stroke-free participants (mean age 65.8, SD 10.2; 57.9% women) of the population-based Rotterdam Study. Participants were followed from baseline (1997-2005) up to 2013 for the occurrence of ICH. A subset of 4,179 participants underwent brain MRI for microbleed assessment between 2005 and 2011. We computed genetic risk scores (GRS) for the joint effect of lipid variants. Cox proportional hazards and logistic regression models were used to investigate the association of GRS of lipid fractions with ICH and microbleeds.

Results: After a mean follow-up of 8.7 (SD 4.1) years, 67 (0.7%) participants suffered an ICH. Microbleed prevalence was 19.6%. Higher genetic load for high serum total and LDL cholesterol was associated with an increased risk of ICH. Higher genetic load for high serum LDL cholesterol was also associated with a higher prevalence of multiple lobar microbleeds.

Conclusions: Genetic susceptibility for high serum total and LDL cholesterol is positively associated with incident ICH and multiple lobar microbleeds. We did not find a genetic basis for the previously reported inverse association between serum lipid levels and ICH.

Introduction

Hypercholesterolemia is an established modifiable risk factor for ischemic cardiovascular disease, including ischemic stroke. Paradoxically, high serum total cholesterol has been associated inversely with clinical intracerebral hemorrhages (ICH)[1-4] and their potential subclinical precursor, cerebral microbleeds.[5-8] Profound insight into this seemingly contradictory association is essential given the growing concern of adverse ICH events in persons vigorously treated with lipid-lowering medication.[9-11]

To date, studies have focused solely on serum lipid levels to investigate the inverse association with ICH. Some studies reported that low total cholesterol drove this association,[1,2,4] whilst others pointed towards a specific lipid fraction.[3,5,6] Results from these studies are, however, limited by the fact that serum lipid levels were measured only once, and associations may partly be explained by residual confounding due to unmeasured determinants, such as diet. No study reported on a potential genetic basis for the inverse association of serum lipids with ICH. Studying genes that influence serum lipid levels may provide more robust, unconfounded associations, as genes should not be susceptible to changes in lifestyle or environment.

We investigated 161 known genetic loci for serum total, HDL, and LDL cholesterol, and triglycerides and studied their associations with risk of ICH and with presence of their potential subclinical precursor, cerebral microbleeds.

Methods

Study Population

This study was conducted within the Rotterdam Study, a prospective population-based cohort study aimed at investigating determinants and consequences of chronic diseases in an aging population.[12] In 1990, 7,983 persons (78% of invitees) were included in the initial study wave (RS-I). In 1999, the cohort was expanded by 3,011 participants (67% of invitees) (RS-II). The cohort expanded a second time in 2006 with 3,932 participants (65% of invitees) (RS-III). The total of 14,926 participants enrolled, were invited to undergo home interviews and various physical and laboratory examination at the research center every 4 years. Genotyping was done in 1997 (RS-I), 1999 (RS-II), and 2006 (RS-III). Of the 14,926 participants, 9,011 were genotyped and stroke-free at baseline (Figure). From 2005 onwards, brain MRI, including microbleeds assessment, was performed in those without MRI contraindications (pacemakers,

claustrophobia).[13,14] Of the 9,011 stroke-free participants who were included at baseline, 4,179 underwent brain MRI scanning between 2005 and 2011.

Genotyping

Participants were genotyped using Illumina HumanHap 550 Duo BeadChip or the Illumina Infinium II HumanHap 610 Quad Arrays. Genotyping was done at the Human Genotyping Facility Genetic Laboratory department of Internal Medicine, Erasmus MC, Rotterdam, the Netherlands.[14] Variant-specific quality controls included filters for call rate (>98%), minor allele frequency (>0.1%), Hardy-Weinberg equilibrium (*P* values>10-6), and differential missingness by outcome or genotype (mishap test in PLINK, http://pngu.mgh.harvard.edu/purcell/plink/). Markov Chain Haplotyping (MaCH) package (http://www.sph.umich.edu/csg/abecasis/MACH, version 1.0.15 or 1.0.16 software) was used for imputation to the 1000 Genomes Phase I Version 3 reference panel (all populations). For each imputed variant, quality of imputation was estimated as the ratio of the empirically observed dosage variance to the expected binomial dosage variance. For this study, we extracted data on 161 variants, 73 which have been related to serum concentrations of total cholesterol, 56 to LDL, 71 to

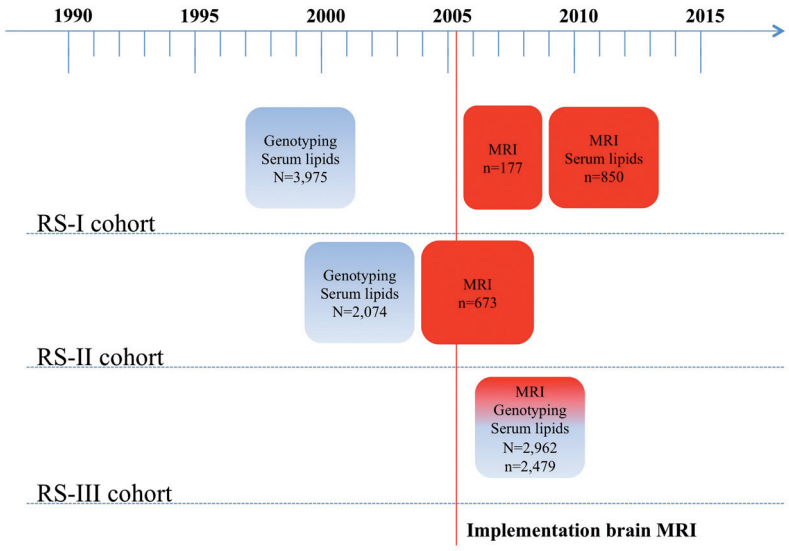

Figure. *Schematic overview of the study population.*
Blue boxes: population at risk of intracerebral hemorrhages (ICH) (baseline). Red boxes: subgroup of the population at risk of ICH that underwent MRI for the first time. Note that in some cases serum lipid levels were assessed at visits preceding MRI.
N= total population for ICH analyses; n= total population for cerebral microbleed analyses.

HDL, and 39 to triglycerides.[15] Imputation quality (R^2) for the serum lipid variants was >0.60 (mean=0.96).

Assessment of Stroke

Stroke was defined as a syndrome of rapidly developing clinical signs of focal or global disturbance of cerebral function, with symptoms lasting 24 hours or leading to death, with no apparent cause other than of vascular origin.[16] A history of stroke was assessed in all participants upon study entry using home interviews and was confirmed by reviewing medical records. Participants were subsequently followed for stroke occurrence through automated linkage of general practitioners' medical records with the study database. Medical records from nursing homes and from general practitioners of participants who moved out of the study area were checked on a regular basis. Research physicians reviewed all potential strokes using hospital discharge letters and information from general practitioners. An experienced vascular neurologist verified the stroke diagnoses.[17] Computed tomography reports were used to distinguish intracerebral hemorrhages from ischemic strokes. Strokes were classified as unspecified if neuroimaging was absent. Follow-up was complete until January 1st 2013 accounting for 77,991 (98.2% of potential) person-years.

Brain MRI and Microbleed Assessment

A multi-sequence MRI protocol was used on a 1.5-Tesla MRI scanner (GE Healthcare, Milwaukee, WI).[13] Cortical infarcts were defined as focal lesions affecting the cortical gray matter on FLAIR, T1-weighted, and T2-weighted sequences. Microbleeds were detected using a custom-made accelerated 3-dimensional T2*-weighted gradient-recalled echo sequence (repetition time= 45ms, echo time= 31, matrix size= 320x244, flip angle= 13, field-of-view= 25x17.5cm2, parallel imaging acceleration factor = 2, 3D acquisition with 96 slices encoded with a slice thickness of 1.6mm zero padded to 192 slices of 0.8mm, acquisition time 5min 55sec).[18] Microbleeds were defined as small, round areas of signal loss on T2*-weighted images and their presence, number, and location were rated by trained research-physicians.[18]

Covariates

Serum total cholesterol, HDL cholesterol, and triglyceride levels were determined using an automated enzymatic procedure (Hitachi analyzer, Roche Diagnostics, Washington DC). LDL cholesterol was calculated using the Friedwald formula (LDL cholesterol= total cholesterol – HDL cholesterol – triglycerides / 2.17), in

those with triglyceride levels ≤4.51 mmol/L.[19] If serum lipids levels were not assessed at time of MRI, serum lipid levels of a preceding visit were used (Figure). Records on lipid-lowering medication use (ATC-code C10) were retrieved from local pharmacies serving the study area. Blood pressures measurements were averaged over 2 readings measured within a single center visit using a random-zero sphygmomanometer. Smoking habits were defined as ever versus never smoking. Diabetes mellitus was defined as having fasting glucose levels of ≥7.0 mmol/L or the use of glucose-lowering medication. Body-mass-index was calculated as weight (in kilograms) divided by height (in meters) squared. Blood pressure-lowering and antithrombotic medication was assessed by interview during home visits.

Statistical Analysis

We computed weighted genetic risk scores (GRS) for total, LDL, and HDL cholesterol, and triglycerides by summing the number of serum lipid fraction alleles and weighting them by the reported effect estimate of each lipid variant (73 variants for total cholesterol, 56 variants for LDL cholesterol, 71 variants for HDL cholesterol, and 39 variants for triglycerides using results from the GWAS on serum lipids).[15] Note that most variants were pleiotropic and thus there was overlap across the risk scores.

Cox proportional hazards models were used to compute the estimated hazard ratios (HR) and 95% confidence intervals (CI) for the association of serum lipid fractions and GRS of lipid fractions with incident ICH. Logistic regression models were used to estimate odds ratios (OR) and 95% CI for the association of serum lipid fractions and GRS of lipid fractions with presence of cerebral microbleeds on MRI. Microbleeds were categorized by their location and count (single and multiple strictly lobar and deep or mixed microbleeds versus no microbleeds).[18] We fitted 3 models for the main analyses, adjusting for age and sex in the first model, additionally for serum lipid levels and lipid-lowering medication in the second model, and cardiovascular risk factors (blood pressures, smoking habits, diabetes mellitus, body-mass-index, blood pressure-lowering and antithrombotic medication use) in the third model. For microbleeds, analyses were repeated after excluding participants with cortical infarcts on MRI (n=151). Also, we repeated the analysis for ICH and cerebral microbleeds after excluding *APOE* alleles from the GRS.

Missing cardiovascular covariate data (≤7%) were imputed based on sex, age, and cardiovascular risk factors using logistic regression models. Analyses were done using IBM SPSS statistic for Windows, Version 21.0 (IBM Corp., Armonk, NY), using an α-value of 0.05.

Results

Baseline characteristics of the study population are presented in Table 1. During a mean follow-up of 8.7 years (SD 4.1), 67 (0.7%) participants suffered an ICH. The prevalence of lobar microbleeds and the prevalence of deep or infratentorial microbleeds in those who underwent MRI (n=4,179) was respectively 12.9% and 6.7%. GRS of total, LDL, and HDL cholesterol and triglycerides were strongly associated with their corresponding serum lipid fraction (respectively $P=6.2x10^{-83}$ for total cholesterol, $P=5.0x10^{-71}$ for LDL cholesterol, $P=2.4x10^{-102}$ for HDL cholesterol, and $P=7.0x10^{-96}$ for triglycerides) (Supplementary Table 1). Associations of fasting serum lipid levels with ICH and microbleeds are shown in Supplementary Table 2 and 3. Although not significant, serum triglycerides associated inversely with ICH risk and microbleed presence. Additionally, serum HDL cholesterol was inversely related to lobar microbleeds, whereas LDL cholesterol was inversely associated with deep or infratentorial microbleeds.

Table 2 shows the association of GRS of serum lipid fractions with incident ICH. Higher GRS of total cholesterol was associated with an increased risk of ICH, even after adjusting for serum total cholesterol, lipid-lowering medication, and cardiovascular risk factors (HR 1.31, 95% CI 1.02-1.68). Per standard deviation increase in the GRS of LDL cholesterol the risk of ICH increased significantly (age and sex adjusted HR 1.32, 95% CI 1.04-1.68). No associations were found for GRS of HDL cholesterol and triglycerides with ICH. GRS of LDL was associated with ICH even after excluding *APOE* alleles from the risk score (age and sex adjusted HR: 1.27, 95% CI 1.00-1.62).

The association between GRS of lipid fractions and cerebral microbleeds is presented in Table 3. Higher GRS of LDL cholesterol associated with a higher prevalence of multiple lobar microbleeds (OR 1.17, 95% CI 1.00-1.38). GRS of lipid fractions were not associated with deep or infratentorial microbleeds. Additional adjustments for serum lipid fractions, lipid-lowering medication, and cardiovascular risk factors did not alter the results (data not shown). The association between GRS of LDL and multiple lobar microbleeds disappeared after excluding *APOE* alleles from the GRS (age and sex adjusted OR 1.03, 95% CI 0.88-1.21).

Table 1. *Baseline characteristics of the study population*

	Complete cohort N=9011	Set with MRI N=4179
Age, years	65.8 (10.2)	64.2 (11.0)
Women	5214 (57.9)	2286 (54.7)
Serum total cholesterol, mmol/L	5.7 (1.0)	5.5 (1.7)
Serum LDL cholesterol, mmol/L	3.7 (0.9)	3.4 (0.9)
Serum HDL cholesterol, mmol/L	1.4 (0.4)	1.5 (2.4)
Serum triglycerides, mmol/L*	1.4 (1.0-1.8)	1.3 (1.0-1.8)
Use of lipid-lowering medication	1346 (14.9)	1020 (24.4)
Systolic blood pressure, mmHg	140.1 (20.8)	139.2 (21.3)
Diastolic blood pressure, mmHg	78.5 (11.3)	82.3 (10.9)
Use of blood pressure-lowering medication	2088 (23.2)	1460 (34.9)
Use of antithrombotic medication	1564 (17.4)	1227 (29.4)
Smoking	6337 (70.3)	2944 (70.4)
Diabetes mellitus	906 (10.1)	352 (8.4)
Body mass index, kg/m^2	27.2 (4.1)	27.4 (4.1)

*Values represent mean (standard deviation) or *median [interquartile range] for continuous variables, and number (percentage) for categorical variables.*

Table 2. *Genetic risk scores of lipid fractions and the risk of intracerebral hemorrhage*

		Intracerebral hemorrhages Hazard ratios (95% confidence interval)		
Genetic risk scores	Events/number at risk	Model 1	Model 2	Model 3
Total cholesterol	67/9011	1.24 (0.98-1.58)	1.32 (1.03-1.69)	1.31 (1.02-1.68)
LDL cholesterol	67/9011	1.32 (1.04-1.68)	1.38 (1.08-1.77)	1.38 (1.07-1.77)
HDL cholesterol	67/9011	1.04 (0.82-1.32)	1.01 (0.79-1.30)	1.02 (0.80-1.31)
Triglycerides	67/9011	0.89 (0.70-1.13)	0.94 (0.73-1.20)	0.95 (0.74-1.21)

Values represent adjusted hazard ratios for intracerebral hemorrhages in relation to genetic risk scores of lipid fractions (increase per Z-scores).
Model 1: adjusted for age and sex.
Model 2: adjusted for age, sex, corresponding lipid fraction (serum total cholesterol for total cholesterol risk score, LDL for LDL risk score, serum HDL for HDL risk score, serum triglyceride for triglyceride risk score), and lipid-lowering medication.
Model 3: as model 2, additionally adjusted for blood pressures, ever smoking, diabetes mellitus, body-mass-index, blood-pressure lowering medication, and antithrombotic medication use.
Abbreviations: LDL= low-density lipoprotein, HDL= high-density lipoprotein.

Table 3. *Genetic risk scores of lipid fractions and microbleeds by location and count*

	Microbleeds Odds ratios (95% confidence interval)					
	Events/ total population	**Yes versus No**	Events/ total population	**Single versus No**	Events/ total population	**Multiple versus No**
Genetic risk scores	**Strictly lobar microbleeds**					
Total cholesterol	539/3900	1.01 (0.92-1.11)	379/3740	0.96 (0.86-1.07)	160/3521	1.14 (0.97-1.34)
LDL cholesterol	539/3900	1.07 (0.97-1.17)	379/3740	1.02 (0.92-1.14)	160/3521	1.17 (1.00-1.38)
HDL cholesterol	539/3900	1.01 (0.92-1.11)	379/3740	1.00 (0.98-1.11)	160/3521	1.06 (0.90-1.25)
Triglycerides	539/3900	0.93 (0.85-1.02)	379/3740	0.91 (0.82-1.02)	160/3521	0.98 (0.84-1.15)
	Deep or infratentorial microbleeds					
Total cholesterol	279/3640	1.01 (0.89-1.15)	107/3468	0.92 (0.75-1.12)	172/3533	1.08 (0.92-1.27)
LDL cholesterol	279/3640	1.00 (0.88-1.14)	107/3468	0.87 (0.71-1.06)	172/3533	1.10 (0.94-1.30)
HDL cholesterol	279/3640	1.04 (0.92-1.18)	107/3468	1.15 (0.95-1.39)	172/3533	0.97 (0.83-1.14)
Triglycerides	279/3640	0.93 (0.82-1.06)	107/3468	0.88 (0.72-1.06)	172/3533	0.97 (0.83-1.14)

Values represent age and sex adjusted odd ratios for categories of single and multiple microbleeds in relation to genetic risk scores for lipid fractions (increase per Z-scores). Abbreviations: LDL= low-density lipoprotein, HDL= high-density lipoprotein.

Discussion

In this population-based study, we found that genetic susceptibility for high total and LDL cholesterol associated with an increased risk of ICH. In addition, higher GRS of LDL cholesterol associated with a higher prevalence of multiple lobar microbleeds. Associations remained unchanged after adjusting for serum lipid concentrations, lipid-lowering medication, and cardiovascular risk.

Strengths of this study include the prospective population-based design aiding generalizability, the large number of participants, which enhances statistical power, and the virtually complete follow-up for ICH. Our findings have to be interpreted in light of some limitations. First, strokes were classified as unspecified in the absence of neuroimaging, thus some ICH cases may have been misclassified. Second, our study population comprised mostly persons of European ancestry. Third, the RS-I and RS-II subcohorts used in this study were part of the GWAS that identified the serum lipid genes, and accounted for 7.3% of the entire discovery cohort. Fourth, for 850 participants we adjusted for serum lipid levels that were measured in visits preceding brain MRI (Figure). Yet, it should be noted that as we cannot date microbleed occurrence on baseline MRI scans, measuring lipid concentrations at the same time as microbleed assessment does not necessarily lead to better adjustments of potential confounding.

In our study, a higher genetic load for high serum LDL was associated with an increased risk of ICH. Although, our findings are in line with another observational study that investigated serum lipid levels and incident ICH,[20] we were unable to corroborate findings from the majority of studies reporting inverse associations of serum lipid fractions with ICH.[1-3,5,21,22] This includes findings from a meta-analysis of 23 prospective cohort and nested prospective case-control studies accounting for 7,960 ICH cases (5.6% of the population),[3] which reported that lower serum total and LDL cholesterol related to an increased risk of ICH. No associations were found for HDL cholesterol or triglycerides. The mechanism by which low serum lipids would influence the pathogenesis of ICH is unclear but it has been suggested that low lipid levels negatively affect the preservation of vessel wall integrity.[23-25] Low total cholesterol levels may cause smooth muscle cells to degenerate, which weakens the endothelial layer of intracerebral vessel walls. This causes vascular hyperpermeability and precipitates the extravasation of erythrocytes.[23-25] On the other hand, low total cholesterol may also be a secondary phenomenon in ICH patients with hypertension and excessive alcohol usage.[4]

Our study provides no evidence for a genetic basis for the inverse association of lipid fractions and incident ICH. The disparity between our findings and that of

previous studies may partly be explained by the fact that serum lipid fractions were typically measured only once, whereas lipid genes provide a more reliable lifetime exposure risk to elevated serum lipids levels. Also, individuals at highest risk of ICH may have had more aggressive risk factor management (i.e., lipid-lowering medication use, low-fat diet, increased physical activity). Insufficient adjustment for these factors in data analysis may have led to confounding in studies investigating serum lipid levels and ICH. Additionally, residual confounding due to unmeasured factors may also have influenced their findings to some extent. Our results provide more robust associations as genes are not susceptible to changes in lifestyle or environment. Finally, most studies investigating serum lipid levels did not control for competing risk of ischemic stroke, coronary heart disease or mortality. Thus, in these studies, less people with elevated serum lipid levels may have been at risk for ICH due to prior occurrence of other cardiovascular events or death.

Associations between GRS of lipid fractions and multiple lobar microbleeds were comparable to those found for ICH, providing additional support for a common pathophysiological pathway for silent and overt cerebral hemorrhages. If we presume lobar microbleeds to be precursors of ICH, our findings suggest that high genetic load for high serum LDL cholesterol facilitates the progression of cerebral vasculopathy. This progression may particularly be mediated by the presence of *APOE* risk alleles, as we only found an association between GRS of LDL and multiple lobar microbleeds once we included *APOE* alleles in the GRS. After excluding *APOE* alleles, we found no reason to assume that genetic susceptibility for high serum lipids differs across cerebral vasculopathies, as results were similar for microbleeds in regions typically affected by cerebral amyloid angiopathy (lobar regions) and for microbleeds in regions characteristically affected by hypertensive arteriopathy (deep or infratentorial regions).

In conclusion, in a large population-based cohort we found that higher genetic load for high serum total and LDL cholesterol increases the risk of ICH and associates with a higher prevalence of multiple lobar cerebral microbleeds. Our results do not support findings from previous observational studies proposing a paradoxical association of serum lipid fractions with ischemic stroke and ICH. Our findings suggest that high LDL cholesterol is amongst the modifiable risk factors for ICH, and individuals with hypercholesterolemia may benefit from lipid-lowering treatment.

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Supplementary Table 1. *Genetic risk scores of lipid fractions and serum lipid levels*

	Serum lipid levels Mean differences (95% confidence interval)							
Genetic risk scores	Total cholesterol	*P*	LDL cholesterol	*P*	HDL cholesterol	*P*	Triglycerides	*P*
Total cholesterol	0.21 (0.19;0.23)	<0.001	0.19 (0.16;0.21)	<0.001	0.03 (0.01;0.05)	0.010	0.10 (0.07;0.12)	<0.001
LDL cholesterol	0.19 (0.16;0.21)	<0.001	0.20 (0.18;0.22)	<0.001	-0.04 (-0.06;-0.02)	0.001	0.06 (0.04;0.08)	<0.001
HDL cholesterol	0.03 (0.01;0.05)	0.015	-0.04 (-0.06;-0.01)	0.001	0.23 (0.21;0.25)	<0.001	-0.11 (-0.13;-0.09)	<0.001
Triglycerides	0.06 (0.04;0.08)	<0.001	0.03 (0.01;0.06)	0.002	-0.12 (-0.14;-0.09)	<0.001	0.22 (0.20;0.25)	<0.001

Values represent mean differences for standardized serum lipid levels in relation to genetic risk scores of lipid fractions (increase per Z-scores). Triglycerides were natural log transformed.
Abbreviations: LDL= low-density lipoprotein, HDL= high-density lipoprotein.

Supplementary Table 2. *Serum lipid levels and the risk of intracerebral hemorrhage*

	Intracerebral hemorrhage Hazard ratios (95% confidence interval)			
Serum lipid levels	Events/number at risk	Model 1	Model 2	Model 3
Total cholesterol	58/7859	0.92 (0.69;1.22)	0.89 (0.67;1.19)	
LDL cholesterol	58/7859	0.96 (0.73;1.26)	0.94 (0.71;1.24)	0.99 (0.74;1.33)
HDL cholesterol	58/7859	1.05 (0.80;1.37)	1.03 (0.78;1.35)	0.91 (0.65;1.27)
Triglycerides	58/7859	0.84 (0.63;1.13)	0.81 (0.60;1.09)	0.77 (0.54;1.10)

Values represent adjusted hazard ratios for intracerebral hemorrhages in relation to serum lipid levels (increase per Z-scores). Triglycerides were natural log transformed.
Model 1: adjusted for age, sex, and lipid-lowering medication.
Model 2: as model 1, additionally adjusted for blood pressures, ever smoking, diabetes mellitus, body-mass-index, blood-pressure lowering medication, and antithrombotic medication use.
Model 3: as model 2, additionally adjusted for lipid fractions other than the one under investigation.
Note that the number of events and total number of participants at risk differs from main Table 2 because serum lipid levels (here the exposure variable) were not imputed in this analysis. Total cholesterol was missing in 576 participants, LDL cholesterol in 1119 participants, HDL cholesterol in 637 participants, and triglycerides in 658 participants.
Abbreviations: LDL= low-density lipoprotein, HDL= high-density lipoprotein.

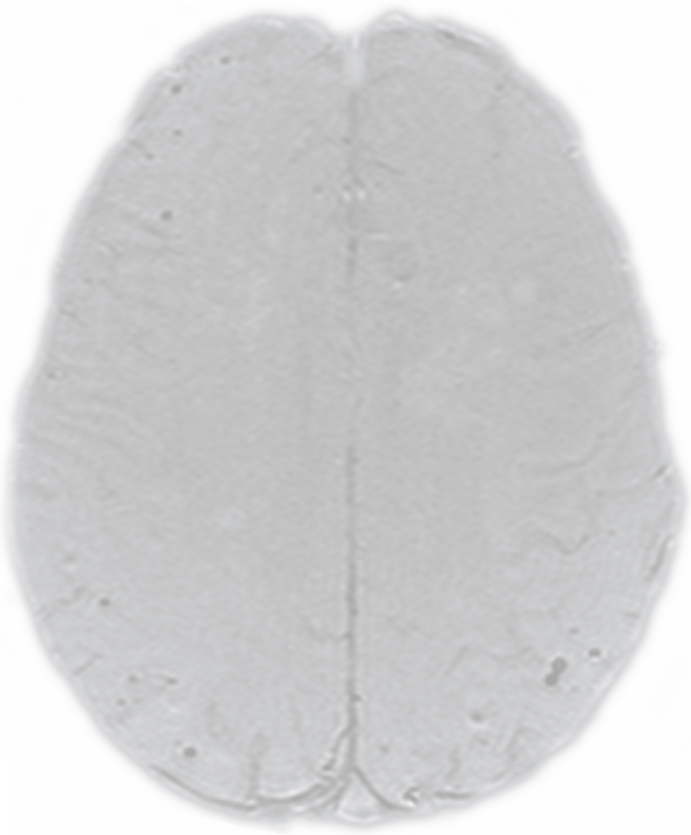
Supplementary Table 3. *Serum lipid levels and cerebral microbleeds*

		Strictly lobar microbleeds Odds ratios (95% confidence interval)		
Serum lipid levels	Events/total population	Model 1	Model 2	Model 3
Total cholesterol	512/3746	0.97 (0.88;1.06)	0.97 (0.88;1.07)	
LDL cholesterol	495/3647	1.00 (0.90;1.12)	1.02 (0.91;1.13)	1.03 (0.92;1.15)
HDL cholesterol	495/3647	0.87 (0.67;1.14)	0.81 (0.62;1.08)	0.71 (0.52;0.97)
Triglycerides	495/3647	0.90 (0.77;1.05)	0.92 (0.78;1.08)	0.83 (0.69;1.00)
		Deep or infratentorial microbleeds Odds ratios (95% confidence interval)		
Serum lipid levels	Events/total population	Model 1	Model 2	Model 3
Total cholesterol	252/3486	0.80 (0.70;0.92)	0.83 (0.72;0.95)	
LDL cholesterol	246/3398	0.77 (0.66;0.90)	0.81 (0.69;0.95)	0.82 (0.70;0.97)
HDL cholesterol	246/3398	1.08 (0.75;1.56)	1.05 (0.71;1.54)	0.91 (0.59;1.40)
Triglycerides	246/3398	0.82 (0.66;1.03)	0.84 (0.67;1.07)	0.87 (0.67;1.12)

Values represent adjusted odds ratios for microbleeds relation to serum lipid levels (increase per Z-scores). Triglycerides were natural log transformed.
Model 1: adjusted for age, sex, and lipid-lowering medication.
Model 2: as model 1, additionally adjusted for blood pressures, ever smoking, diabetes mellitus, body-mass-index, blood-pressure lowering medication, and antithrombotic medication use.
Model 3: as model 2, additionally adjusted for lipid fractions other than the one under investigation.
Note that the number of events and total number of participants differs from main Table 3 because serum lipid levels (here the exposure variable) were not imputed in this analysis. Total cholesterol was missing in 181 participants, LDL cholesterol in 286 participants, HDL cholesterol in 183 participants, and triglycerides in 183 participants.
Abbreviations: LDL= low-density lipoprotein, HDL= high-density lipoprotein.

CHAPTER 3

Pharmacological Drug Use and Cerebral Microbleeds



3.1

Use of coumarin anticoagulant drugs and cerebral microbleeds

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Abstract

Background: It remains undetermined whether the use of coumarin anticoagulants associates with cerebral microbleeds in the general population. We investigated whether: 1) coumarin use relates to higher prevalence and incidence of microbleeds, 2) microbleeds are more frequent in people with higher maximum international normalized ratios (INR), 3) among coumarin users, variability in INR associates with microbleed presence.

Methods: From the population-based Rotterdam Study, 4,945 participants aged ≥45 years were included in the cross-sectional analysis, and 3,069 participants had follow-up brain MRI. Information on coumarin use was obtained from automated pharmacy records. Coumarin users were monitored, and INR values were measured in consecutive visits. Presence and location of microbleeds were rated on brain MRI. We investigated the association of coumarin use with microbleeds using multivariable logistic regression.

Results: Overall, 8.6% had used coumarin anticoagulants before the first MRI, and 5.9% before follow-up MRI. The prevalence of microbleeds was 19.4%, and the incidence 6.9% over 3.9 years (SD 0.5). Compared to never-users, coumarin users had a higher prevalence of deep or infratentorial microbleeds and a higher incidence of any microbleeds, although statistical significance was not reached in the latter. A higher maximum INR was associated with deep or infratentorial microbleeds. Among coumarin users, a greater variability in INR associated with a higher prevalence of microbleeds.

Conclusions: Coumarin use is associated with microbleeds. Associations were strongest for people with greater variability in INR.

Introduction

Oral coumarin anticoagulants are widely used to treat patients with (risk of) thromboembolic diseases. Bleeding is a serious adverse effect of all oral anticoagulants,[1] and intracerebral hemorrhages (ICH) are among the most feared complications because of high morbidity and mortality rates.[2]

Analogous to the increased risk of ICH,[3] it is conceivable that oral coumarin anticoagulants also increase the frequency of smaller, subclinical hemorrhages. These so-called cerebral microbleeds are recognized as hypointense foci on brain magnetic resonance imaging (MRI) and are thought to represent hemosiderin depositions.[4]

In the population-based Rotterdam Study we investigated, firstly, whether coumarin users had a higher prevalence and/or incidence of microbleeds compared to never-users. Secondly, we studied whether microbleeds were more frequent in participants with a higher maximum international normalized ratio (INR) value. Thirdly, among coumarin users, we studied if variability in INR was associated with microbleed presence.

Methods

Participants

The study was conducted within the Rotterdam Study, a large population-based cohort study.[5] Between 2005 and 2011, 6,367 participants were invited to undergo a baseline brain MRI-scan.[6] In total 5,735 non-demented participants were eligible to take part in the study, and 5,074 actually participated. Data on 4,945 participants were available for cross-sectional analyses, after excluding incomplete scans (n=72) or scan of inadequate quality (n=57). Of those, 3,069 participants had complete follow-up MRI examinations, and were used in longitudinal analyses on incident microbleeds.

Oral anticoagulant drug use

Approximately 99% of participants were registered with one or more of seven pharmacies serving the study area. Complete automated records of all outpatient filled prescriptions were available for these participants from January 1st 1991 onwards. Information on prescription included the product name, international non-proprietary name, anatomical therapeutic chemical (ATC) code, total number of delivered units, prescribed daily number of units, date of delivery, and drug dosage. The oral anticoagulants under study were coumarins (ATC

code B01AA04 acenocoumarol, and B01AA07 phenprocoumon). Other antithrombotic medications of which the use was recorded were heparins (B01AB) and platelet aggregation inhibitors (B01AC). Overall, 427 participants had used coumarin anticoagulants at some time before baseline MRI, of whom 147 for the indication atrial fibrillation, 207 for the treatment or prophylaxis of deep venous thrombosis/pulmonary embolism, 24 for peripheral arterial occlusive disease, 47 for cardiac diseases (including coronary heart disease, heart failure, and valvular dysfunction), 1 person because of factor V Leiden mutation, and 1 person for antiphospholipid syndrome.

INR

Prothrombin times were monitored by a regional anticoagulant clinic (Star Medical Diagnostic Center) every 1-6 weeks, depending on target levels and stability of INR values. For each participant we extracted the highest measured INR value before baseline brain MRI, as well as INR values measured in up to 10 consecutive visits after initiation of treatment. The formula for variance growth rate as described by Fihn et al[7] was applied to calculate INR variability from single INR values measured in two consecutive visits (i.e., the variability was calculated for INR measured at visit 1 and 2, visit 2 and 3, et cetera). A higher variance growth rate indicates greater fluctuations in INR values across consecutive measurements, irrespective of the target INR.[8]

Microbleed Rating

All participants were scanned on the same 1.5-Tesla MRI scanner (GE,), using a multisequence protocol that included a T1-weighted, proton density-weighted and fluid-attenuated inversion recovery sequence.[6] For microbleed detection we used a T2*-weighted gradient-recalled echo sequence (T2*GRE),[9] optimized to increase the conspicuity of cerebral microbleeds. Microbleeds, defined as focal areas of low signal intensity on T2*GRE, were scored on presence, number, and location by 1 of 5 trained raters, using a protocol that was defined at the baseline of the study with good interobserver and intraobserver reliability.[9] All baseline and follow-up scans that were rated positive for microbleeds were included in a side-by-side comparison blinded to the time-point of the scans to assess the final number and location of microbleeds in each scan.

Infarcts on MRI

The presence of infarcts, i.e., lacunes, subcortical and cortical infarcts, was rated on MRI as described before.[9]

Assessment of Cardiovascular Risk Factors

We considered various cardiovascular risk factors as potential confounders, namely blood pressure, serum total and high-density lipoprotein (HDL) cholesterol, smoking ("ever" versus "never"), diabetes mellitus (fasting glucose levels ≥7.0 mmol/L, ≥11.1 mmol/L if fasting samples were unavailable, or the use of any glucose-lowering medication), use of lipid-lowering and blood-pressure-lowering medication (assessed by interviews during home visits), and *Apolipoprotein E* (*APOE*) genotyping which was performed on coded genomic DNA samples.

Statistical Analysis

Participants were grouped into ever versus never-users of coumarin anticoagulants. Microbleed presence was investigated dichotomously (present versus absent) and by location. We categorized microbleeds as strictly lobar (presumed to reflect cerebral amyloid angiopathy [CAA]),[9] or as deep or infratentorial microbleeds irrespective of the presence of any lobar microbleeds (presumed to reflect hypertensive arteriopathy).[9] Logistic regression was used in all analyses to obtain odds ratios (OR) and 95% confidence intervals (CI).

In the first analysis, we used three models to study the relation between coumarin anticoagulant use, the prevalence of microbleeds, and the risk of microbleeds. Model 1 was adjusted for age and sex. Model 2 was additionally adjusted for cardiovascular risk factors associated with deep or infratentorial microbleeds, namely systolic and diastolic blood pressures, serum total and HDL cholesterol, smoking, diabetes mellitus, lipid-lowering and antihypertensive medication. Model 3 was adjusted for age, sex, total cholesterol, lipid-lowering medication and *APOE* genotype, as *APOE* ε4 carriership is a strong risk factor for lobar microbleeds and because of the role of *APOE* in lipid metabolism. To address potential confounding by indication in our study we repeated the analyses described above in model 1, 2, and 3 whilst also adjusting for the indication of coumarin anticoagulant drug use. Sensitivity analyses were done excluding participants who had used other antithrombotic agents (e.g., heparin, aspirin) to demonstrate the pure association of oral coumarin anticoagulants and microbleeds. Also, to further reduce potential confounding by indication, all analyses were repeated after excluding participants with infarcts on MRI.

In the second analysis, we investigated the relation between maximum INR values and microbleed presence cross-sectionally after adjustments for age sex, and duration of coumarin use. To this end, we categorized participants who used coumarin anticoagulants into groups of maximum INR values 1-4 (including 4);

4-6 (including 6); >6, depending on their highest measured INR, and compared them to a reference category of never-users (assumed INR value of 1). The cut-off points were chosen pre-hoc using Dutch guidelines for managing anticoagulant therapy. The cut-off of 4 was chosen based on what was considered to be the upper therapeutic range for the most common indications when using anticoagulants, and INR of 6 was chosen because based on previous literature an increased risk of ICH was particularly shown when INR exceeded this level.[10]

The third analysis was restricted to participants who had used coumarin anticoagulants. We investigated the association of variability in INR with the presence of microbleeds after adjusting for age, sex, and duration of coumarin use. We calculated tertiles of variability in INR and compared highest to the lowest tertiles.

Analyses were done using the statistical software package IBM SPSS Statistics version 20.0 using an α-value of 0.05.

Results

A total of 427 (8.6%) participants had used oral coumarin anticoagulants at some time before the first MRI. One or more microbleeds were seen on the baseline MRI scans of 957 (19.4%) participants (median number of microbleeds 1 (range 1-111), 91% had ≤6 microbleeds). Of 3,069 participants with follow-up MRI 181 (5.9%) participants had used coumarin anticoagulant drugs at some time before the second MRI scan. The cumulative incidence of microbleeds was 6.9% over a mean follow-up of 3.9 years (SD 0.5) (Table 1).

Participants who used coumarin anticoagulants had a higher prevalence of deep or infratentorial microbleeds (with or without lobar microbleeds) compared to never-users (age and sex-adjusted OR for deep or infratentorial microbleeds 1.70, 95% CI 1.24-2.34) (Table 2). Although statistical significance was not reached, it seemed that compared to never-users, coumarin users were at increased risk of developing new microbleeds (OR for any microbleeds 1.44, 95% CI 0.89-2.32). Associations remained similar after additional adjustments for cardiovascular risk factors. After taking into account the indication for coumarin use, we still found an association between coumarin anticoagulants and prevalent deep or infratentorial microbleeds (Supplementary Table 1). In a post-hoc analysis, we found a higher frequency of both strictly deep or infratentorial (age and sex-adjusted OR 1.66, 95% CI 1.06-2.60) and mixed microbleeds (deep or infratentorial with lobar microbleeds) (OR 1.71, 95% CI 1.14-2.58) in coumarin users compared to never-users.

Table 1. *Baseline characteristics of the study population*

	People with baseline MRI (N=4945)	People with follow-up MRI (N=3069)
Age, years	64.0 (11.0)	59.7 (8.1)
Women	2724 (55.1)	1657 (54.0)
Ever use of coumarin anticoagulants before MRI	427 (8.6)	181 (5.9)
Exclusive use of coumarin anticoagulants*	186 (3.8)	89 (2.9)
INR values at check-up visits		
Always 1-4	151 (3.1)	60 (2.0)
At least once 4-6	210 (4.2)	94 (3.1)
At least once >6	66 (1.3)	27 (0.9)
Systolic blood pressure, mmHg	138.9 (21.2)	134.9 (19.2)
Diastolic blood pressure, mmHg	82.2 (10.9)	81.9 (10.7)
Total cholesterol, mmol/L	5.5 (1.1)	5.6 (1.0)
High-density lipoprotein cholesterol, mmol/L	1.4 (0.4)	1.4 (0.4)
Ever smoker	3436 (69.5)	2125 (69.2)
Diabetes mellitus	433 (8.8)	224 (7.3)
Lipid-lowering medication use	1185 (24.0)	653 (21.3)
Antihypertensive medication use	1696 (34.3)	816 (26.6)
Apolipoprotein E ε4 carriership	1314 (26.6)	801 (26.1)
Lacunar infarcts	368 (7.4)	150 (4.9)
Subcortical infarcts†	9 (0.2)	2 (0.1)
Cortical infarcts	165 (3.3)	67 (2.2)

Continuous variables are presented as mean (standard deviation) and categorical variables as number (percentage). All values presented are baseline values.

**Participants who used other antithrombotic agents (i.e., subcutaneous anticoagulants and platelet aggregation inhibitors) were excluded.*

†Subcortical infarcts were defined as lacunar infarcts ≥ 15mm in size.

INR= international normalized ratio.

The following variables had missing data in the analysis of people with baseline MRI: blood pressures (n=21), total cholesterol (n=74), high-density lipoprotein cholesterol (n=76), smoking (n=26), diabetes mellitus (n=81), lipid-lowering and antihypertensive medication (n=42), Apolipoprotein E genotype (n=347).

The following variables had missing data in the analysis of people with follow-up MRI: blood pressures (n=12), total (n=33) and high-density lipoprotein cholesterol (n=35), smoking (n=9), diabetes mellitus (n=58), lipid-lowering and antihypertensive medication (n=31), Apolipoprotein E genotype (n=206).

Excluding participants who had used other antithrombotic agents besides coumarin anticoagulants did not change the results meaningfully (OR for prevalent deep or infratentorial microbleeds 2.03, 95% CI 1.23-3.36). Finally, excluding participants with infarcts on MRI did not alter the results (OR for prevalent deep or infratentorial microbleeds 1.93, 95% CI 1.32-2.82).

Deep or infratentorial microbleeds seemed more frequent in participants with a higher maximum INR value when compared to never-users (linear trend tests across INR categories for deep or infratentorial microbleeds *P* value=0.073) (Figure 1).

Within the group of oral coumarin anticoagulant users, those within the highest tertile of variability in INR values after initiation of coumarin anticoagulants, had a higher prevalence of deep or infratentorial microbleeds compared to those within the lowest tertile of variability in INR values (linear trend test across tertiles for deep or infratentorial microbleeds *P* value=0.048) (Supplementary Table 2). A greater variability in INR was associated with deep or infratentorial microbleeds, particularly for the first five measurements (Figure 2).

Table 2. *Use of coumarin anticoagulants and microbleeds*

	n/N	Any microbleeds (yes versus no)	n/N	Strictly lobar microbleeds (yes versus no)	n/N	Deep or infratentorial microbleeds (yes versus no)
				Prevalent microbleeds		
Model 1						
Never use	819/4518	Reference	555/4254	Reference	264/3963	Reference
Ever use	138/427	1.29 (1.02-1.62)*	74/363	1.07 (0.80-1.42)	64/353	1.70 (1.24-2.34)*
Model 2						
Never use	791/4384	Reference	535/4128	Reference	256/3849	Reference
Ever use	134/412	1.25 (0.98-1.59)	72/350	1.06 (0.79-1.42)	62/340	1.57 (1.13-2.19)*
Model 3						
Never use	667/3649	Reference	453/3435	Reference	214/3196	Reference
Ever use	120/345	1.33 (1.03- 1.72)*	67/292	1.18 (0.86-1.60)	53/278	1.57 (1.10-2.25)*
				Incident microbleeds		
Model 1						
Never use	189/2888	Reference	136/2835	Reference	53/2752	Reference
Ever use	24/181	1.44 (0.89-2.32)	14/171	1.25 (0.69-2.27)	10/167	1.85 (0.89-3.88)
Model 2						
Never use	181/2812	Reference	129/2760	Reference	52/2683	Reference
Ever use	23/174	1.54 (0.94-2.54)	13/164	1.34 (0.72-2.50)	10/161	2.08 (0.97-4.48)
Model 3						
Never use	145/2346	Reference	102/2303	Reference	43/2244	Reference
Ever use	19/134	1.67 (0.96-2.89)	11/126	1.44 (0.73-2.85)	8/123	2.22 (0.96-5.14)

*Values represent estimated odds ratios (95% confidence interval) for prevalent and incident microbleeds in ever-users versus never-users of coumarin anticoagulants. *Indicates P value <0.05. n/N= number of microbleed cases per exposure category/ total study population within the exposure category.*
Model 1: age, sex adjusted.
Model 2: age, sex, systolic and diastolic blood pressure, total and high-density lipoprotein cholesterol, smoking, diabetes mellitus, lipid-lowering and antihypertensive medication adjusted. (Complete-case analysis).
Model 3: age, sex, total cholesterol, lipid-lowering medication, and apolipoprotein E ε4 carriership adjusted. (Complete-case analysis).

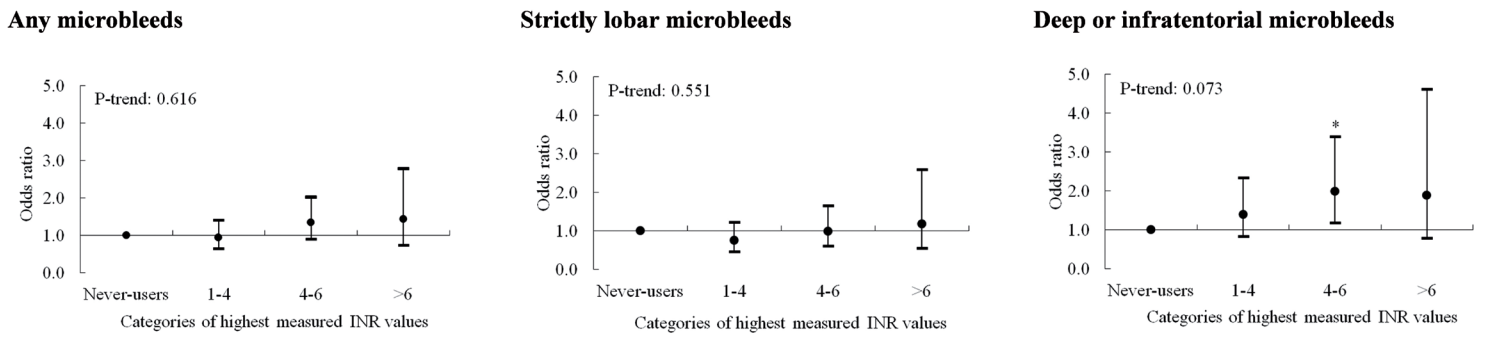

Figure 1. *Categories of highest measured INR values and microbleed presence.*
Age, sex, and duration of coumarin use adjusted odds ratios for microbleed presence in relation to the highest measured INR value in ever-users of coumarin anticoagulants, compared to never-users (assumed INR value of 1). Error bars represent 95% confidence intervals.
**Indicates P value <0.05. INR= international standardized ratio.*

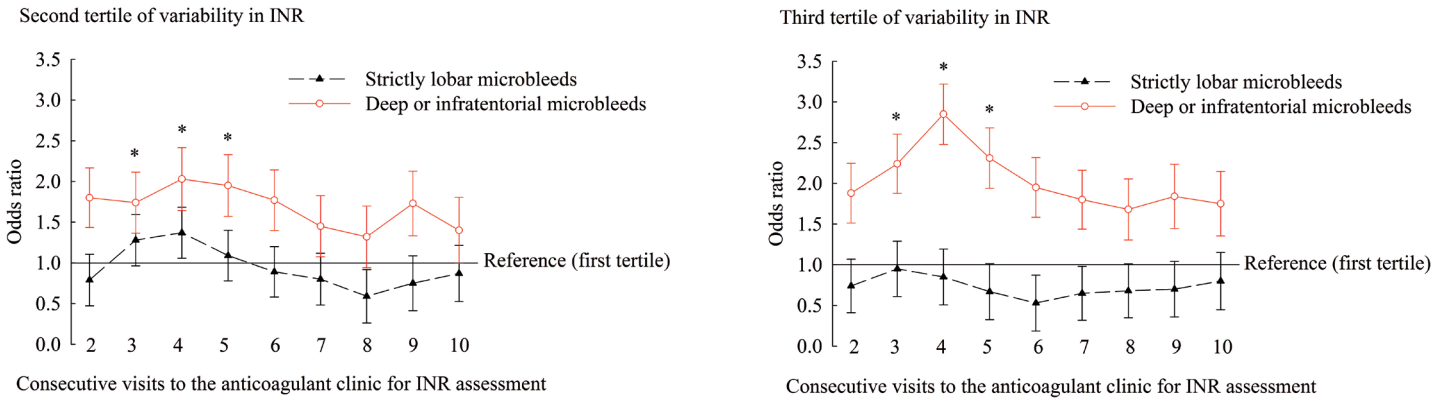

Figure 2.*Variability in INR and microbleed presence.*
*Values represent age and sex-adjusted odds ratios for microbleed presence in relation to the variability in INR. Variability in INR was calculated for every two consecutive visits of INR assessment, and is shown here for the first ten visits. The second tertile (left graph) and third tertile (right graph) of variability in INR were compared to the first tertile (the horizontal 1.0 line=least variability in INR). Error bars show standard errors. *Indicates P value <0.05. INR= international normalized ratio.*

Discussion

In a large sample of people from the general population, we found that compared to never-users, users of oral coumarin anticoagulants had more deep or infratentorial cerebral microbleeds on MRI and seemed to have an increased risk of developing new microbleeds. In addition, a greater variability in INR shortly after the initiation of coumarins was associated with the presence of deep or infratentorial microbleeds.

Strengths of our study are its population-based design, which increases generalizability, and the availability of longitudinal data, which offers insight into temporality of associations. Some methodological considerations need to be addressed. Imaging does not provide information on the date that cerebral microbleeds developed, and therefore it is possible that microbleeds may have occurred before the use of anticoagulant drugs. Also, confounding by indication may pose a problem in our observational study, as anticoagulants are prescribed more often to people with or at increased risk of cardiovascular disease, which in turn is related to cerebral microbleeds, especially deep or infratentorial microbleeds.[9,11] We tried to minimize confounding of our results in several ways. Firstly, we adjusted for important cardiovascular risk factors. Note that the number of incident microbleeds cases was small, and results from multivariate analyses should be interpreted with caution. Secondly, we excluded participants with infarcts on MRI. Thirdly, we took into account the indication of anticoagulant drug use. Fourthly, we used variability in INR as a measurement less prone to confounding by indication, as this measure does not depend on the target INR but on whether the INR, regardless of its value, was stable across consecutive measurements.[8] Our study specifically focused on the relationship between coumarin anticoagulants and microbleeds, and we acknowledge that our findings may not be generalizable to populations using non-coumarin oral anticoagulants. As a final limitation we would like to mention that we did not study clinical events and thus cannot comment on the risk of anticoagulant related major bleeding complications.

Our finding that coumarin anticoagulant users had a higher prevalence of microbleeds is in line with previous studies in ischemic or hemorrhagic stroke patients.[12-16] Compared to these clinical reports, our study now provides additional insight into the subclinical bleeding complications of oral coumarin anticoagulants in stroke-free persons. We also showed that the use of coumarin anticoagulants seemed to increase the risk of developing new microbleeds. Microbleed development is thought to result from leakage of blood through the walls of small brain vessels that are damaged by either CAA or hypertensive

arteriosclerosis.[4,9,17] It has been hypothesized that this process can be halted by adequate hemostatic mechanisms,[18] and the use of anticoagulants inhibits this mechanism. Thus anticoagulant drug use may serve as a catalyst in the presence of CAA or hypertensive arteriopathy to expand the number of microbleeds.[18]

To date, it remains inconclusive if anticoagulant drug use associates differently with microbleeds or ICH at various sites in the brain. We found that the association of coumarin use and microbleeds was primarily driven by the presence of deep or infratentorial microbleeds. This is in contrast to the majority of clinical studies that often found an association with lobar microbleeds.[14,19-21] It should be noted that although lobar microbleeds have mainly been implicated in cerebral amyloid angiopathy and deep microbleeds in hypertensive arteriopathy, both types of pathologies may coexist and cause for a more severe mixed pathology. In our study, participants categorized as deep or infratentorial were allowed to also have microbleeds in lobar brain regions. Therefore, this group was more likely to have multiple microbleeds and potentially also mixed pathology, which could explain why associations were strongest for participants with deep or infratentorial microbleeds in our study.

Lack of statistical power is the most likely reason why we did not find a significant association between coumarin use and incident microbleeds. Nevertheless, our longitudinal analyses suggested an association between anticoagulant drug use and incident lobar microbleeds, which further supports the idea that the associations presented are not merely a reflection of confounding by indication.

Among oral coumarin anticoagulant users, the intensity of anticoagulation was shown to be a strong risk factor for bleeding complications.[1,7,22-25] Regardless of the INR values, microbleeds were more frequently found in people with a greater variability in INR, particularly in the early phase after anticoagulant therapy initiation. A more vigilant monitoring of INR values and dosage requirements may become beneficial if future longitudinal studies show that microbleeds are indeed precursors of major ICH.

In conclusion, we found that oral coumarin anticoagulant use is associated with cerebral microbleeds. This association was particularly present for people with higher INR values and greater variability in INR.

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Supplementary Table 1. *Coumarin use and microbleeds, adjusted for indication of coumarin use*

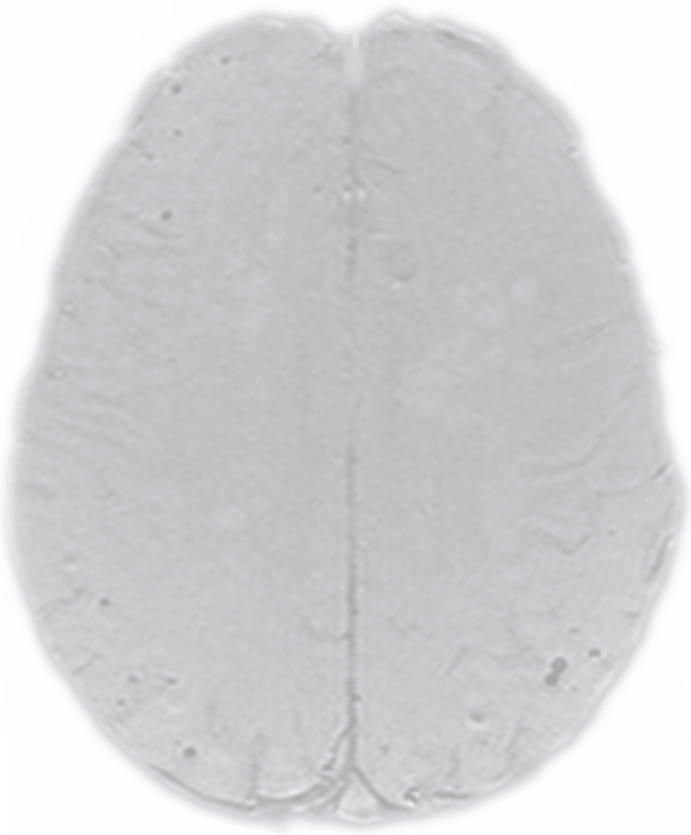
	n/N	Prevalent any microbleeds (yes versus no)	n/N	Prevalent strictly lobar microbleeds (yes versus no)	n/N	Prevalent deep or infratentorial microbleeds (yes versus no)
Model 1						
Never use	819/4518	Reference	555/4254	Reference	264/3963	Reference
Ever use	138/427	1.51 (0.92-2.50)	74/363	1.10 (0.58-2.09)	64/353	2.09 (1.11-3.97)*
Model 2						
Never use	791/4384	Reference	535/4128	Reference	256/3849	Reference
Ever use	134/412	1.38 (0.82-2.33)	72/350	1.00 (0.51-1.97)	62/340	1.79 (0.92-3.45)
Model 3						
Never use	667/3649	Reference	453/3435	Reference	214/3196	Reference
Ever use	120/345	1.77 (1.03-3.04)*	67/292	1.32 (0.67-2.59)	53/278	2.21 (1.12-4.38)*

Model 1: age, sex, and indication of anticoagulant drug use adjusted.
Model 2: age, sex, systolic and diastolic blood pressure, total and high-density lipoprotein cholesterol, smoking, diabetes mellitus, lipid-lowering and antihypertensive medication, and indication of anticoagulant drug use adjusted. (Complete-case analysis).
Model 3: age, sex, total cholesterol, lipid-lowering medication, and apolipoprotein E ε4 carriership, and indication of anticoagulant drug use adjusted. (Complete-case analysis).
*Values represent estimated odds ratios (95% confidence interval) for microbleeds in ever versus never-users of coumarin anticoagulants. *Indicates P value < 0.05. n/N= number of microbleed cases per exposure category/ total study population within the exposure category.*

Supplementary Table 2. *Variability in INR and microbleed presence*

	n/N	Prevalent any microbleeds (yes versus no)	n/N	Prevalent strictly lobar microbleeds (yes versus no)	n/N	Prevalent deep or infratentorial microbleeds (yes versus no)
1st tertile	44/142	Reference	27/125	Reference	17/115	Reference
2nd tertile	43/144	0.98 (0.58-1.66)	25/126	0.92 (0.49-1.74)	18/119	1.08 (0.51-2.26)
3rd tertile	51/140	1.40 (0.84-2.34)	22/111	0.97 (0.51-1.85)	29/118	1.95 (0.99-3.86)

Values represent age, sex, and duration of coumarin use adjusted odds ratios (95% confidence interval) for microbleed presence in relation to tertiles of variability in INR among ever-users of oral coumarin anticoagulants. The 1st tertile consists of coumarin anticoagulant users with the lowest variability in INR. INR= international standardized ratio. n/N= number of people using coumarin anticoagulants and had microbleeds/ total number of people in the tertile.



3.2

Inhibition of serotonin reuptake by antidepressants and cerebral microbleeds

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Abstract

Background: Serotonin reuptake inhibiting antidepressants decrease platelet aggregation. This may cause an increased risk of intracerebral hemorrhage. However, the risk of subclinical microbleeds, which are highly prevalent in middle-aged and elderly people, is unknown. We studied whether serotonin reuptake inhibiting antidepressants increase the frequency of cerebral microbleeds and secondarily whether they lower the presence of ischemic vascular damage.

Methods: Within the population-based Rotterdam Study, information on antidepressant use was obtained from continuously monitored pharmacy records. Brain MRI was available in 4,945 participants (55% women, mean age 64 years) between 2005-2011. We categorized antidepressants based on affinity for the serotonin transporter: high, intermediate or low. Microbleeds (presence and location) and ischemic lesions (lacunes, white matter lesions) were rated on brain MRI. Logistic and linear regression, adjusted for age, sex, depressive symptoms and cardiovascular risk were used to study the association of antidepressants with microbleeds, and ischemic vascular lesions.

Results: Antidepressant use with strong serotonin reuptake inhibition was not associated with microbleed presence (odds ratio compared to non-use: 1.03, 95% confidence interval: 0.75; 1.39) irrespective of microbleed location in the brain. Exclusion of antithrombotic users or persons with cortical infarcts did not change our results. Furthermore, serotonin reuptake inhibition was not related to ischemic vascular brain damage.

Conclusions: In the general population, use of serotonin reuptake inhibiting antidepressants is not related to presence of cerebral microbleeds. This strengthens the idea that the platelet inhibitor effects of antidepressant drugs with affinity for serotonin are minimal, and further supports the safety of SSRIs for non-gastrointestinal bleedings.

Introduction

The use of antidepressant medication in the general population has increased considerably in past decades, in particular the use of selective serotonin reuptake inhibitors (SSRIs).[1,2] This increase in SSRI use may be explained by a broadened indication of SSRI, a different adverse effect profile, and a lower toxicity compared with classic tricyclic antidepressants.[3-5]

Yet, despite a more favorable adverse effect profile, the use of SSRIs is not entirely risk free.[6-9] SSRIs block the reuptake of serotonin by platelets and decrease serotonin platelet concentration, which may lead to impaired aggregation and prolonged bleeding times.[10-14] SSRIs have, therefore, extensively been studied in relation to intracerebral hemorrhages,[15-22] and a recent meta-analysis of controlled observational studies showed an increased risk of intracerebral hemorrhages in SSRI users compared with nonusers.[23]

In addition, via the same pathophysiological pathway of reducing platelet aggregation, antidepressants with a high inhibition for serotonin reuptake may also reduce the risk of ischemic stroke, although to date this hypothesis is scarcely supported by literature.[15,16,21,24,25]

Apart from major cerebrovascular events, it has not yet been investigated whether SSRIs or strong inhibitors of serotonin reuptake are associated with subclinical cerebrovascular lesions and more particularly with subclinical bleedings. Cerebral microbleeds have increasingly been recognized on MRI in stroke patients and mostly in association with larger intracerebral hemorrhages.[26-28] Yet, microbleeds are also highly prevalent in the general population, and microbleeds may similarly represent bleeding-prone vessels in these people. Support for this is provided in our previous studies in which we showed an association between antiplatelet drugs use and the presence of cerebral microbleeds in the general population.[29,30]

Given the association of microbleeds with symptomatic bleeds and antiplatelet drug use, we hypothesized that people who use antidepressants with a great inhibition of serotonin reuptake may have a higher prevalence of cerebral microbleeds than nonusers and users of antidepressant with a low serotonin affinity. Moreover, we secondarily investigated whether the use of these drugs is associated with the presence of ischemic vascular damage on MRI, in particular a lower frequency of lacunes of presumed vascular origin[31] and lower white matter lesion (WML) volume.

Methods

Participants

The Rotterdam Study is a prospective population-based cohort study, within Ommoord, a suburb in Rotterdam, the Netherlands. The study comprises 14,926 participants and investigates the prevalence, incidence of, and risk factors for diseases in an aging population.[32] The study started in 1990, and after baseline examination, follow-up assessments were conducted every 4 to 5 years including interviews and an extensive set of examinations. From 2005 onward, brain MRI was embedded within the core protocol of the Rotterdam Study to investigate age-related brain changes on imaging.[33] The institutional review board approved the study. Between 2005 and 2011, 5,735 participants visiting the study center in that period were eligible to undergo a brain MRI. After informed consent was signed, a total of 5,074 non-demented people were scanned. After excluding participants in whom MRI was not completed (n=72) and scans with low quality (n=57), data on 4945 participants were available for analyses.

Assessment of Antidepressant Drug Use

We determined antidepressant drug use prior to brain MRI based on fully computerized pharmacy records from the 7 pharmacies in the Ommoord district. More than 99% of the participants have their drug prescriptions filled at these regional pharmacies. Medication records were continuously monitored from January 1st, 1991, onward. Records included the date of prescribing, the total amount of drug units per prescription, the prescribed daily number of units, the product name of the drugs, and the anatomic therapeutic chemical code. The duration of treatment was calculated by counting the number of prescription days. The average prescribed daily dose was expressed in standardized defined daily doses calculated by summing up the total number of prescribed defined daily doses from all prescriptions divided by the total duration.

We classified antidepressants based on their degree of serotonin reuptake inhibition. The classification is based on the dissociation constant (K) for the serotonin transporter. A lower dissociation constant reflects a higher affinity for the serotonin transporter and, therefore, a higher inhibition of serotonin reuptake. Based on previous literature, we categorized antidepressants into high (paroxetine, clomipramine, sertraline, duloxetine, fluoxetine), intermediate (escitalopram, citalopram, imipramine, fluvoxamine, amitriptyline, venlafaxine), and low (desimipramine, opipramol, nortriptyline, doxepin, dosulepin, maprotiline, moclobemide, mianserin, trazodone, nefazodone, mirtazapine) degrees of serotonin reuptake inhibition.[17,34-38]

People who used multiple antidepressants from the different groups were excluded from the main analyses (n=268), to secure a pure exposure. These users were considered switchers and were analyzed in subsequent analyses.

Brain MRI and Assessments of MRI Markers

Brain MRI scans were performed on a 1.5-Tesla MRI scanner (GE Healthcare, Milwaukee, WI).[33] Our multisequence MRI protocol included the following scans: T1-weighted, proton-density-weighted, T2-weighted, and fluid-attenuated inversion recovery. For microbleed detection, we used a custom-made accelerated 3-dimensional T2*-weighted gradient-recalled echo sequence with high spatial resolution and long echo-time to enhance the detection of microbleeds.[39]

Microbleeds were defined as focal areas of low signal intensity on T2*-weighted imaging. Their presence, location, and numbers were scored by 1 of 5 trained research physicians, with good intraobserver and interobserver agreement.[40] We categorized microbleeds based on their presumed underlying cause into lobar microbleeds (presumably reflective of cerebral amyloid angiopathy) and deep or infratentorial microbleeds (presumably reflective of hypertensive arteriopathy).[40]

Lacunes and cortical infarcts were rated on fluid-attenuated inversion recovery, proton-density-weighted, and T1-weighted sequences by the same raters of microbleeds. Lacunes were defined as focal lesions between ≥3 and <15 mm in size.[40] Infarcts showing involvement of gray matter were classified as cortical infarcts. Brain tissue was segmented into gray matter, white matter, and cerebrospinal fluid, using validated automated post-processing steps that include conventional k-nearest-neighbor brain tissue classifier extended with WML segmentation.[41,42]

Assessment of Covariables

We addressed potential confounders by characterizing depressive symptoms, cardiovascular risk factors, and cardiovascular medication use in our study population. Antidepressant drugs are mainly prescribed for depressive disorders. Depression has a bidirectional association with cardiovascular disease, and cardiovascular disease is related to the presence of microbleeds.[43,44]

Presence of depressive symptoms was evaluated using the Center for Epidemiological Studies Depression Scale.[45] A score of 16 or higher was indicative of participants with clinically relevant depressive symptoms. A high sensitivity for major depression for this score was reported in older adults in the Netherlands.[46]

Participants' cardiovascular risk was assessed during the center visit preceding MRI, using interview, laboratory, and physical examinations.[47] This included presence of diabetes mellitus, smoking status (ever versus never), serum total cholesterol levels, serum high- density lipoprotein cholesterol levels, and systolic and diastolic blood pressure. Finally, use of lipid-lowering drugs (C10), antihypertensive drugs (C02, C03, C07, C08, and C09), and antithrombotic drugs (B01AA, B01AB, B01AC, and B01AX) was assessed from pharmacy records during follow-up before MRI.

Statistical Analysis

We analyzed the association between use of antidepressants (the degree of serotonin reuptake inhibition (high, intermediate, low)) with the presence of cerebral microbleeds (present versus absent) using multiple logistic regression, taking nonusers as reference category. Analyses were repeated for microbleeds at different locations in the brain, namely strictly lobar regions and deep or infratentorial regions (with or without lobar microbleeds). Furthermore, we repeated all analyses using low and intermediate serotonin reuptake inhibition antidepressant users as reference category. Switchers were excluded from the main analyses, and the subsequent analyses were repeated including switchers.

All analyses were adjusted for age and sex. We additionally adjusted for presence of depressive symptoms, diabetes mellitus, smoking, total and high-density lipoprotein cholesterol, systolic and diastolic blood pressure, use of lipid-lowering medication, antihypertensive medication, and antithrombotic agents. Sensitivity analyses were performed with exclusion of MRI-defined cortical infarcts or exclusion of antithrombotic drug users. Moreover, analyses were stratified for sex, the exposure was dichotomized based on the duration of treatment (cutoff was 90 days), and interaction tests with antithrombotic drug users were performed. The average prescribed daily dose, expressed in standardized defined daily dose, was also studied dichotomized on 1.00 defined daily dose as the cutoff to look at an effect of dose.

Furthermore, we studied the association between the degree of serotonin reuptake inhibition of antidepressants and the presence of lacunes and WML volume with, respectively, multiple logistic and linear regression. People with cortical infarcts were excluded from these analyses. Analyses were adjusted for the same factors as described above. Analyses of WML volume were additionally adjusted for intracranial volume. WML was log-transformed because of the skewed distribution.

We considered a *P* value <0.05 as statistically significant, and analyses were performed with a commercially available software program (IBM SPSS Statistics for Windows, Version 21.0).

Results

Characteristics of the study population are presented in Table 1. Mean age was 64.0 years (SD, 11.0), and 2,724 (55.1%) were women. A total of 930 (18.8%) persons had a history of antidepressant use before MRI, and 311 (6.2%) had exclusively used antidepressants with a high degree of serotonin reuptake inhibition, 304 (6.1%) of an intermediate, and 47 (1.0%) antidepressants of a low degree. Among users, 268 (5.4%) switched between the different antidepressant drug categories. In the total study population, 957 (19.4%) had microbleeds, of whom 629 had strictly lobar and 328 deep or infratentorial microbleeds. In the group of antidepressant drug users (n=930), 18.9% had microbleeds, which did not significantly differ from the 19.5% in the population of nonusers. Of all participants in our study, lacunes were present in 370 (7.5%), and median WML volume was 3.0 mL.

Compared to non-use, the use of antidepressants with a high serotonin reuptake inhibitory potential was not associated with cerebral microbleed presence (age, sex-adjusted odds ratio [OR]: 1.03; 95% CI 0.75; 1.39). In addition, no association was found for low (OR: 0.76; 95% CI 0.36; 1.62) or intermediate (OR: 1.04; 95% CI 0.77; 1.39) serotonin affinity antidepressants. Compared to non-use, the use of antidepressant medication with either high, intermediate, or low affinity for serotonin was neither related to lobar, nor to deep or infratentorial microbleeds (Table 2). Additionally, no association between antidepressants use and microbleeds was found for people who switched between different antidepressant drugs (OR: 0.95; 95% CI 0.68; 1.33). Additional adjustments for cardiovascular risk factors, cardiovascular medication and depressive symptoms did not change any of the results significantly (Table 2). Excluding participants with MRI-defined cortical infarcts (n=158), and excluding ever antithrombotic drug users (n=1,326) also did not materially change our results (data not shown). Moreover, the exposure split by duration and average prescribed daily dose of antidepressant drug treatment and stratification by gender did not significantly change our results (data not shown). Effect modification of antidepressant drug exposure by antithrombotic drugs was not present (*P*=0.96).

We did not find a higher frequency of cerebral microbleeds, irrespective of their location in the brain, when comparing the high affinity group with the combined intermediate and low affinity group (OR: 1.03; 95% CI: 0.68; 1.56) (Table 3).

Finally, we did not find a lower frequency of lacunes (OR: 1.14; 95% CI 0.67; 1.94) nor a smaller WML volume (mean difference of WML volume: 0.06; 95% CI -0.03; 0.15) for use of antidepressants with a high serotonin reuptake inhibition potential compared to non-use, neither did we find a relation when investigating the use of low and intermediate degree of serotonin reuptake inhibition (Table 4).

Table 1. *Baseline characteristics of the study population*

	N=4945
Age, years	64.0 (11.0)
Females	2724 (55.1)
Depressive symptoms	417 (8.6)
Diabetes mellitus	433 (8.9)
Smoking	3436 (69.8)
Antidepressant drug users	
High degree of inhibition*	311 (6.2)
Intermediate degree of inhibition*	304 (6.1)
Low degree of inhibition*	47 (1.0)
Switchers	268 (5.4)
Presence of cerebral microbleeds	957 (19.4)
Strictly lobar	629 (13.6)
Deep or infratentorial	328 (7.6)
White matter lesion volume, mL	3.0 (1.6 – 6.5)
Lacunes	370 (7.5)
Cortical infarcts	165 (3.3)
Total cholesterol, mmol/L	5.5 (1.1)
High-density lipoprotein cholesterol, mmol/L	1.4 (0.4)
Systolic blood pressure, mmHg	138.9 (21.2)
Diastolic blood pressure, mmHg	82.2 (10.9)
History of lipid lowering drug use	1185 (24.2)
History of antihypertensive drug use	1696 (34.6)
History of antithrombotic drug use	1415 (28.6)

Values represent mean (standard deviation) or number (percentage). White matter lesion volume is represented as median (interquartile range).

** Degree of serotonin reuptake inhibition: High = paroxetine, clomipramine, sertraline, duloxetine, fluoxetine. Intermediate = escitalopram, citalopram, impramine, fluvoxamine, amitriptyline, venlafaxine. Low = desimipramine, opipramol, nortriptyline, doxepin, dosulepin, maprotiline, moclobemide, mianserin, trazodone, nefazodone, mirtazapine.*

Table 2. *Degree of serotonin reuptake inhibition for antidepressant drugs and the presence of cerebral microbleeds*

Degree of serotonin reuptake inhibition	Any microbleeds		Deep or infratentorial microbleeds		Strictly lobar microbleeds	
	n/N	Odds ratio (95% CI)	n/N	Odds ratio (95% CI)	n/N	Odds ratio (95% CI)
Model 1						
Non-use	781 / 4015	1.00 (Reference)	270 / 3504	1.00 (Reference)	511 / 3745	1.00 (Reference)
Low	9 / 47	0.76 (0.36;1.62)	2 / 40	0.51 (0.12;2.15)	7/ 45	0.92 (0.40;2.10)
Intermediate	65 / 304	1.04 (0.77;1.39)	23 / 262	1.07 (0.67;1.70)	42 / 281	1.04 (0.73;1.47)
High	53 / 311	1.03 (0.75;1.39)	20 / 278	1.17 (0.72;1.90)	33 / 291	0.93 (0.64;1.36)
Model 2						
Non-use	741 / 3850	1.00 (Reference)	257 / 3366	1.00 (Reference)	484 / 3593	1.00 (Reference)
Low	8 / 45	0.70 (0.32;1.57)	2 / 39	0.50 (0.11;2.15)	6 / 43	0.80 (0.33;1.96)
Intermediate	60 / 291	0.96 (0.70;1.30)	21 / 252	0.94 (0.58;1.54)	39 / 270	0.98 (0.68;1.41)
High	51 / 298	0.97 (0.70;1.35)	19 / 266	1.10 (0.66;1.84)	32 / 279	0.88 (0.59;1.30)

Values represent odds ratios for microbleeds in relation to antidepressant drugs with low, intermediate, and high affinity for serotonin. Non-users are the reference population for all analyses presented in Table 2.

Model 1: adjusted for age and sex.

Model 2: adjusted for age, sex, depression, diabetes mellitus, smoking, total and high-density lipoprotein cholesterol, systolic and diastolic blood pressure, ever use of lipid-lowering drugs, antihypertensive drugs, and antithrombotic drugs. Number of cases/total population deviate from model 1, as we performed a complete caseset analysis.

Abbreviations: n= number of cases; N= total population within the exposure category.

Table 3. *Degree of serotonin reuptake inhibition for antidepressant drugs and the presence of cerebral microbleeds within drug users*

Degree of serotonin reuptake inhibition	Any microbleeds		Deep or infratentorial microbleeds		Strictly lobar microbleeds	
	n/N	Odds ratio (95% CI)	n N	Odds ratio (95% CI)	n/N	Odds ratio (95% CI)
Low/intermediate	74/351	1.00 (Reference)	25/302	1.00 (Reference)	49/326	1.00 (Reference)
Model 1						
High	53/311	1.03 (0.68;1.56)	20/278	1.14 (0.60;2.16)	33/291	0.93 (0.56;1.52)
Model 2						
High	51/298	1.02 (0.66;1.58)	19/266	1.16 (0.59;2.31)	32/279	0.91 (0.54;1.52)

Values represent odds ratios for microbleeds in relation to antidepressant drugs with high affinity for serotonin. Users of low and intermediate degree of serotonin reuptake inhibition antidepressants are the reference population for the presented analyses in Table 3.

Model 1: adjusted for age and sex.

Model 2: adjusted for age, sex, depression, diabetes, smoking, total and high-density lipoprotein cholesterol, systolic and diastolic blood pressure, ever use of lipid-lowering drugs, antihypertensive drugs, and antithrombotic drugs. Number of cases/total population deviate from model 1, as we performed a complete caseset analysis.

Abbreviations: n= number of cases; N= total population within the exposure category.

Table 4. *Degree of serotonin reuptake inhibition for antidepressant drugs and the presence of lacunes and white matter lesion volume*

Degree of serotonin reuptake inhibition	Lacunes		White matter lesion volume	
	n/N	Odds ratio (95% CI)	N	Difference in mean (95% CI)
Model 1				
Non-use	260 / 3888	1.00 (Reference)	3881	0.00 (Reference)
Low	3 / 45	0.82 (0.25;2.74)	45	-0.05 (-0.28;0.18)
Intermediate	23 / 288	1.20 (0.76;1.90)	288	0.08 (-0.02;0.17)
High	16 / 298	1.14 (0.67;1.94)	298	0.06 (-0.03;0.15)
Model 2				
Non-use	247 / 3728	1.00 (Reference)	3722	0.00 (Reference)
Low	3 / 43	0.89 (0.26;3.02)	43	-0.04 (-0.27;0.19)
Intermediate	22 / 275	1.13 (0.70;1.82)	275	0.06 (-0.03;0.16)
High	15 / 285	1.05 (0.60;1.86)	285	0.06 (-0.03;0.15)

Lacunes: values represent odds ratios for lacunes in antidepressant drug users with low, medium, and high affinity for serotonin compared to non-users.
White matter lesions volume: values represent differences in mean log transformed white matter lesion volumes (ml) in antidepressant drug users with low, medium, and high affinity for serotonin compared to non-users.
Model 1: adjusted for age and sex.
Model 2: adjusted for age, sex, depression, diabetes, smoking, total and HDL cholesterol, systolic and diastolic blood pressure, ever use of lipid-lowering drugs, antihypertensive drugs, and antithrombotic drugs. Number of cases/total population deviate from model 1, as we performed a complete caseset analysis.
White matter lesion volume analyses were additionally adjusted for intracranial volume and participants with unreliable white matter lesion segmentations were excluded from analyses.
Abbreviations: n= number of cases; N= total population within the exposure category.

Discussion

In the general population, we did not find an association between antidepressant drug use with a greater inhibition of serotonin reuptake and the presence of cerebral microbleeds. In addition, the degree of serotonin reuptake inhibition was not associated with presence of lacunes or WML volume.

Microbleeds are thought to precede the onset of large symptomatic hemorrhages, and may thus reflect a clinically relevant preclinical imaging marker, although evidence from longitudinal studies is still limited.[26,48,49] The novelty of our study lays in the fact that we investigated the use of SSRIs in relation to subclinical hemorrhagic brain lesions in the general population, in contrast to clinical studies investigating symptomatic hemorrhage. We did not observe an association between degree of serotonin reuptake inhibition and the presence of microbleeds. This is in line with the majority of previous studies on SSRIs and symptomatic brain hemorrhages,[15,18,20] although a recent meta-analysis did find an increased risk of brain hemorrhages in SSRI users (OR cohort studies, 1.68; 95% CI: 1.04; 2.51).[23] As a methodological consideration, heterogeneity in sample size, quality of the individual studies, and different approaches to handle the influence of confounding may have influenced the validity of the meta-analysis to a certain degree.[50]

SSRIs might increase the risk of clinical or subclinical bleedings via the following main biological mechanism. Damage to endothelial layers leads to activation of hemostatic mechanisms, and platelets adhere to damaged vessel walls. Intracellular serotonin is subsequently released into the blood stream and promotes clot formation and vasoconstriction at the site of injury. SSRIs inhibit the reuptake of serotonin by platelets from the blood, reduce intracellular serotonin concentrations, thereby decrease platelet aggregation and increase the risk of bleeding.[12,13] Moreover, a second mechanism proposes that some SSRIs may inhibit cytochrome 450 enzymes such as cytochrome 1A2, 2D6, 3A4, and 2C9. This may increase the bleeding risk by inhibition of the metabolism of certain drugs that have anticoagulant properties such as NSAIDS and antithrombotic drugs.[13,14] Nonetheless, for both mechanisms, we could argue that diminishing intraplatelet serotonin levels only affects hemostasis to a limited extent and thus that remaining platelet function is sufficient to halt significant bleeding. Depletion of serotonin levels in platelets may well be compensated for by other adequately working hemostatic mechanisms. This would partly explain why SSRI use was more consistently associated with extracranial bleeds, in particular gastrointestinal bleedings. Here, SSRI use increases serotonin levels and stimulates the production of gastric acid, which

increases the risk of gastrointestinal bleedings. Bleeding complications may, therefore, be induced by a third mechanism, which does not necessarily involve platelet dysfunction.[14]

No association was found for antidepressants with an affinity for serotonin with microbleeds in either lobar or deep or infratentorial regions of the brain. Although microbleeds at both locations are representative of bleeding-prone vessels, their causes differ. Microbleeds in lobar regions are more likely to result from blood leaking from destructed vessel walls containing amyloid, whereas deep or infratentorial microbleeds most likely represent hemosiderin deposits as a consequence of hypertensive arteriopathy.[28,40,43,51]

Our findings suggest that regardless of the underlying pathology, the decrease of intracellular serotonin platelets caused by antidepressants with a strong serotonin reuptake potential is insufficient to increase the frequency of small, asymptomatic bleedings.

Finally, we did not find a protective effect of antidepressant drugs, with a high affinity for the serotonin transporter, on ischemic vascular brain lesions. This is in line with findings from previous studies in patients with ischemic stroke[15,21] and strengthens the idea that the platelet inhibitor effects of antidepressant drug with affinity for serotonin are minimal. Two previous studies showed an increased risk of ischemic stroke in current SSRI users.[16,24] This increased risk could be explained by a different biological mechanism, which postulates that serotonin induces vasoconstriction of large vessel, and may lead to thromboembolic ischemic stroke in the presence of atherosclerosis.[52] However, in our study, we focused on silent ischemic vascular lesions, involving the small cerebral arteries, which are typically not caused by thromboembolic events.

The strengths of our study are the large sample size, population-based character of our study, and the prospectively gathered electronic pharmacy records that we used to determine antidepressant drug use. Based on a 19.5% prevalence of microbleeds in unexposed subjects, with a 2-sided significance of 0.05, we had sufficient power (80%) to detect an OR of 1.22 or greater. Less strong associations may not have been detected in our study, although based on the recent meta-analysis on SSRIs and symptomatic brain hemorrhages we would expect an estimate of at least this magnitude for subclinical bleedings.[23] Some limitations of our study need to be considered. The cross-sectional design of our study limits our conclusions on a causal pathway. MRI does not provide information on the timing of when cerebral microbleeds occurred because cerebral microbleeds remain visible in the brain for an undefined period. Therefore, there is a possibility that cerebral microbleeds occurred before antidepressant use was

initiated. This may have led to an underestimation of the true association presented because of non-differential misclassification of SSRI users, and further longitudinal investigations are warranted.

Furthermore, confounding by indication and contraindication poses a problem in our observational study. Depression, the most important indication to prescribe antidepressants, has a bidirectional association with cardiovascular disease, and cardiovascular diseases are associated with an increased number of microbleeds. Moreover, tricyclic antidepressants are relatively contraindicated for patients with cardiovascular disease. We minimized these forms of confounding by adjusting for presence of depressive symptoms, cardiovascular risk factors, and cardiovascular medication. Also, we reclassified the antidepressant drugs based on their affinity to the serotonin reuptake transporter. Although we aimed to address all potential confounders in our study, residual confounding cannot be ruled out and may have affected our results to an extent that associations may have been overestimated.

In conclusion, this study adds important information to the previous reports on antidepressant drug use and bleeding risk. We report that, in the general population, the use of antidepressant drugs that inhibit serotonin reuptake is not related to the presence of cerebral microbleeds. This further supports the safety of these antidepressants for non-gastrointestinal bleedings. Because these results are cross-sectional, further longitudinal research on antidepressant drug use and the risk of microbleeds in relation to major intracerebral hemorrhage is of high interest.

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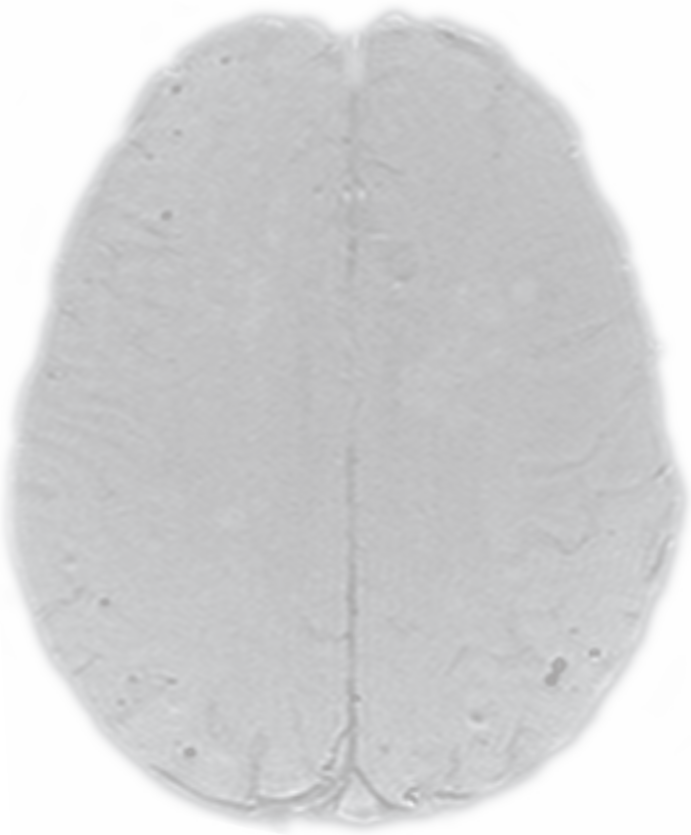

3.3

ANTIDEPRESSANT USE AND INCIDENT CEREBRAL MICROBLEEDS

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Abstract

Background: Serotonin specific antidepressants may increase the risk of adverse bleeding events. In a previous cross-sectional study, we observed no association between antidepressant use and presence of subclinical cerebral bleedings. In the current study we investigated longitudinally whether antidepressant use increases the risk of new subclinical cerebral microbleeds.

Methods: In total, 2,559 participants aged ≥45 years of the population-based Rotterdam Study, all without microbleeds at baseline, underwent baseline and repeat brain MRI between 2005 and 2013 (mean time interval 3.9 years, SD 0.5) to determine the incidence of microbleeds. Antidepressant use (yes versus no) was assessed between baseline and follow-up scan. In additional analysis antidepressants were classified as low, intermediate, or high affinity for the serotonin transporter, and alternatively as selective serotonin reuptake inhibitors (SSRIs) or non-SSRIs. We used multivariable logistic regression models to investigate the association of antidepressants with incident microbleeds.

Results: Antidepressant use was associated with higher cerebral microbleed incidence (odds ratio [OR] 2.22, 95% CI 1.31; 3.76) than non-use. When stratified by affinity for the serotonin transporter, intermediate serotonin affinity antidepressant use was associated with an increased risk of developing microbleeds (OR 3.07, 95% CI 1.53; 6.17). Finally, SSRIs and non-SSRIs use were both associated with increased microbleed incidence.

Conclusions: Antidepressant use associated with an increased risk of developing microbleeds. Our results may support findings from previous clinical studies regarding increased intracranial and extracranial bleeding risk in antidepressant users.

Introduction

Observational studies suggest that the biological effects[1] of antidepressants predispose users to symptomatic hemorrhagic adverse events, such as gastrointestinal and intracerebral hemorrhages.[2-4] Symptomatic hemorrhages are presumably just the tip of the iceberg, and more widespread subclinical bleedings may be present.

In the brain, reduced platelet activation due to antiplatelet drug use has been associated with a higher prevalence of subclinical microbleeds.[5] The question arises whether antidepressants have comparable effects on platelet function, as it is shown that these drugs also impair platelet aggregation. We previously showed that in the general population, use of antidepressants, with a high serotonin affinity, did not associate with a higher frequency of subclinical microbleeds on brain MRI.[6] This study however was limited by its cross-sectional design. Therefore, we now investigated the association between antidepressant use (and their degree of serotonin reuptake inhibition) and incident cerebral microbleeds.

Methods

Participants

This study was embedded within the Rotterdam Study, a large prospective population-based cohort. Study details were described extensively before.[7,8] The Rotterdam Study has been approved by the Medical Ethics Committee of the Erasmus MC and by the Ministry of Health, Welfare and Sport of the Netherlands, implementing the "Wet Bevolkingsonderzoek: ERGO (Population Studies Act: Rotterdam Study)".

For this study, we included 3,054 participants who were affiliated with one of the pharmacies serving the study area, and underwent both baseline and repeat brain MRI between August 2005 and July 2013. Participants with microbleeds on baseline MRI were excluded (n=495), leaving a total of 2,559 participants for analyses.

Antidepressant Drugs

Pharmacy records were available from 1991 onwards and provided information on prescription date of antidepressants, number of drug units, prescribed daily number of units, and Anatomical Therapeutic Chemical (ATC) code. Exposure was defined as antidepressants use between baseline and follow-up MRI,

irrespective of previous antidepressant use. Non-use between the MRIs was defined as the reference group. Antidepressants were categorized based on their affinity for the serotonin transporter into low, intermediate, and high degree of serotonin reuptake inhibition, as described previously.[6] Further, antidepressants were categorized into SSRIs (ATC-code='N06AB') and non-SSRIs (respectively, all other 'N06A'). Also, the duration of treatment and the average number of prescribed defined daily doses were assessed.

Brain MRI

Participants were scanned at both time points on the same 1.5-Tesla MRI scanner (GE Healthcare, Milwaukee, WI), as described previously.[8] In short, cerebral microbleeds were rated by trained research-physicians and defined as focal areas of low signal intensity on an accelerated 3-dimensional T2*-weighted gradient-recalled echo sequence.[9] Scans rated positive for microbleeds were included in a side-by-side comparison to determine the incidence of microbleeds.[10]

Statistical Analysis

We used multivariable logistic regression to investigate if any antidepressant use between baseline and follow-up MRI was associated with an increased risk of incident microbleeds, when compared with non-users. We repeated the analysis for the degree of serotonin reuptake inhibition (high, intermediate, low),[6] and for SSRIs and non-SSRIs, after exclusion of participants who used antidepressants from more than one group (switchers, n=43, n=39, respectively). All analyses were adjusted for age, sex, and time (in years) between baseline and repeat MRI scan. We additionally adjusted for potential confounders at baseline, including the presence of depressive symptoms based on the Center for Epidemiological Studies Depression Scale (CES-D), and a propensity score of cardiovascular risk (diabetes mellitus, smoking, total and high-density lipoprotein cholesterol, systolic and diastolic blood pressure, use of lipid-lowering (ATC-code C10), blood pressure-lowering (ATC-codes C02, C03, C07 through C09), and antithrombotic drugs (ATC-codes B01AA, B01AB, B01AC, and B01AX)). Post-hoc analyses were done to additionally adjust for duration of treatment, for average number of prescribed defined daily doses and to test the interaction of antidepressant with antithrombotic drug use between baseline and follow-up scan. Analyses were performed using IBM SPSS Statistics for Windows, Version 21.0, using an α-value of 0.05.

Results

Baseline characteristics of the study population are presented in Table 1. The mean age was 59.0 years (SD 7.8) and 1,403 (54.8) were women. The incidence of microbleeds over 3.9 years (SD 0.5) of follow-up was 3.7%.

Antidepressant use was associated with incident cerebral microbleeds (age, sex, and scan interval-adjusted odds ratio [OR] 2.22, 95% confidence interval [CI] 1.31; 3.76), compared to non-use (Table 2). When categorized by affinity for the serotonin transporter, only intermediate serotonin affinity antidepressant use was associated with an increased risk of developing microbleeds (OR 3.07, 95% CI 1.53; 6.17), also after additional adjustment for depressive symptoms and a propensity score of cardiovascular risk and cardiovascular drugs (OR 3.29, 95%CI 1.59; 6.79). Antidepressants with a high serotonin affinity were associated with incidence of microbleeds, though results did not reach statistical significance (OR 2.18, 95%CI 0.90; 5.29) (Table 2). Both SSRIs and non-SSRIs were associated with an increased risk of incident cerebral microbleeds, compared to non-use (Table 2). Additional adjustments for duration and dose of antidepressant treatment only marginally changed effect estimates, although associations were non-significant, possibly due to loss of statistical power (results not shown). We found no effect modification by antithrombotic drug use in a post-hoc analysis on the association of antidepressant use with incident microbleeds (*P* value=0.772).

Table 1. *Baseline characteristics*

	N= 2559
Age, years	59.0 (7.8)
Women	1,403 (54.8)
Depression score	3.0 [1.0 – 6.0]
Ever smoker	1,755 (68.8)
Diabetes mellitus	181 (7.2)
Total cholesterol, mmol/L	5.6 (1.0)
High-density lipoprotein cholesterol, mmol/L	1.4 (0.4)
Systolic blood pressure, mmHg	134.2 (18.9)
Diastolic blood pressure, mmHg	81.9 (10.5)
Lipid-lowering drug use	527 (20.8)
Blood pressure-lowering drug use	656 (25.9)
Antithrombotic drug use	422 (16.5)

Values represent mean (standard deviation) and median [interquartile range] for continuous variables and number (percentage) for categorical variables.
Depression score was based on the Center for Epidemiological Studies Depression Scale (range 0-60).
ATC code for lipid-lowering (C10), blood pressure-lowering (C02, C03, C07 through C09), and antithrombotic drugs (B01AA, B01AB, B01AC, B01AX).

Table 2. *Antidepressant use and the risk of cerebral microbleeds*

	Model 1		Model 2	
	n/N	Odds ratio (95% CI)	n/N	Odds ratio (95% CI)
Non-use	75/2260	Reference	72/2194	Reference
Antidepressant use				
Any use	19/299	2.22 (1.31; 3.76)	19/290	2.29 (1.31; 4.02)
Degree of inhibition of serotonin reuptake				
Low	0/35	Not applicable	0/34	Not applicable
Intermediate	10/120	3.07 (1.53; 6.17)	10/116	3.29 (1.59; 6.79)
High	6/101	2.03 (0.86; 4.81)	6/97	2.18 (0.90; 5.29)
Type of antidepressant				
SSRI	8/123	2.27 (1.06; 4.85)	8/118	2.39 (1.09; 5.25)
Non-SSRI	9/137	2.28 (1.11; 4.68)	9/133	2.37 (1.13; 4.97)

Values represent odds ratios (95% confidence intervals [CI]) for incident microbleeds in antidepressant users compared to non-users.
Model 1: adjusted for age, sex and scan interval.
Model 2: adjusted for age, sex, scan interval, depression score based on Center for Epidemiological Studies Depression scale, and a propensity score comprised of diabetes mellitus, smoking, total and high-density lipoprotein cholesterol, systolic and diastolic blood pressure, use of lipid-lowering drugs, blood-pressure lowering drugs, and antithrombotic drugs. Complete case analysis.
Abbreviations: n= number of cases; N= total population within the exposure category.
Number after stratifications does not add up to total number of antidepressant drug users because of exclusion of switchers.

Discussion

In this population-based study, we found that antidepressant use was associated with an increased risk of incident first-ever microbleeds after four years of follow-up. Associations were similar for different categories of antidepressants users, and persisted after adjusting for depressive symptoms and cardiovascular risk.

The increased risk of developing microbleeds in antidepressant users might be a direct consequence of the inhibiting effects on the serotonin transport by antidepressants. Platelet motility may decrease due to reduced intra-platelet serotonin concentrations.[1] In line with this, we expected strongest associations for users in the group with a high affinity for the serotonin transporter and in SSRIs, as they selectively block serotonin reuptake and impair platelet aggregation most. Stratification on the degree of serotonin reuptake inhibition, however, yielded small subgroups and hampered our ability to study these trends. Since we found that both SSRIs and non-SSRIs increased the risk of

incident microbleeds with similar effect sizes, this suggests that the association may not be due to the affinity for the serotonin transporter. We have to consider the possibility of reverse causality as incident microbleeds could have occurred at some time before antidepressant drugs use during follow-up, and biologically it has been hypothesized that microbleeds may contribute to the progression of depression.[11]

In our previous cross-sectional study we did not find an association between antidepressants and microbleed presence.[6] An explanation for this could be that our previous results were underestimated because of non-differential misclassification of antidepressant drug exposure, as microbleeds might have occurred before antidepressant use. Although this issue may also be present in the current study, we believe that using a narrow antidepressant drug exposure window and identifying first-ever incident microbleeds made our current longitudinal results more robust than the previous cross-sectional results.

The results of our study should be interpreted in light of some limitations. The number of incident microbleed cases during follow-up was small and limited our ability to perform microbleed subgroup analysis, for example regarding their location in the brain. Also, our results may be confounded by the indication for treatment, since depression has a bidirectional association with cardiovascular disease, and cardiovascular diseases are associated with microbleeds. We adjusted for depressive symptoms at baseline, but because we lacked data to control for depressive symptoms during follow-up potential residual confounding by depression may still be present. Also, as we mentioned reverse causality may have been present in our study. We tried to minimize the effects of reverse causality by excluding participants with prevalent microbleeds at baseline and by studying first-ever incident microbleeds.

In conclusion, antidepressant use was associated with an increased risk of developing microbleeds. Our results support findings from previous clinical studies regarding bleeding risk in antidepressants, and suggest that these risks may also apply to subclinical bleeding manifestations.

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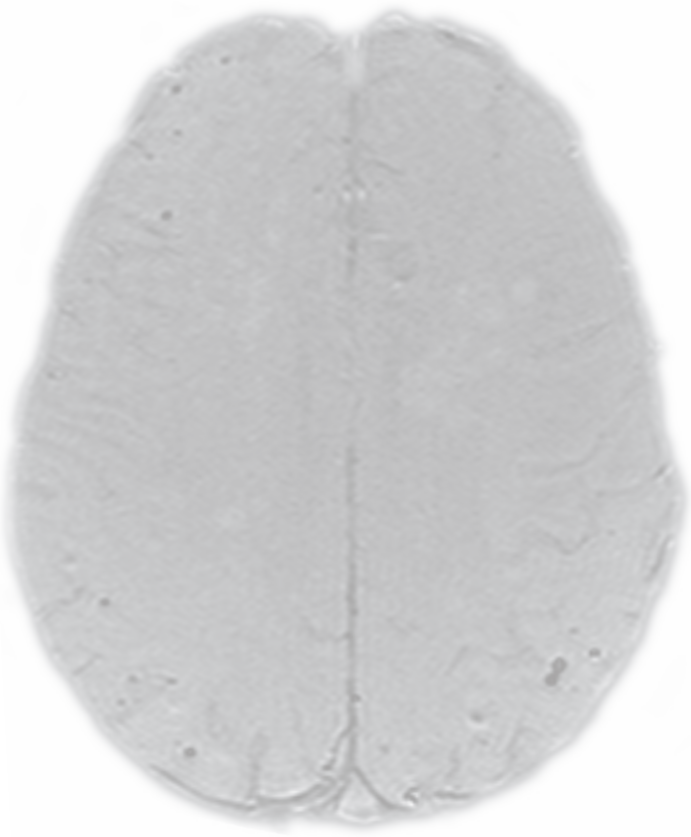
CHAPTER 4

Clinical Implications of Cerebral Microbleeds



4.1

Cerebral microbleeds and the risk of mortality

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Abstract

Background: Presence of cerebral microbleeds indicates underlying vascular brain disease and has been implicated in lobar hemorrhages and dementia. However, it remains unknown whether microbleeds also reflect more systemic vascular burden. We investigated the association of microbleeds with all-cause and cardiovascular related mortality in the general population.

Methods: We rated the brain MRI scans of 3,979 Rotterdam Scan Study participants to determine presence, number, and location of microbleeds. Cox proportional hazards models, adjusted for age, sex, subcohort, vascular risk factors, and other MRI markers of cerebral vascular disease, were applied to quantify the association of microbleeds with mortality.

Results: After a mean follow-up of 5.2 (± 1.1) years, 172 (4.3%) persons had died. Presence of microbleeds, and particularly deep or infratentorial microbleeds, was significantly associated with an increased risk of all-cause mortality (sex-, age-, subcohort adjusted hazard ratio (HR) 2.27; CI 1.50- 3.45), independent of vascular risk factors (HR 1.87; 95% CI 1.20- 2.92). The presence of deep or infratentorial microbleeds was strongly associated with the risk of cardiovascular related mortality (HR 4.08; CI 1.78- 9.39). Mortality risk increased with increasing number of microbleeds.

Conclusions: The presence of microbleeds, particularly multiple microbleeds and those in deep or infratentorial regions, indicates an increased risk of mortality, independent of other MRI markers of cerebral vascular disease. Our data suggest that microbleeds may mark severe underlying vascular pathology associated with poorer survival.

Introduction

Vascular diseases are worldwide the leading cause of mortality, currently accounting for 30% of all deaths.[1] With the dramatic surge of vascular risk factors expected in the developing world, the incidence of vascular mortality will only further increase in coming decades.[2] In the brain, vascular disease may manifest itself not only as stroke, but also as cognitive decline and dementia,[3] all of which are related to shorter survival.[4-7] Before vascular disease becomes clinically apparent, usually a long preclinical phase has passed during which vascular pathology steadily accumulates. A cornerstone of preventive research has been to identify markers that are indicative of such pre-clinical pathology and thus predict poor survival.[8] For vascular disease in the brain, several pre-clinical markers have been identified and include white matter lesions (WML) and lacunar infarcts, which can both be visualized on magnetic resonance imaging (MRI).[9] Indeed, previous studies have established that presence of these markers relates to shorter survival.[10-12]

In recent years, cerebral microbleeds have emerged as a novel MRI marker of vascular brain disease, and microbleeds are highly prevalent in the general population.[13,14] In contrast to WML and lacunar infarcts, which reflect ischemic changes in the brain, cerebral microbleeds are thought to be small foci of leakage of blood cells.[15] It remains unknown to what extent microbleeds increase the risk of mortality. Also, it is unclear whether presence of microbleeds only reflects vascular disease in the brain, or whether it actually indicates systemic vascular disease.

Until now, two patient-based studies have investigated the association between microbleeds and mortality. One study in memory clinic patients found that presence of microbleeds was associated with risk of mortality independently of other MRI markers of vascular brain disease,[10] whilst another study in patients with increased risk of cardiovascular disease reported that it was primarily the number of microbleeds that was related to mortality.[16] We were particularly interested to see whether we could extend these findings to a large group of presumably asymptomatic individuals, since the prevalence of microbleeds is high in the general population. We therefore investigated the association between presence, number and location of microbleeds with regard to all-cause, and cardiovascular related mortality in the general middle-aged and elderly population.

Methods

Study Design and Participants

The study is embedded in the Rotterdam Study, an ongoing prospective population-based cohort study aimed at assessing the incidence, prevalence and determinants of chronic diseases in the elderly. The initial cohort was extended with participants enrolled at a younger age in subsequent years of the study. Rationale and study design have been described previously.[17,18] The institutional review board approved the study. From 2005 until 2009, a total of 4,898 participants of the Rotterdam Study were invited to undergo brain MRI in the context of the Rotterdam Scan Study.[18] Participants with prevalent dementia (n=30), and MRI contraindications (n=389) were considered ineligible. Of the eligible participants, 4,082 (91 %) gave written informed consent. After excluding participants with physical inabilities to complete MRI (n=44), and MR images of low quality due to artifacts (n=59), ratings of microbleeds were available for 3,979 participants.

Brain MRI, Assessment of MRI Markers

We used a multisequence MRI protocol on the same 1.5-Tesla scanner (GE Healthcare Milwaukee, WI) in all participants.[18] Microbleeds were rated as focal areas of signal loss on 3D T2* GRE-weighted MR imaging (repetition time= 45 ms, echo time= 31, matrix size= 320x244, flip angle= 13, field-of-view= 25x17.5 cm^2, parallel imaging acceleration factor= 2, 96 slices encoded with a slice thickness of 1.6 mm zero padded to 192 slices of 0.8 mm, acquisition time 5 min 55 sec).[19] The presence, number and location of cerebral microbleeds were recorded. Microbleeds were categorized into lobar, deep, and infratentorial, as described previously.[14] The presence of lacunar and cortical infarcts was rated on FLAIR, proton density weighted and T1-weighted sequences.[14] WML and total brain volume were fully automatically segmented and quantified by summation of all voxels of each tissue class across the whole brain.[20]

Follow-up for Mortality

In the Rotterdam Study, information on vital status is obtained from municipal health authorities in Rotterdam on a monthly basis, and from general practitioners in the study area on a continuous basis. For each reported event, information on cause of death is obtained from general practitioners, and by checking medical records. Deaths due to cardiovascular causes were identified using the 'Rotterdam Study cardiovascular mortality classification'.[21] For all-cause mortality participants were followed from date of entry until date of death,

lost to follow-up or July 1st 2012, whichever came first. Information on cause specific mortality was available up to January 1st 2011. Accordingly, for cause specific mortality participants were followed from date of entry until date of death, lost to follow-up or January 1st 2011, whichever came first.

Assessments of Vascular Covariates

Potential vascular risk factors were selected based on previous literature published on microbleeds.[13,14] Research nurses collected data on all participants attending the research center by structured interviews, physical and laboratory examinations.[17] Blood pressure was measured in two readings using a random zero sphygmomanometer in sitting position, and both measures were averaged. Serum total cholesterol and high-density lipoprotein were measured using an automated enzymatic procedure.[17] Smoking behavior was defined as "ever" or "never" smoked. Diabetes was defined as having fasting blood glucose of ≥7.0 mmol/L, and/or the use of any glucose lowering medication. The use of any medication was assessed during home visits by standardized interviews. *APOE* genotyping was performed on coded genomic DNA samples.[22] Distribution of *APOE* genotype and allele frequencies in this population were in Hardy-Weinberg equilibrium.

Statistical Analysis

Baseline characteristics of the study population, by microbleed status, were investigated cross-sectionally using Student t-tests and Chi square tests to determine differences. WML volume was natural log transformed due to its skewed distribution.

Cox proportional hazards models were fitted to obtain the estimated hazard ratios and 95% confidence intervals on all-cause, and cardiovascular related mortality associated with the presence of one or more microbleeds. In addition, to determine the presence of a dose response association, we preformed analyses by categories of microbleed count assigning participants as having no (reference category), one, two through four, and five or more microbleeds.[23,24] Finally, since microbleeds at different locations of the brain are considered to relate to different etiology, we explored the risk of mortality associated with the presence of microbleeds at different locations of the brain (i.e., strictly lobar versus no microbleeds, and deep or infratentorial microbleeds with or without presence of lobar microbleeds versus no microbleeds). We constructed four statistical models. In Model 1, we adjusted only for age, sex and Rotterdam Study subcohort. In Model 2, we additionally adjusted for conventional vascular risk factors, namely

systolic and diastolic blood pressure, total cholesterol, high-density lipoprotein cholesterol, lipid lowering medication, antihypertensive medication, diabetes, and smoking. These were selected because of their strong association with deep or infratentorial microbleeds. In Model 3, we focused on reported risk factors for lobar microbleeds. Because *APOE* carriership is strongly related to lobar microbleeds we adjusted this model for *APOE* genotype.[14] Since *APOE* genotype affects lipid metabolism we additionally adjusted model 3 for cholesterol levels and lipid lowering medication. Finally, in Model 4 we extended Model 1 by adjustments for other imaging markers of vascular brain disease, specifically brain infarcts, WML and total brain volume [adjusted for intracranial volume (ICV)]. The proportional hazards assumption was tested using Schoenfeld residuals. Kaplan-Meier survival curves were constructed for categories of microbleed count for all- cause mortality. All analyses were conducted using statistical software package SPSS (version 20.0). *P* values <0.05 were considered statistically significant.

Results

Baseline characteristics of the population are shown in Table 1. Of the 3,979 Rotterdam Scan Study participants, 609 (15.3 %) had one or more cerebral microbleeds at baseline MRI. Of these, 413 (67.8 %) had strictly lobar microbleeds, and 196 (32.2 %) had microbleeds in deep or infratentorial regions. As previously reported,[13] higher age, systolic blood pressure, smoking, *APOE* ε4carriership, lacunar infarcts and white-matter lesions on MRI, and total brain volume were significantly associated with the presence of cerebral microbleeds. After a mean follow-up of 5.2 (±1.1) years, a total of 172 (4.3 %) people had died. Of the 124 participants who died before January 1st 2011, cause of death was known, and 36 (29.0 %) died of cardiovascular causes: 19 participants died of coronary heart disease, 6 due to ischemic stroke, 3 due to hemorrhagic stroke, 2 participants died of unspecified stroke, and 6 of other atherosclerotic or cardiovascular diseases.

Table 2 shows the estimated hazard ratios for presence of cerebral microbleeds in relation to all-cause, cardiovascular, stroke, and non-cardiovascular related mortality after basic adjustments for age, sex, and Rotterdam Study subcohort. After these adjustments, the presence of microbleeds related to a higher risk of all-cause (hazard ratio [HR] 1.56; 95% CI 1.12–2.17), and cardiovascular related mortality (HR 2.37; 95% CI 1.19–4.70). When investigating microbleeds with respect to their location in the brain, we found that microbleeds located deep or infratentorial in the brain were strongly associated with all-cause (HR 2.27; 95% CI 1.50–3.45), cardiovascular (HR 4.08; 95% CI 1.78–9.39), stroke (HR 5.02; 95%

Table 1. *Baseline characteristics of study participants*

	Total (N= 3979)	
	Microbleeds (N=609)	**No microbleeds (N=3370)**
Age (yrs)*	64.5 (9.8)	59.5 (8.3)
Female	322 (52.9)	1842 (54.7)
Duration of follow-up, deaths all-cause (yrs)	5.1 (1.3)	5.2 (1.1)
Duration of follow-up, deaths cause specific (yrs)	3.8 (1.1)	3.7 (1.0)
Systolic blood pressure (mmHg)*	140.3 (20.8)	134.4 (19.2)
Diastolic blood pressure (mmHg)*	82.1 (11.3)	81.7 (10.7)
Use of blood pressure lowering medication	212 (35.5)	921 (27.6)
Total cholesterol (mmol/l)	5.6 (1.1)	5.6 (1.0)
HDL cholesterol (mmol/l)	1.4 (0.4)	1.4 (0.4)
Use of Lipid lowering medication	145 (24.2)	713 (21.4)
Diabetes	54 (9.1)	263 (8.0)
Ever smoked*	453 (74.8)	2342 (69.9)
APOE-e4 carriership*	188 (37.7)	890 (32.5)
Lacunar infarcts on MRI*	75 (12.3)	138 (4.1)
Cortical infarcts on MRI	22 (3.6)	89 (2.6)
White matter lesion volume (ml)*	8.1 (12.6)	4.1 (6.2)
Total brain volume (ml)*	933.6 (103.6)	945.4 (106.0)

Data presented as mean (standard deviation) for continuous variables and number (percentage) for categorical variables.
** P-value <0.05, indicates significant difference between people with and without microbleeds.*
Abbreviations: HDL= high-density lipoprotein, APOE-e4= Apo lipoprotein E4,
APOE-e4 carriership status was missing in n= 742, mainly due to insufficient blood draw for DNA extraction.

CI 1.33–18.91), and non-cardiovascular related mortality (HR 1.81; 95% CI 0.99–3.29), whilst strictly lobar microbleeds were not related to mortality (Table 2). In a post hoc analysis we found that people with strictly deep or infratentorial microbleeds had an increased risk of all-cause mortality compared with people without microbleeds (age, sex, subcohort adjusted HR: 1.84; 95% CI 0.93–3.64).

Table 3 and Figure show the association between microbleed count and the risk of mortality. The risk of all-cause mortality increased gradually with larger numbers of microbleeds in the brain (Figure). Participants with five or more cerebral microbleeds, had a 2.8 times (95%CI 1.65–4.62) higher risk of dying, compared to people without cerebral microbleeds, whereas participants with one cerebral microbleed had a 1.3 times (95% CI 0.84–2.02) higher risk (Table 3). Testing for linear trends in the hazard of microbleed count per category showed

Table 2. *Cerebral microbleeds and the risk of mortality*

	All-cause mortality		Cardiovascular mortality		Stroke mortality		Non-cardiovascular mortality	
	n/N	HR (95% CI)	n/N	HR (95% CI)	n/N	HR (95% CI)	n/N	HR (95% CI)
No microbleeds	115/3370	Ref.	20/3370	Ref.	6/3370	Ref.	62/3370	Ref.
Any microbleeds	57/609	1.56 (1.12- 2.17)	16/609	2.37 (1.19- 4.70)	5/609	1.98 (0.59- 6.70)	26/609	1.30 (0.81- 2.09)
Deep or infratentorial	30/196	2.27 (1.50- 3.45)	9/196	4.08 (1.78- 9.39)	4/196	5.02 (1.33- 18.91)	14/196	1.81 (0.99- 3.29)
Strictly lobar	27/413	1.16 (0.76- 1.79)	7/413	1.73 (0.71- 4.19)	1/413	1.00 (0.10- 10.32)	12/413	0.96 (0.51- 1.80)

Values represent hazard ratios (HR) with 95% confidence interval (CI).
Adjusted for age, gender, and Rotterdam Study subcohort.
Follow-up for all-cause mortality was until 01.07.2012, and a total of 172 people had died.
Follow-up for cardiovascular (including stroke related) and non-cardiovascular mortality was until 01.01.2011, and a total of 124 people had died.
Abbreviations: n= deaths, N= number of subjects.

Table 3. *Cerebral microbleeds per category of microbleed count and risk of mortality*

		All-cause mortality		Cardiovascular mortality		Stroke mortality	
		n/N	HR (95% CI)	n/N	HR (95% CI)	n/N	HR (95% CI)
No microbleeds		115/3370	Ref.	14/3370	Ref.	6/3370	Ref.
Categories	**1**	25/395	1.30 (0.84- 2.02)	5/395	1.48 (0.55- 3.99)	0/395	No cases
	2 thr. 4	14/143	1.34 (0.76- 2.35)	4/143	2.02 (0.67- 6.09)	2/143	2.62(0.50- 13.65)
	≥5	18/71	2.76 (1.65- 4.62)	7/71	5.64 (2.27- 13.99)	3/71	6.87 (1.62- 29.07)

Values represent hazard ratios (HR) with 95% confidence interval (CI).
Adjusted for age, gender, and Rotterdam Study subcohort.
Follow-up for all-cause mortality was until 01.07.2012, and a total of 172 people had died.
Follow-up for cardiovascular (including stroke related) mortality was until 01.01.2011, and a total of 124 people had died.
Abbreviations: n= deaths, N= number of subjects.

a *P* value of 0.001. Also, the risk of cardiovascular, and stroke related mortality was higher when the number of microbleeds increased (Table 3).

After adjusting for vascular risk factors the presence of microbleeds, and especially deep or infratentorial microbleeds, associated strongly with all-cause mortality (Table 4, model 2 and 3). Similar results were found after adjustment for infarcts, WML load and brain volume (Table 4, model 4). Particularly the presence of 5 or more microbleeds indicated an increased risk of all-cause mortality after adjusting for vascular risk factors (HR 2.41; 95% CI 1.37–4.24), and after adjusting for other MRI markers of vascular brain disease (HR 2.01; 95% CI 1.16–3.48).

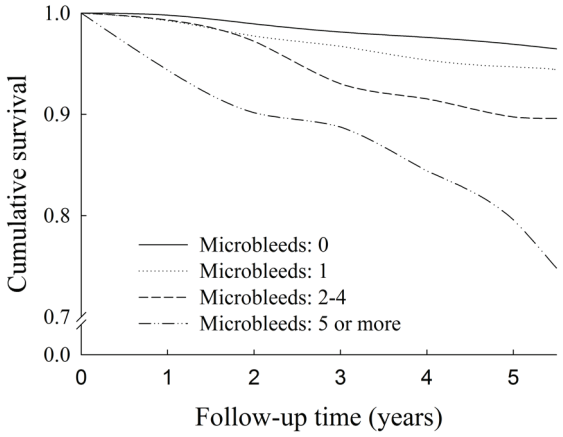

Figure. *Kaplan-Meier survival curves of cerebral microbleeds per category of microbleed count.*

Table 4. *Cerebral microbleeds and risk of all-cause mortality*

	Model 1	Model 2	Model 3	Model 4
	HR (95% CI)	HR (95% CI)	HR (95% CI)	HR (95% CI)
No microbleeds	Ref.	Ref.	Ref.	Ref.
Any microbleeds	1.56 (1.12- 2.17)	1.37 (0.96- 1.94)	1.29 (0.86- 1.93)	1.35 (0.96- 1.91)
Deep or infratentorial	2.27 (1.50- 3.45)	1.87 (1.20- 2.92)	1.76 (1.05- 2.94)	1.90 (1.20- 3.00)
Strictly lobar	1.16 (0.76- 1.79)	1.04 (0.65- 1.66)	1.00 (0.59- 1.69)	1.05 (0.67- 1.64)

Values represent hazard ratios (HR) with 95% confidence interval (CI).
Model 1: adjusted for age, gender, Rotterdam Study subcohort.
Model 2: as Model 1, and additionally adjusted for systolic and diastolic blood pressure, total cholesterol, high-density lipoprotein cholesterol, lipid lowering- and antihypertensive medication, diabetes, smoking.
Model 3: as Model 1, and additionally adjusted for apolipoprotein E ε4 carriership, total cholesterol, high-density lipoprotein cholesterol, lipid lowering medication.
Model 4: as Model 1, and additionally adjusted for presence of lacunar and cortical brain infarcts, white matter lesion volume (ln transformed, per standard deviation increase), total brain volume and intracranial volume.

Discussion

We found that the presence of deep or infratentorial microbleeds, but not lobar microbleeds, was related to increased risk of all-cause mortality. These associations were strongest with cardiovascular mortality. In people with more than five microbleeds, these associations were independent from vascular risk factors and other MRI markers of vascular brain disease.

Strengths of our study include the population-based setting, which aids generalizability, and the large sample size, facilitating various subgroup analyses. Linkage with data from municipal health authorities ensured virtually complete follow up for mortality. Also, we were able to investigate the association between microbleeds and risk of mortality independent of vascular risk factors and imaging markers due to detailed assessment in the Rotterdam Study. Our scan protocol was optimized to increase conspicuity of microbleeds.[19] Care should be taken that the number of microbleeds detected may differ in various scan settings as it strongly depends on imaging parameters used.[19] We categorized microbleeds as done conventionally into strictly lobar versus deep or infratentorial microbleeds based on their presumable underlying pathology.[14] However, such categorization might suffer from the fact that people with deep or infratentorial microbleeds may have more microbleeds than those with only lobar microbleeds. It is thus possible that the association between deep or infratentorial microbleeds and mortality is driven by numbers of microbleeds rather than location. To address this issue, we performed a post hoc analysis, comparing people with strictly deep or infratentorial microbleeds with people without microbleeds. We found that strictly deep or infratentorial microbleeds still increased the risk of mortality by 84% (age, sex, subcohort adjusted HR: 1.84; 95 % CI 0.93–3.64). It is noteworthy that the number of cardiovascular deaths (including strokes) in our study group was relatively small. One explanation is that non-participants (e.g., those with MRI contraindications, such as pacemaker, metal implants) have a high likelihood of cardiovascular death. Furthermore, over two-thirds of the cardiovascular deaths (including strokes) occur beyond the age of 75.[25] In our study group, 92.3% of the population was younger than 75 years, and thus less likely to die of cardiovascular causes.

We found that presence of microbleeds associates with an increased risk of mortality. These findings are in line with previous studies conducted in a memory clinic population,[10] and in a population of people with an increased risk of developing cardiovascular disease (PROSPER study).[16] We now show that these findings can be extended to people of the general population, emphasizing the role of microbleeds as a preclinical marker of vascular disease in asymptomatic individuals. People with microbleeds were not only more likely to die from vascular brain disease (i.e., strokes) but also from vascular disease outside the brain (i.e., myocardial infarction, heart failure). Previously, it has been suggested

that vascular brain disease may to a large extent reflect systemic vascular disease.[11,26-28] It is therefore viable that microbleeds not only reflect changes in cerebral vessels but may also reflect systemic vascular changes that indicate poorer survival. Further evidence for this notion comes from our observation that especially deep or infratentorial microbleeds drove the associations.[16] Indeed, deep or infratentorial microbleeds are thought to reflect hypertensive arteriopathy,[14] which overlaps considerably with systemic vascular disease, whereas lobar microbleeds are more indicative of cerebral amyloid angiopathy, which is primarily restricted to the brain.[29] This might also explain findings from a previous study that showed lobar microbleeds to be associated with stroke-specific mortality.[16] It is likely that these strokes were intracerebral hemorrhages, a major clinical sequel of lobar microbleeds.[29] In our study, the overall number of stroke related mortality was small, precluding robust observations.

The association between deep or infratentorial microbleeds and non-cardiovascular deaths may be explained by deaths due to other neurological causes (i.e., neurodegenerative disease). Another potential explanation may be misclassification of underlying cause of death in people dying from presumably non-vascular causes (70% of non-cardiovascular deaths was attributed to death from cancer).

We found that the associations between microbleeds and all-cause mortality weakened after adjustment for vascular risk factors. Still, we found that deep or infratentorial microbleeds relate to a higher risk of cardiovascular mortality, even after adjustment for vascular risk factors. This suggests that microbleeds reflect risk factors for vascular brain disease that we did not measure in our study (e.g., genetic factors), or microbleeds may be a more sensitive marker of mortality than these concomitantly measured vascular risk factors. Finally, we showed that multiple microbleeds associate with mortality independent of other MRI markers of vascular brain disease. Therefore, (multiple) microbleeds may be used in addition to other imaging markers such as brain infarcts, WML to identify presence and severity of cerebral vascular disease, and to indicate poorer survival.[10-12] Future studies should indicate whether implementing MRI techniques as a screening method will be cost-effective.

In conclusion, our study shows that in the general population the presence of deep or infratentorial microbleeds is associated with an increased risk of mortality, especially cardiovascular mortality. This implies that microbleeds may be a novel sensitive marker of severe vascular disease and thus an increased risk of death.

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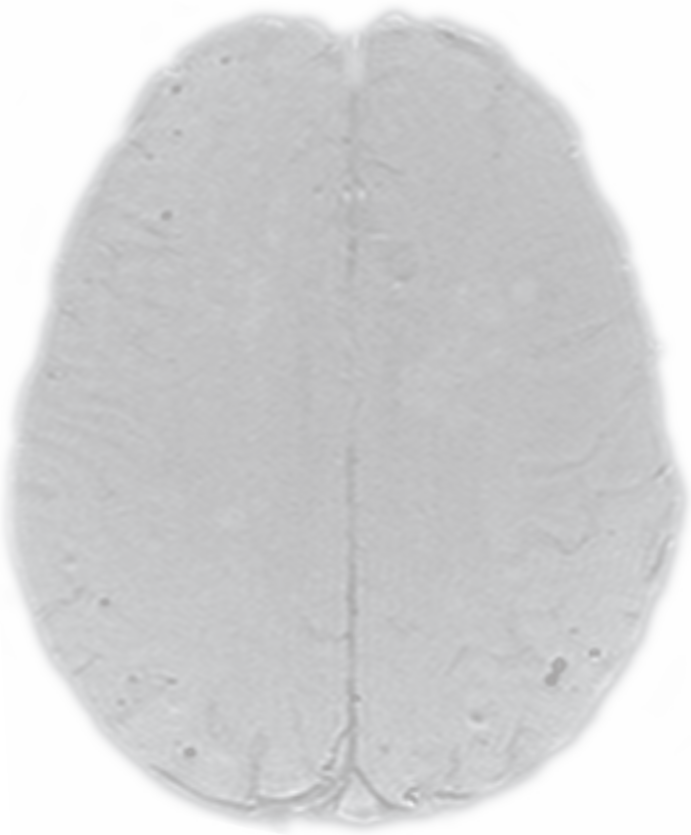

4.2

Cerebral microbleeds and the risk of stroke

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Abstract

Background: Cerebral microbleeds are highly prevalent in people with clinically manifest cerebrovascular disease, and have been shown to increase the risk of stroke recurrence. Microbleeds are also frequently found in healthy elderly, a population in which the clinical implication of microbleeds is unknown.
Methods: In the population-based Rotterdam Study, presence, number, and location of microbleeds were assessed at baseline on brain MRI of 4,759 participants aged ≥45 years. Participants were followed for incident stroke throughout the study period (2005 until 2013). We used Cox proportional hazards to investigate if people with microbleeds were at increased risk of stroke compared to those without microbleeds, adjusting for demographic, genetic, and cardiovascular risk, and cerebrovascular imaging markers.
Results: Microbleed prevalence was 18.7% (median count 1 [1-111]). During mean follow-up of 4.9 years (SD 1.6) 93 strokes occurred (72 ischemic, 11 hemorrhagic, and 10 unspecified). Microbleed presence was associated with an increased risk of all strokes (HR 1.93, 95% CI 1.25-2.99). The risk increased with greater microbleed count. Compared to those without microbleeds, participants with microbleeds in locations suggestive of CAA (lobar with or without cerebellar microbleeds) were at increased risk of intracerebral hemorrhage (HR 5.27, 95% CI 1.38-20.23). Microbleeds at other locations were associated with an increased risk of both ischemic stroke and intracerebral hemorrhage.
Conclusions: Microbleeds on MRI are associated with an increased risk of stroke in the general population. Our results strengthen the notion that microbleeds mark progression of cerebrovascular pathology and represent a precursor of stroke.

Introduction

Stroke is the second leading cause of death worldwide and the third most common cause of disability-adjusted life-years.[1,2] Although stroke has an acute onset, there is abundant evidence for a long subclinical period in which cerebrovascular pathology accumulates and can be visualized on non-invasive brain imaging. Markers of subclinical cerebrovascular pathology that are known to increase the risk of ischemic stroke are white matter lesions and lacunes.[3] In the last decade, punctuate hemorrhagic lesions in the brain parenchyma, so called cerebral microbleeds, have emerged as another manifestation of subclinical cerebrovascular pathology.[4] In contrast to white matter lesions and lacunes, microbleeds are thought to reflect the presence of both ischemic and hemorrhagic brain vasculopathy.[5,6] In addition, evidence suggests that the location of cerebral microbleeds provides more information on the type of underlying vasculopathy, i.e., cerebral amyloid angiopathy (CAA) in the presence of lobar microbleeds and hypertensive arteriopathy when deep or mixed microbleeds are seen.[4,7,8]

There is a growing need to clarify whether microbleed presence indicates an increased risk of stroke, as this may provide new insights in the link between subclinical cerebrovascular pathology and stroke. So far, it is known that in patients with a history of stroke microbleed presence increases the risk of recurrent stroke, either hemorrhagic or ischemic.[9-18] Whether microbleeds are associated with an increased risk of stroke in community-dwelling elderly without a history of stroke remains unclear. Only two prospective studies investigated the association of microbleeds with subsequent stroke in people without a history of cerebrovascular events.[19,20] Both studies found that microbleed presence was associated with an increased risk of stroke, although important limitations were small sample size, absence of microbleed subgroup analysis, and lack of generalizability due to an exclusive focus on Asian populations.[19,20]

In the population-based Rotterdam Study, we investigated whether cerebral microbleeds at various locations are associated with an increased risk of recurrent and first-ever ischemic stroke and intracerebral hemorrhage.

Methods

Study Population

The Rotterdam Study is an ongoing prospective population-based cohort that studies occurrence, determinants and consequences of diseases in an aging population.[21] The Rotterdam Study has been approved by the medical ethics

committee according to the Population Study Act Rotterdam Study, executed by the Ministry of Health, Welfare and Sports of the Netherlands. The cohort originated in 1990, was expanded twice in 2000 and 2006, and now comprises 14,926 participants aged ≥45 years, predominantly of Caucasian origin. All participants gave written informed consent. In 2005, brain MRI, including microbleed assessment, was implemented within the core protocol of the study.[22] Between January 1st 2005 and January 1st 2013, 5,074 out of 5,735 eligible participants (88.5%) underwent brain scanning. Participants with scans that were incomplete (n=72) or of insufficient quality for microbleed rating (n=57) were excluded. Also, we excluded participants (n=186) in whom follow-up for incident stroke ended before date of MRI due to the absence of automatic linkages between the general practitioners office and our study database, leaving a total of 4,759 participants for analyses.

Brain MRI and Microbleeds

A 1.5-Tesla MRI scanner (GE Healthcare, Milwaukee, WI) was used to obtain T1-weighted, T2-weighted, fluid-attenuated inversion recovery (FLAIR), and T2*-weighted sequences.[22] Cerebral microbleeds were recognized as focal, small, round to ovoid areas of signal loss on T2*-weighted images (gradient-recalled echo with repetition time= 45ms, echo time= 31, matrix size= 320x244, flip angle= 13, field-of-view= 25x17.5cm2, parallel imaging acceleration factor= 2, 3D acquisition with 96 slices encoded with a slice thickness of 1.6mm zero padded to 192 slices of 0.8mm, acquisition time 5min 55sec).[23] Their presence, number, and location were scored by 1 of 5 trained research physicians using a protocol that is in place since initiation of the study in 2005 with good intraobserver (k=0.87) and interobserver agreement (k=0.85). Signal voids caused by sulcal vessels, calcifications, and signal averaging from bone were considered mimics of microbleeds.[4] Raters were blinded to all clinical data. Microbleeds were classified based on their location in the brain into groups most befitting their presumed etiology. Microbleeds at locations where CAA is highly prevalent (strictly lobar or lobar with cerebellar locations) were termed "CAA related" microbleeds, and microbleeds at other locations (deep gray matter, deep white matter, and brainstem with or without lobar microbleeds; cerebellar with or without deep or brainstem microbleeds) were termed "non-CAA related" microbleeds.

Aside from microbleeds we assessed the presence of infarcts and the load of white matter lesions. Lacunes were defined as focal lesions between ≥3 and <15mm seen on FLAIR, T1-weighted, and T2-weighted sequences. If lacunes were ≥15mm they were defined as subcortical infarcts, and if cortical gray matter was affected they were classified as cortical infarcts. Brain tissue was segmented

into gray matter, white matter, and cerebrospinal fluid using automated post-processing tools that included conventional k-nearest-neighbor brain tissue classifier extended with white matter lesion segmentation.[24] Intracranial volume was defined as the sum of gray matter, white matter, and white matter lesion volume, and cerebrospinal fluid.

Stroke Assessment and Follow-up

Upon study entry, history of stroke was assessed using home interviews and by reviewing medical records. Subsequently, participants were continuously followed-up for occurrence of stroke through automated linkage of general practitioners' medical records with the study database.[25] For participants who moved outside the study district or lived in nursing homes, medical records were regularly checked by contacting their treating physicians. Research physicians reviewed all potential strokes using hospital discharge letters, information from general practitioners and from nursing home physicians. An experienced vascular neurologist verified the stroke diagnoses. In accordance with World Health Organization (WHO) criteria, stroke was defined as a syndrome of rapidly emerging clinical signs of focal or global disturbance of cerebral function. Symptoms should last ≥24 hours or cause death, with no apparent cause other than of vascular origin. Strokes were classified into ischemic strokes or spontaneous intracerebral hemorrhages based on neuroimaging reports (computed tomography). In the absence of neuroimaging, strokes were classified as unspecified.

Follow-up started on the date that participants came for brain MRI. Participants were followed until date of (fatal or non-fatal) stroke occurrence, date of death, date of last contact in case of loss to follow-up, or January 1st 2013 (end of the study period), whichever came first. Follow-up was complete for 23,356 (94.9%) of potential person-years.

Assessments of Covariates

Participants' demographics, genetic and cardiovascular risk factors were assessed during the center visit preceding the brain MRI, using structured interviews, physical, and laboratory examinations. We defined various potential cardiovascular confounders. Blood pressure measurements were averaged over two readings using a random zero sphygmomanometer. Hypertension was defined as a blood pressure of >140 systolic or >90 diastolic, or the use of blood pressure-lowering medication. Serum total and high-density lipoprotein (HDL) cholesterol were measured using an automated enzymatic procedure. Smoking behavior was defined as smoked 'ever' versus 'never'. Participants were

considered diabetic when fasting blood glucose levels were ≥7.0 mmol/L or when they used glucose-lowering medication. Blood pressure-lowering and lipid-lowering medication use was assessed in home interviews. Pharmacy records were used to determine the use of antithrombotic medication (ATC code B01AA04 acenocoumarol, B01AA07 phenprocoumon, B01AB heparins, and B01AC platelet aggregation inhibitors) between baseline MRI and stroke event, death or January 1st 2013, whichever came first. Finally, *APOE* genotyping was done on coded genomic DNA samples.

Statistical Analysis

Student's *t*-test and Fisher's exact test were used to compare characteristics of study participants by microbleed status. Cox proportional hazards models were fitted to obtain the estimated hazard ratios (HR) and 95% confidence intervals for the association of microbleeds with stroke (all fatal and non-fatal strokes, fatal and non-fatal ischemic strokes, and fatal and non-fatal intracerebral hemorrhages). We first investigated the risk of recurrent and first-ever stroke combined. Afterwards we excluded participants with a history of stroke at baseline (n=8) and separately investigated the risk of first-ever stroke. Analyses were repeated for location of microbleeds ("CAA related" and "non-CAA related" locations versus no microbleed), and for microbleed count to investigate a potential dose-response effect (predefined categories of 1, 2-4, and >4 microbleeds versus no microbleeds).[26-28] We constructed 4 models to adjust for potential confounding. In order not to overfit the statistical models we used logistic regression to compute propensity scores for cardiovascular risk. In these, microbleed status (yes versus no) was considered the dependent variable and the following cardiovascular risk factors were considered independent covariates: hypertension, total and HDL cholesterol, smoking status, diabetes mellitus status, use of lipid-lowering and antithrombotic medication. The estimated propensity score was the derived predicted value of this equation. Missing covariate data (≤7.0%) were imputed based on sex, age, and cardiovascular risk factors using regression models. The first model was adjusted for age-squared, sex, and Rotterdam Study subcohort. The second model was additionally adjusted for *APOE* ε4 carriership. The third model was adjusted for age-squared, sex, Rotterdam Study subcohort and the propensity score described above. Finally, the fourth model equaled model 1 with additional adjustments for lacunes, white matter lesions, and total intracranial volume. An additional 77 participants were excluded in the analysis of model 4 because the white matter lesion segmentation of these MRI scans was considered unreliable. The proportionality assumption was tested using Schoenfeld residuals.

Kaplan-Meier incident stroke-free survival curves were constructed for groups with microbleed count 0, 1, 2-4, >4, and compared using the log-rank test. In a sensitivity analysis, we repeated the analysis of model 1 after stratification by antithrombotic drug use (ever versus never), and applied formal interaction tests to determine significant differences in subgroups.

Finally, we examined all research and hospital scans of incident intracerebral hemorrhage cases, and visually correlated the locations of baseline microbleeds and incident hemorrhages, since it was hypothesized that symptomatic hemorrhages would occur in close proximity of microbleeds.

Analyses were done using IBM SPSS statistics for windows, version 21.0 (IBM Corp., Armonk, NY), using an α-value of 0.05.

Results

Characteristics of the study population are presented in Table 1. Of the total 4,759 participants 55.3% were women and the average age at baseline was 63.8 years (SD 10.9). Microbleed prevalence was 18.7% (median microbleed count 1 [range 1-111]). Older age, hypertension, lower total cholesterol, smoking, use of lipid-lowering and antithrombotic medication, and ischemic vascular lesions on brain MRI were all significantly associated with microbleed presence. At baseline, 8 (0.2%) participants had a history of stroke (six ischemic and two unspecified). Over a mean follow-up of 4.9 years (SD 1.6), 93 (2.0%) participants suffered a stroke, of whom 72 ischemic strokes (3.1 per 1,000 person-years), 11 intracerebral hemorrhages (0.5 per 1,000 person-years), and 10 unspecified strokes (0.4 per 1,000 person-years).

People with microbleeds at baseline were at increased risk of developing new strokes compared with those without microbleeds (age-squared and sex adjusted HR 1.93, 95% CI 1.25-2.99) (Table 2). Risk was highest for participants with microbleeds in brain locations not typically affected by CAA (HR 3.35, 95% CI 1.94-5.78). For subtypes of strokes, we found that microbleeds in locations not typically affected by CAA associated with an increased risk of both ischemic stroke (HR 3.05, 95% CI 1.65-5.63) and intracerebral hemorrhage (HR 5.92, 95% CI 1.07-32.86), whereas microbleeds in locations suggestive of CAA were only associated with an increased risk of intracerebral hemorrhage (HR 5.27, 95% CI 1.38-20.23). Additional adjustments for *APOE* ε4 carriership, cardiovascular risk, and imaging markers of vascular brain disease weakened these associations, but significance remained (Table 2). Microbleed presence was also associated with an increased risk of first-ever stroke, in a similar differential pattern for

Table 1. *Characteristics of the study population*

	Total population (N=4759)	Microbleeds absent (N=3867)	Microbleeds present (N=892)	*P* value
Age, years (mean, SD)	63.8 (10.9)	62.4 (10.5)	69.7 (10.9)*	<0.001
Women (n, %)	2,631 (55.3)	2,155 (55.7)	476 (53.4)	0.204
Hypertension (n, %)	2,914 (61.2)	2,267 (58.6)	647 (72.5)*	<0.001
Total cholesterol, mmol/L (mean, SD)	5.5 (1.0)	5.6 (1.0)	5.4 (1.1)*	0.001
High-density lipoprotein cholesterol, mmol/L (mean, SD)	1.4 (0.4)	1.4 (0.4)	1.4 (0.4)	0.720
Ever smoker (n, %)	3,315 (69.7)	2,665 (68.9)	650 (72.9)*	0.021
Diabetes mellitus (n, %)	410 (8.6)	322 (8.3)	88 (9.9)	0.145
Lipid-lowering medication use (n, %)	1,118 (23.5)	860 (22.2)	258 (28.9)*	<0.001
Antithrombotic medication use (n, %)	1,274 (26.8)	891 (23.0)	383 (42.9)*	<0.001
Apolipoprotein E ε4 carriership (n, %)	1,435 (30.2)	1,137 (29.4)	298 (33.4)*	0.021
White matter lesion volume† (median, IQR)	2.9 (1.6-6.1)	2.7 (1.5-5.1)	5.0 (2.5-11.7)*	<0.001
Lacunes on MRI (n, %)	310 (6.5)	194 (5.0)	116 (13.0)*	<0.001
Subcortical infarcts on MRI (n, %)	8 (0.2)	4 (0.1)	4 (0.4)*	0.045
Cortical infarcts on MRI (n, %)	111 (2.3)	85 (2.2)	26 (2.9)	0.218

*Values presented mean (standard deviation [SD]) for continuous variables and number (%) for categorical variables. †Presented as median (interquartile range [IQR]) and assessed in 4,687 participants. * P value <0.05, indicates significant difference in people with and without microbleeds.*

the various microbleed locations as described above (Table 3). Especially the presence of multiple microbleeds, irrespective of their location, was associated with an increased risk of first-ever stroke (Figure 1). Microbleeds did not associate differently with stroke risk after stratifying on antithrombotic drug use (Supplementary Table 1).

Six participants with microbleeds at baseline developed a first-ever intracerebral hemorrhage during follow-up. All of them presented with multiple microbleeds (median count 8.5, range 2-17), and three had used antithrombotic agents (either platelet inhibitors or oral anticoagulants) during follow-up. The symptomatic hemorrhages were located respectively in lobar (thrice), thalamic (twice), and cerebellar region (once). The location of the symptomatic hemorrhage of five participants correlated exactly with the anatomical location of one of the microbleeds on their prior research scan (Figure 2).

Table 2. *Cerebral microbleeds and the risk of any stroke*

	n/N	All strokes	*P* value	n/N	Ischemic stroke	*P* value	n/N	Intracerebral hemorrhage	*P* value
Model 1									
No microbleeds	59/3867	1.00 (Reference)		49/3867	1.00 (Reference)		5/3867	1.00 (Reference)	
Any microbleeds	34/892	1.93 (1.25-2.99)	0.004	23/892	1.52 (0.91-2.53)	0.124	6/892	5.64 (1.66-19.13)	0.006
Non-CAA related microbleeds	18/259	3.35 (1.94-5.78)	<0.001	14/259	3.05 (1.65-5.63)	<0.001	2/259	5.92 (1.07-32.86)	0.046
CAA related microbleeds	16/633	1.30 (0.74-2.28)	0.383	9/633	0.84 (0.41-1.74)	0.629	4/633	5.27 (1.38-20.23)	0.017
Model 2									
No microbleeds	59/3,867	1.00 (Reference)		49/3867	1.00 (Reference)		5/3867	1.00 (Reference)	
Any microbleeds	34/892	1.87 (1.20-2.90)	0.007	23/892	1.49 (0.89-2.49)	0.145	6/892	5.34 (1.56-18.32)	0.009
Non-CAA related microbleeds	18/259	3.28 (1.89-5.68)	<0.001	14/259	2.97 (1.61-5.51)	0.001	2/259	5.97 (1.07-33.39)	0.046
CAA related microbleeds	16/633	1.24 (0.70-2.19)	0.477	9/633	0.81 (0.39-1.68)	0.560	4/633	5.04 (1.30-19.47)	0.021
Model 3									
No microbleeds	59/3867	1.00 (Reference)		49/3867	1.00 (Reference)		5/3867	1.00 (Reference)	
Any microbleeds	34/892	1.79 (1.16-2.78)	0.010	23/892	1.40 (0.84-2.34)	0.213	6/892	5.41 (1.58-18.46)	0.008
Non-CAA related microbleeds	18/259	2.92 (1.69-5.04)	<0.001	14/259	2.60 (1.41-4.80)	0.003	2/259	5.77 (1.01-32.80)	0.051
CAA related microbleeds	16/633	1.24 (0.71-2.19)	0.469	9/633	0.80 (0.39-1.65)	0.535	4/633	5.25 (1.37-20.17)	0.017
Model 4									
No microbleeds	55/3812	1.00 (Reference)		45/3812	1.00 (Reference)		5/3812	1.00 (Reference)	
Any microbleeds	34/875	1.68 (1.07-2.65)	0.025	23/875	1.28 (0.75-2.17)	0.371	6/875	4.64 (1.33-16.19)	0.017
Non-CAA related microbleeds	18/254	2.34 (1.31-4.19)	0.004	14/254	2.06 (1.07-3.95)	0.031	2/254	3.38 (0.53-21.54)	0.196
CAA related microbleeds	16/621	1.28 (0.72-2.28)	0.399	9/621	0.82 (0.39-1.70)	0.587	4/621	4.88 (1.27-18.83)	0.023

Values represent estimated hazard ratios with 95% confidence interval for any incident stroke (recurrent or first-ever) in participants with microbleeds compared to those without microbleeds. "CAA related" microbleeds included strictly lobar or lobar with cerebellar microbleeds. "Non-CAA related" microbleeds included microbleeds in all other brain locations.

Model 1: adjusted for age-squared, sex, and Rotterdam Study subcohort
Model 2: as model 1, additionally adjusted for APOE ε4 carriership
*Model 3: as model 1, additionally adjusted for propensity score**
Model 4: as model 1, additionally adjusted for lacunes, white matter lesion volume, and intracranial volume.
**Propensity score included: hypertension, total and high-density lipoprotein cholesterol, smoking, diabetes mellitus, lipid-lowering medication, and antithrombotic medication use.*
Abbreviation: n/N= number of people with stroke per exposure category/ total number of participants within the exposure category.

Table 3. *Cerebral microbleeds and the risk of first-ever stroke*

	All strokes			Ischemic stroke			Intracerebral hemorrhage		
	n/N		***P* value**	n/N		***P* value**	n/N		***P* value**
Model 1									
No microbleeds	56/3864	1.00 (Reference)		47/3864	1.00 (Reference)		5/3864	1.00 (Reference)	
Any microbleeds	29/887	1.71 (1.08-2.73)	0.026	19/887	1.28 (0.74-2.22)	0.395	6/887	5.68 (1.68-19.27)	0.006
Non-CAA related microbleeds	15/256	2.89 (1.61-5.20)	<0.001	11/256	2.45 (1.25-4.81)	0.011	2/256	5.98 (1.08-33.16)	0.044
CAA related microbleeds	14/631	1.20 (0.66-2.18)	0.570	8/631	0.78 (0.36-1.67)	0.777	4/631	5.32 (1.39-20.37)	0.016

Values represent estimated age-squared, sex, and Rotterdam Study subcohort adjusted hazard ratios with 95% confidence interval for incident first-ever stroke in participants with microbleeds compared to those without microbleeds.
"CAA related" microbleeds included strictly lobar or lobar with cerebellar microbleeds. "Non-CAA related" microbleeds included microbleeds in all other brain locations.
Abbreviation: n/N= number of people with stroke per exposure category/ total number of participants within exposure category.

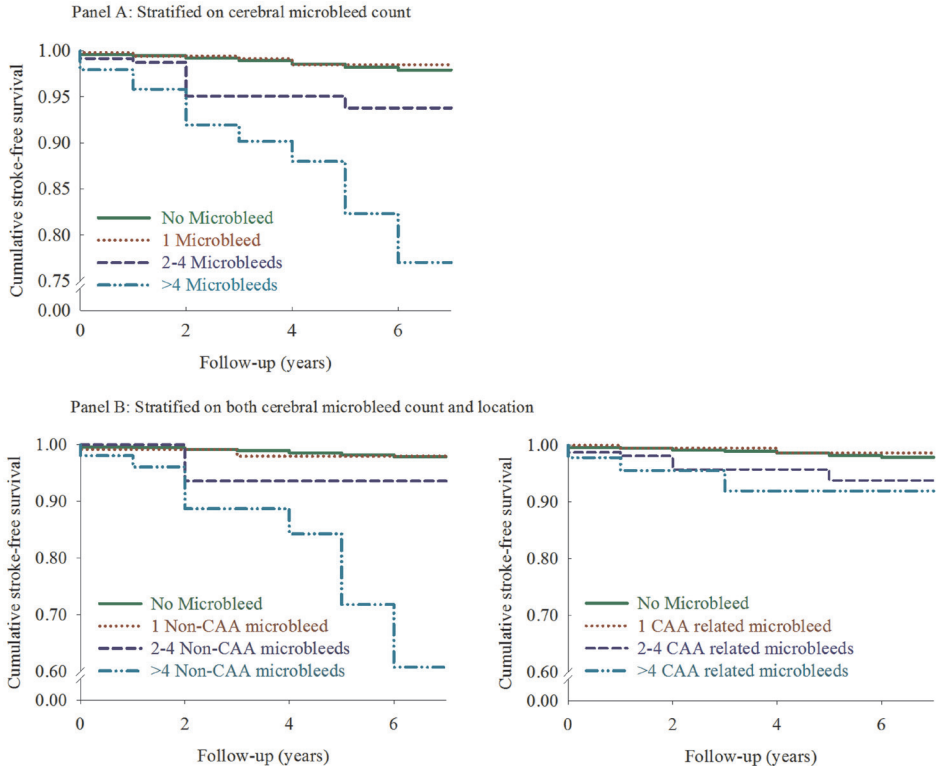

Figure 1. *Kaplan-Meier stroke-free survival curves.*

Panel A of Figure 1 shows the crude cumulative stroke-free survival curves for participants without cerebral microbleeds and with increasing microbleed count over the first 5 years of follow-up. P values for the stroke-free survival curves with 1,2-4, >4 microbleeds compared to no microbleeds were respectively: 0.708; <0.001, <0.001.

Panel B of Figure 1 shows the crude cumulative stroke-free survival curves after additional stratification on cerebral microbleed location. The left graph represents the stroke-free survival curves for participants without cerebral microbleeds and with increasing count of non-CAA related microbleeds over the first 5 years of follow-up. Events per strata/ total number per strata were: no microbleeds: 56 events/3988; 1 microbleed: 2 events/132; 2-4 microbleeds: 4 events/89; >4 microbleeds: 9 events/68. P values for the stroke-free survival curves with 1,2-4,>4 non-CAA related microbleeds compared to no microbleeds were respectively: 0.750; 0.030; <0.001. The right graph represents the stroke-free survival curves for participants without cerebral microbleeds and with increasing count of CAA related microbleeds over the first 5 years of follow-up. Events per strata/ total number per strata were: no microbleeds: 56 events/3988; 1 microbleed: 4 events/445; 2-4 microbleeds: 7 events/167; >4 microbleeds: 3 events/56. P values for the stroke-free survival curves with 1,2-4,>4 CAA related microbleeds compared to no microbleeds were respectively: 0.547; 0.001; 0.001.

"CAA related" microbleeds included strictly lobar or lobar with cerebellar microbleeds. "Non-CAA related" microbleeds included microbleeds in all other brain locations.

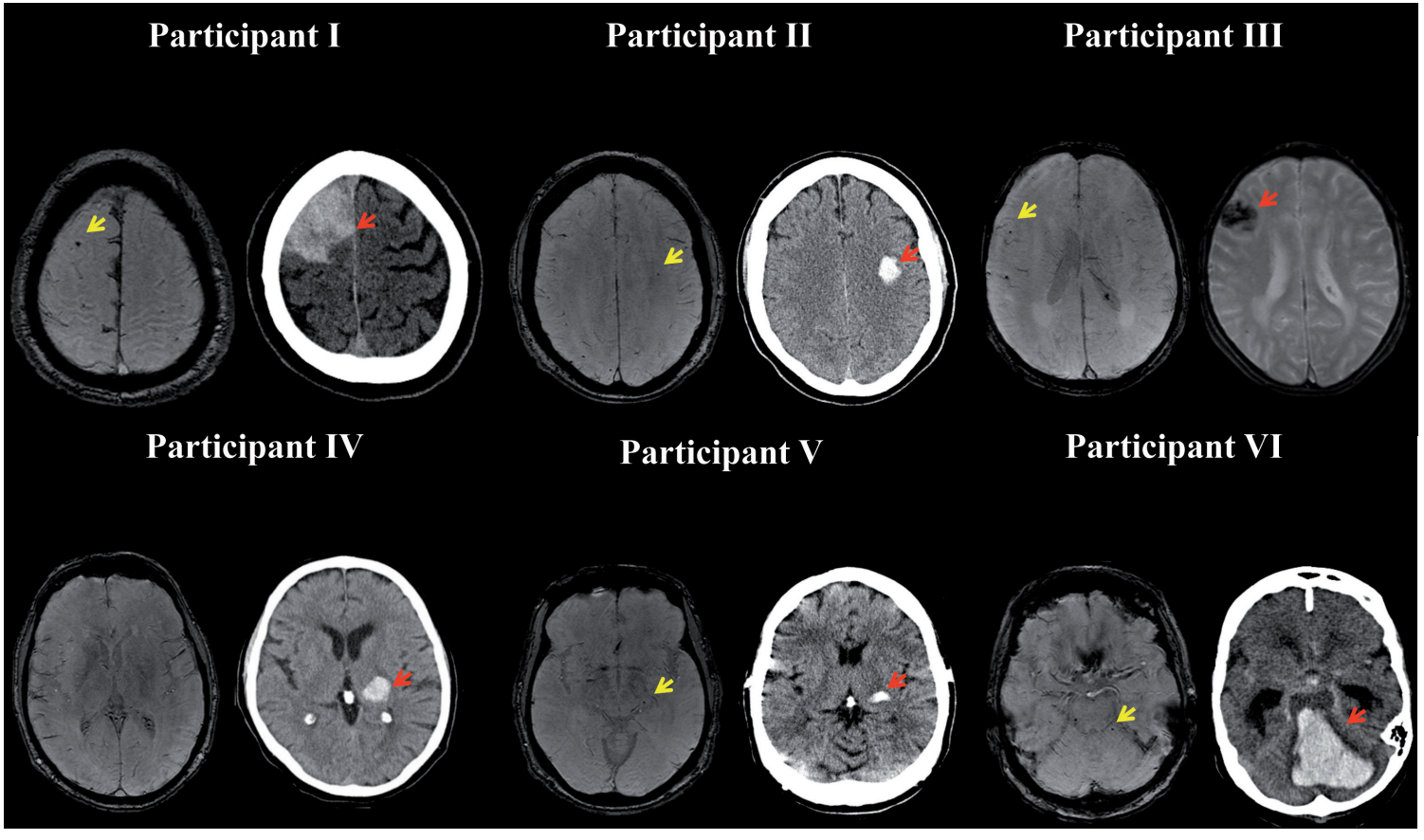

Figure 2. *Co-localization of baseline cerebral microbleeds and subsequent intracerebral hemorrhages.*
For each participant: left image represents baseline research brain MR image co-registered to the hospital CT or MR image (right) which shows an acute intracerebral hemorrhage (red arrow). The yellow arrow represents a microbleed on T2-weighted MRI that preceded the intracerebral hemorrhage.*

Discussion

In the population-based Rotterdam Study we investigated whether microbleed presence on MRI is a determinant of future stroke. We found that the presence of microbleeds, and especially multiple microbleeds, was associated with an increased risk of stroke, including first-ever stroke. Cerebral microbleeds located in regions not typically affected by CAA were associated with an increased risk of both ischemic stroke and intracerebral hemorrhage, whereas microbleeds located in regions where CAA is known to be highly prevalent seemed to particularly associate with an increased risk of intracerebral hemorrhage.

In a population of community-dwelling middle-aged people and elderly we found that microbleeds on MRI associated with an increased risk of both ischemic stroke and intracerebral hemorrhage. The latter has been demonstrated previously in patients with lobar intracerebral hemorrhages due to CAA. These patients were at increased risk of recurrent stroke when microbleeds were present on MRI.[9,17] Evidence that microbleeds also associate with ischemic or occlusive brain disease was found in a recent meta-analysis of clinical studies.[29] They showed that Caucasian patients with microbleeds who were admitted for acute ischemic stroke or transient neurological attack had a 3.87 times (95% CI 0.91-16.4) higher odds of spontaneous intracerebral hemorrhage, and 2.23 times (95 % CI 1.29-3.85) higher odds of ischemic stroke compared to patients without microbleeds. We now showed that these results can be extrapolated to Caucasian community-dwelling persons without a history of stroke.

We found that the risk of stroke subtype differed according to the location of microbleeds in the brain, and thus possibly differs with underlying vasculopathy. Our results suggest that microbleeds, regardless of their presumed underlying pathology (either CAA or hypertensive arteriopathy) strongly associate with intracerebral hemorrhage. Only microbleeds in non-lobar brain regions (suspected of hypertensive arteriopathy) were related to ischemic stroke. It may be that in the general population CAA-related microbleeds do not relate to overt ischemic stroke or that our sample of ischemic stroke cases was too small to detect a significant association. The latter may be more accurate given prior findings from other studies, which suggest that CAA related microbleeds associate with silent and overt ischemic brain lesions.[5,29]

We also observed that intracerebral hemorrhages were predisposed to occur in the same anatomical location as pre-existing microbleeds. Although we previously showed that microbleeds indicate the presence of more widespread small vessel disease,[28] this finding implies that microbleeds may be of value in pinpointing focal areas in the brain with more active vasculopathy. It should be noted,

however, that the number of ICH cases was small and hampered our ability to conduct relevant statistical analysis. Therefore, the overlap in anatomic location between microbleeds and ICH may also be due to chance.

Only two previous studies, both in Asian populations, investigated the association of microbleeds with stroke in elderly without a history of cerebrovascular events.[19,20] One study found an increased risk of ischemic stroke in participants with any microbleeds compared with no microbleeds.[19] The other study found that deep or mixed (i.e., both in deep and lobar brain regions) microbleeds was associated with an increased risk of ischemic stroke and deep intracerebral hemorrhage.[20] Methodological limitations of both studies included small number of stroke cases, limited or no correction for potential confounders, and absence of subgroup analysis for microbleed location. Compared to our study, both studies had an overrepresentation of intracerebral hemorrhage cases compared with ischemic stroke cases, which could be explained by ethnic differences in study population as Asians on average have a two-times higher risk of intracerebral hemorrhages compared with Caucasians.[29,30] In addition, blood vessels in Asian intracerebral hemorrhage patients may more often be affected by fibrohyalinosis rather than β-amyloid deposition, reflecting differences in cardiovascular risk for Asians and Caucasians.[31]

In line with another clinical study,[9] our results suggest a dose-response effect, with higher risk of stroke in people with multiple microbleeds on MRI. We should, however, consider that small vessel pathology progresses gradually and cut-off points are not easily chosen, especially since microbleed detection – and the perceived number – strongly depends on technical imaging parameters.[32] Also, the presence of microbleeds (including just a single microbleed) has been associated with an increased risk of new bleeds, and to relate to more diffuse brain damage, i.e., to white matter.[28,33] Although microbleed burden may thus be used as a severity measure, we note that their presence in itself seems to indicate diffuse vascular brain disease which is progressive in nature.

The use of antithrombotic drugs in people with microbleeds on MRI remains a topic of debate, and is fueled by studies like ours that show that microbleed presence is associated with an increased risk of both ischemic stroke and intracerebral hemorrhage. Our current results suggest that stroke risk associated with microbleeds was not affected by antithrombotic drugs use. However, we were unable to investigate this association separately for intracerebral hemorrhage due to the small number of cases. Results from ongoing clinical trials and cost-benefit studies should settle whether the benefits of ischemic stroke prevention by use of antithrombotic drugs outweigh the risk of intracerebral hemorrhage in people with microbleeds.

Strengths of our study include the population-based character, the longitudinal design, and a systematic stroke detection protocol that allows for thorough collection of fatal and non-fatal stroke events, both in-hospital and outside. Some potential limitations have to be mentioned. First, participants who were eligible and underwent brain MRI scanning were on average younger and had lower cardiovascular risk than those who were eligible for MRI but did not participate.[33] Also, excluding participants with incomplete follow-up for incident stroke may have been a source of bias, as these participants might have been less healthy on average. This potentially induced selection bias in our study and may have led to an underestimation of associations. Second, in accordance with the incidence of intracerebral hemorrhage in the general population the number of intracerebral hemorrhage cases during follow-up in our study was small, and hampered our ability to conduct in depth statistics. Third, microbleeds were rated by trained research physicians. Since microbleed detection depends on rater expertise, our results may not translate directly to routine clinical practice. Fourth, the categorization into "CAA related" and "non-CAA related" microbleeds, though in line with current research and clinical practice, is artificial and does not accurately reflect the multifactorial nature of microbleeds, in whatever location. Therefore, this categorization will inherently have led to misclassification between categories. Nevertheless, it is important to note that the most robust results were found for any versus no microbleeds. In addition, the prevalence of CAA-related microbleeds may have been diluted because we included relatively young participants in our study. Fifth, residual confounding due to unmeasured confounders, e.g., atrial fibrillation, may have affected our results to some extent."

In conclusion, in the general population, microbleed presence on MRI was associated with an increased risk of stroke, both ischemic and hemorrhagic. The risk differs for the subtypes of stroke depending on the location of the cerebral microbleeds. Those with the largest microbleed burden are at highest risk of stroke. Microbleeds may thus present a subclinical precursor of stroke. Future studies should investigate whether microbleed presence contributes to stroke risk on an individual level.

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Supplementary Table 1. *Cerebral microbleeds and the risk of first-ever stroke stratified on use of antithrombotic drugs*

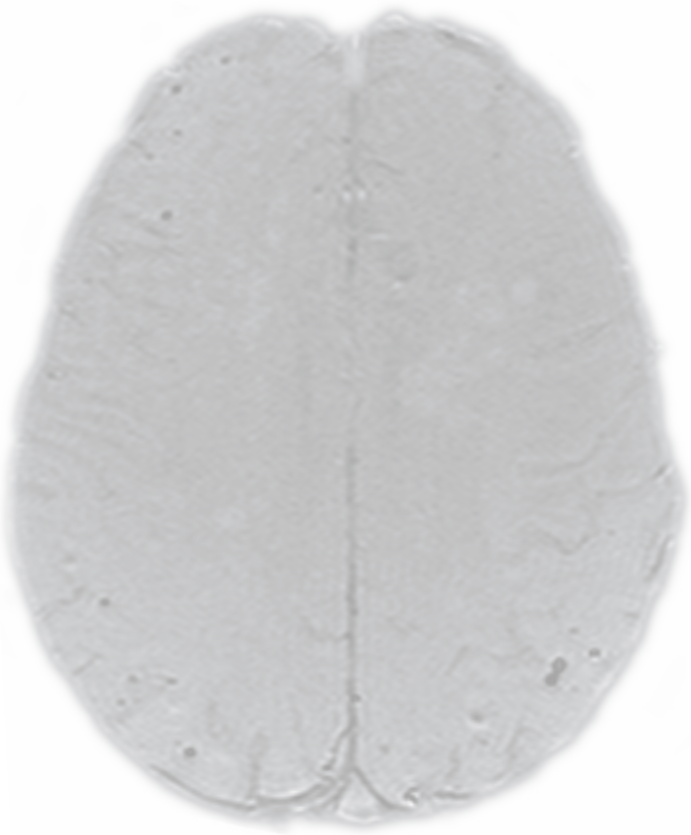
	Non-users of antithrombotic drugs (N=3327)		Users of antithrombotic drugs (N=1424)	
	n/N	All strokes	n/N	All strokes
No microbleeds	30/2818	1.00 (Reference)	26/1046	1.00 (Reference)
Any microbleeds	12/509	1.69 (0.84-3.39)	17/387	1.64 (0.88-3.07)
Non-CAA microbleeds	5/125	2.42 (0.91-6.47)	10/131	2.95 (1.41-6.19)
CAA related microbleeds	7/384	1.41 (0.60-3.28)	7/247	1.02 (0.44-2.39)

Values represent estimated age-squared, sex, and Rotterdam Study subcohort adjusted hazard ratios with 95% confidence interval for incident first-ever stroke in participants with microbleeds compared to those without microbleeds, stratified by antithrombotic drug use (ATC code B01A). Antithrombotic drug use was assessed between baseline MRI and stroke event, death or January 1st 2013. Complete case analysis.

"CAA related" microbleeds included strictly lobar or lobar with cerebellar microbleeds. "Non-CAA related" microbleeds included microbleeds in all other brain locations.

Abbreviation: n/N= number of people with stroke per exposure category/ total number of participants within exposure category.

Formal interaction test for all strokes: any microbleeds P value=0.779, non-CAA related P value=0.946, CAA related P value=0.518.



4.3

Cerebral microbleeds and the risk of dementia

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Abstract

*Background***:** Cerebral microbleeds are hypothesized downstream markers of brain damage caused by both vascular and amyloid pathological mechanisms. We sought to determine whether microbleeds, and more specifically microbleed count and location, associate with an increased risk of cognitive impairment and dementia in the general population.

Methods: In the population-based Rotterdam Study, we assessed presence, number, and location of microbleeds at baseline (2005-2011) on brain MRI of 4,841 participants aged ≥45 years. Participants underwent neuropsychological testing at two time points on average 5.9 years (SD 0.6) apart, and were followed for incident dementia throughout the study period until 2013. The association of microbleeds with cognitive decline and dementia was studied using multiple linear regression, linear mixed effects modeling, and Cox proportional hazards.

Results: Microbleed prevalence was 15.3% (median count 1 [1-88]). A higher count of microbleeds associated with cognitive decline. Microbleeds in brain regions suggestive of cerebral amyloid angiopathy were associated with decline in executive functions, information processing, and memory function, whereas microbleeds in other brain regions were associated with decline in information processing and motor speed. After mean follow-up of 4.8 years (SD 1.4), 72 people developed dementia, of whom 53 Alzheimer's disease. Presence of microbleeds was associated with an increased risk of dementia (age, sex, education adjusted HR 2.02, 95%CI 1.25;3.24), including Alzheimer's dementia (HR 2.10, 95%CI 1.21;3.64).

Conclusions: In the general population, a high microbleed count associated with an increased risk of cognitive deterioration and dementia. Microbleeds thus mark the presence of diffuse vascular and neurodegenerative brain damage.

Introduction

With increasing life expectancy, societies are facing a major public health challenge as the number of people living with cognitive impairments and dementia are growing steadily. There is a growing need to identify early etiological markers of cognitive impairment and dementia, since timely implementation of preventive strategies is key to positively influence the disease course. Accumulating evidence suggests that vascular pathology has a central role in cognitive deterioration.[1] Studies that have investigated pathological changes of cerebral small vessels emphasized a potential role for these vessels in the pathogenesis of cognitive impairment and dementia.[2-4] Arteriosclerosis and amyloid angiopathy are leading causes of cerebral small vessel disease. Consequences of small vessel disease on brain parenchyma can be visualized by neuroimaging. These lesions can either be ischemic (lacunes, white matter lesions) or hemorrhagic (cerebral microbleeds). While the underlying pathogenic cascade of lacunes and white matter lesions mainly revolves around vascular risk factors (i.e., chronic hypertension, smoking, diabetes),[5] microbleed pathogenesis involves vessel wall damage due to both vascular risk factors and accumulation of β-amyloid.[6] As such, it has been suggested that microbleeds may help explain the overlap between cerebrovascular and neurodegenerative pathology in cognitive dysfunction and dementia.

While microbleeds do not appear to affect the rate of cognitive decline in patients with Alzheimer's disease,[7] it remains unclear whether microbleeds play a role in cognitive deterioration in non-cognitively impaired individuals. This is mainly due to the lack of longitudinal data and the heterogeneity of cognitive tests used in previous studies. Thus far, cross-sectional studies in the general population showed that a high microbleed count associated with lower scores on MMSE and on tests sensitive to executive function, processing speed, and motor function.[8-11] Studies in patients with cerebrovascular disease report inconsistent results, with some reporting only associations between microbleeds and global cognition, and others also between microbleeds and specific cognitive domains.[12-15] To date, it also remains unclear whether community-dwelling elderly with microbleeds are at increased risk of dementia, and more particularly Alzheimer's disease. Should microbleeds relate to Alzheimer's dementia, it would highlight the role of vascular pathology in the etiology of the disease and build a bridge between the vascular and amyloid hypothesis.

In the prospective population-based Rotterdam Study, we studied whether presence, number and location of microbleeds marks decline of cognitive functioning, and associates with an increased risk of dementia.

Methods

Study Population

This study was conducted in the prospective population-based Rotterdam Study.[16] After its start in 1990, a total of 7,983 people were included in the initial study wave. In 1999, the cohort was expanded with 3,011 participants, and in 2006 again with 3,932 participants. The total of 14,926 participants enrolled were invited to undergo home interviews and various physical and laboratory examination at the research center every 4 years. Of these, 5,074 (88.5% of invitees) non-demented participants without MRI contraindication underwent brain MRI between 2005 and 2011 (considered as baseline for this study) for the assessment of microbleeds.[17] We excluded participants if scans were incomplete or of inadequate quality (n=129). In addition, we excluded 56 participants with insufficient screening for dementia, and 48 participants for whom follow-up for incident dementia ended before date of MRI due to the absence of automatic linkage between the general practitioners office and our study database. In total, 4,841 participants were included in the dementia analysis. Of these, 3,257 participants (without prevalent or incident dementia) underwent both baseline and follow-up cognitive testing, and were included in the analysis of cognitive decline. Baseline cognition was assessed during the research visit closest to MRI date (2002-2008) and reassessed at a subsequent visit (2009-2014). Follow-up cognitive tests were unavailable for participants who underwent baseline brain MRI between 2009 and 2011. The Rotterdam Study has been approved by the Medical Ethics Committee of the Erasmus MC and by the Ministry of Health, Welfare and Sport of the Netherlands, implementing the "Wet Bevolkingsonderzoek: ERGO (Population Studies Act: Rotterdam Study)". All participants provided written informed consent to participate in the study and to obtain information from their treating physicians.

Brain MRI and Markers of Small Vessel Disease

Participants were scanned on a 1.5-Tesla MRI scanner (GE Healthcare Milwaukee, WI) using a multisequence protocol consistent of T1-weighted, proton density weighted, fluid-attenuated inversion recovery (FLAIR), and T2*-weighted sequences.[17] Trained research physicians, blinded to clinical data, reviewed the MR images. Cerebral microbleeds were defined as small, round to ovoid areas of focal signal loss on T2*-weighted images. Intra-observer (k=0.87) and inter-observer agreement (k=0.85) were good.[6] Infarcts were defined as focal lesions with the same signal intensity as cerebrospinal fluid on all sequences. Infarcts ≥3 and <15mm in size were classified as lacunes, infarcts ≥15 mm as

subcortical infarcts, and infarcts involving cortical gray matter as cortical infarcts. Brain tissue was segmented into gray matter, white matter, and cerebrospinal fluid using automated post-processing tools that included conventional k-nearest-neighbor brain tissue classifier extended with white matter lesion segmentation.[18] Intracranial volume was defined the sum of cerebrospinal fluid, gray matter white matter, and white matter lesion.

Assessment of Cognitive Functioning

The neuropsychological test battery comprised the Mini-Mental State Examination (MMSE), letter-digit-substitution task (LDST), word fluency test (WFT), Stroop test (consisting of reading, color-naming, and interference subtask), 15-word verbal learning test (15-WLT) and Perdue Pegboard test.[19] We computed compound scores for global cognition (average Z-score of the Stroop interference subtask, LDST, WFT, delayed recall of the 15-WLT, and Perdue Pegboard), executive functioning (average Z-score of Stroop interference subtask, LDST, WFT), information-processing speed (average Z-score of Stroop reading and color-naming subtask, and LDST), memory (average Z-score of immediate and delayed recall of the 15-WLT), and motor speed (average Z-score of Perdue Pegboard).

Assessment of Dementia

A three-step protocol was used to screen for prevalent and incident dementia. All participants underwent the MMSE and the Geriatric Mental Schedule (GMS) organic level. Those who screened positive on either test, MMSE <26 or GMS organic level >0, also underwent an examination and informant interview with the Cambridge Examination for Mental Disorders in the Elderly. Those allegedly suffering from dementia underwent further neuropsychological testing if necessary. In addition, all participants were continuously monitored for dementia by linking the study database to digitized medical records from general practitioners and the Regional Institute for Outpatient Mental Health Care. If available, clinical neuroimages were used in the diagnostic process. The final diagnosis was made in accordance with international criteria and determined by a consensus panel led by a neurologist.[20,21] Follow-up for incident dementia was complete until January 1st, 2013, for 23177 (98.5%) of potential person-years.

Assessment of Covariates

Covariates were assessed during the same visit in which baseline cognition was tested. Blood pressure was measured in two readings using a random zero

sphygmomanometer in sitting position, and both measures were averaged. Hypertension was defined as a systolic blood pressure of ≥140 mmHg, or diastolic blood pressure of ≥90 mmHg, or the use of blood pressure-lowering medication. Serum total and high-density lipoprotein cholesterol were measured using an automated enzymatic procedure. Smoking behavior was classified as "ever" versus "never" smoked. People were considered diabetic when fasting blood glucose levels were ≥7.0 mmol/L or when they used glucose-lowering medication. Medication use (glucose-lowering, blood pressure-lowering, and lipid-lowering medication) and education level was assessed during home visits by standardized interviews. *APOE* genotyping was performed on coded genomic DNA samples. Distribution of *APOE* genotype and allele frequencies in this population were in Hardy–Weinberg equilibrium.

Statistical Analysis

We investigated the association of microbleed presence, location, and number with cognitive decline and dementia, using people without microbleeds as a reference group. Microbleeds were classified according to their presumed underlying etiology as microbleeds located in brain regions suggestive of cerebral amyloid angiopathy ("CAA-related", lobar brain regions with or without cerebellar microbleeds), and other brain regions ("non-CAA-related" microbleeds). In addition, we classified every microbleed according to its topographic distribution in the brain (frontal, temporal, parietal, and occipital lobe, infratentorial, and deep).

We first used multiple linear regression to investigate the association of microbleeds with cognitive decline. We examined microbleeds (as yes versus no and as pre-defined categories of 0,1,2-4,>4) in relation to individual neuropsychological tests and afterwards with specific cognitive domains. Z-scores of baseline and follow-up cognitive tests were calculated for each participant. Decline in cognitive scores was studied by using cognitive scores at follow-up as dependent variable and adjusting for baseline test scores in the linear regression models.

Second, we used linear mixed models with added random effects to determine the relationship between microbleed count per topographic distribution in the brain and cognitive decline in specific domains.

Third, Cox proportional hazards were used to study the relation between microbleeds and dementia, including Alzheimer's dementia.

All analyses were adjusted for age, sex, and education. Regression models that

studied microbleeds with decline in cognitive domains were additionally adjusted for *APOE* ε4, a propensity score of cardiovascular risk (hypertension, total and HDL cholesterol, smoking status, diabetes mellitus, lipid-lowering and antithrombotic medication use), intracranial volume and other imaging markers of cerebral small vessel disease (lacunes and white matter lesions). Logistic regression was used to compute propensity scores for cardiovascular risk. Here, microbleed status (yes versus no) was defined the dependent variable and the above-mentioned cardiovascular risk factors were considered independent covariates. The estimated propensity score was the derived predicted value of the equation.

Participants with unreliable segmentations of white matter lesions volume (n=127) were excluded in the analysis involving white matter lesion volume.

Results

Microbleeds and Cognitive Decline

In total, 3,257 participants (mean age 59.6 years (SD 7.8), 54.7% women) without prevalent or incident dementia underwent baseline and repeat cognitive testing on average 5.9 years apart (Table 1). The prevalence of CAA related and non-CAA related microbleeds was respectively 10.9% and 3.8%. The topographic distribution of cerebral microbleeds was as follows: 5.3 % had at least 1 microbleeds in frontal lobe, 5.5% in temporal lobe, 5.1% in parietal lobe, 3.4% in occipital lobe, 3.2% in infratentorial regions, and 3.5% in deep hemispheric regions.

Compared with no microbleeds, the presence of any microbleeds did not associate with decline in cognition. We did, however, observe that the presence of more than 4 microbleeds was associated with worse performance on LDST, WFT, Stroop reading and naming, immediate WLT, and Perdue Pegboard neuropsychological testing during follow-up. Furthermore, presence of multiple CAA related microbleeds specifically associated with worse performance on WFT, Stroop reading and naming, and immediate WLT. Presence of multiple non-CAA-related microbleeds associated with worse performance on Perdue Pegboard (Table 2). In accordance, CAA related microbleeds were the strongest determinant for decline in information processing speed, whereas non-CAA related microbleeds were most strongly associated with decline in motor speed (Figure). Adjusting for *APOE* ε4, cardiovascular risk factors, and other imaging markers of cerebral small vessel disease weakened these associations (Supplementary Table 1).

Table 1. *Characteristics of the study population*

	Cognitive decline analysis N=3257	Incident dementia analysis N=4841
Age, years	59.6 (7.8)	63.8 (10.9)
Women	1782 (54.7)	2663 (55.0)
Education level		
Primary education	244 (7.5)	436 (9.0)
Lower/intermediate general education	1188 (36.5)	1792 (37.0)
Intermediate vocational education	960 (29.5)	1460 (30.2)
Higher vocational education	838 (25.7)	1115 (23.0)
Hypertension	1743 (53.5)	2982 (61.6)
Total cholesterol, mmol/L	5.6 (1.0)	5.5 (1.0)
High-density lipoprotein cholesterol, mmol/L	1.4 (0.4)	1.4 (0.4)
Smoking	2251 (69.1)	3384 (69.9)
Diabetes mellitus	247 (7.6)	428 (8.8)
APOE ε4 carriers	876 (26.9)	1117 (23.1)
Lipid-lowering medication	693 (21.3)	1187 (24.5)
Antithrombotic medication	663 (20.4)	1370 (28.3)
Lacunes	149 (4.6)	356 (7.4)
Intracranial volume, mL	1126.4 (119.9)	1124.3 (121.3)
White matter lesions volume, mL (median [IQR])*	2.3 (1.4;4.1)	2.9 (1.6;6.0)

*Values represent mean (standard deviation) for continuous variables and number (percentage) for categorical variables. The following variables had missing values: education level (n=27), hypertension (n=22), total cholesterol (n=29), HDL cholesterol (n=31), smoking (n=13), diabetes mellitus (n=51), APOE genotype (n=202), lipid-lowering medication (n=27). *Calculated in 3,130 participants with reliable white matter lesion volume segmentations.*

Regarding the topographic distribution of cerebral microbleeds, microbleeds in distinct anatomical brain regions associated non-specifically with decline in various cognitive domains (Table 3).

Microbleeds and Dementia

Follow-up for dementia was complete in 4,841 participants (50.0% women, mean age 63.8 years) (Table 1). During a mean follow-up of 4.8 years (SD 1.4), 72 participants developed dementia, of whom 53 Alzheimer's dementia. The presence of microbleeds, both CAA related and non-CAA related, was associated with an increased risk of dementia (age, sex, and education adjusted HR for dementia in people with any microbleeds 2.02, 95% CI 1.25;3.24) (Table 4). Strong associations remained after censoring for stroke (HR 1.70, 95% CI 1.00;2.87). CAA related and non-CAA related microbleeds associated with an increased risk of Alzheimer's dementia in the same magnitude as that of non-Alzheimer's dementia (Table 4).

Table 2. *Cerebral microbleeds and cognitive decline*

	Nr.	LDST	WFT	Stroop reading	Stroop naming	Stroop interference	WLT immediate	WLT delayed	WLT recognition	Perdue Pegboard
No microbleeds	2780	Reference	Reference	Reference	Reference	Reference	Reference	Reference	Reference	Reference
Microbleed, any	477	-0.05 (-0.12;0.02)	0.02 (-0.06;0.11)	0.01 (-0.08;010)	-0.03 (-0.10;0.04)	-0.07 (-0.14;0.00)	-0.01 (-0.09;0.08)	0.04 (-0.07;1.14)	0.03 (-0.07;0.14)	-0.03 (-0.13;0.06)
Single	326	-0.02 (-0.09;0.06)	0.04 (-0.06;0.14)	0.06 (-0.04;0.17)	-0.02 (-0.10;0.06)	-0.07 (-0.15;0.01)	0.0004 (-0.10;0.10)	0.08 (-0.04;0.20)	0.04 (-0.10;0.17)	-0.01 (-0.12;0.11)
2-4	107	-0.05 (-0.18;0.09)	0.09 (-0.08;0.26)	0.07 (-0.11;0.25)	0.05 (-0.08;0.19)	-0.02 (-0.16;0.11)	0.08 (-0.09;0.25)	-0.01 (-0.21;0.20)	-0.09 (-0.31;0.14)	-0.01 (-0.20;0.18)
>4	44	**-0.32 (-0.53;-0.10)**	**-0.35 (-0.62;-0.08)**	**-0.60 (-0.89;-0.31)**	**-0.35 (-0.57;-0.13)**	-0.20 (-0.42;0.02)	**-0.31 (-0.58;-0.03)**	-0.20 (-0.54;0.14)	-0.13 (-0.49;0.24)	**-0.33 (-0.64;-0.03)**
CAA related	354	-0.05 (-0.13;0.03)	0.001 (-0.10;0.10)	-0.01 (-0.11;0.10)	-0.03 (-0.11;0.05)	-0.06 (-0.13;0.02)	-0.01 (-0.11;0.09)	0.04 (-0.08;0.17)	0.06 (-0.06;0.19)	-0.05 (-0.16;0.06)
Single	253	-0.02 (-0.11;0.07)	0.03 (-0.08;0.14)	0.05 (-0.07;0.17)	-0.05 (-0.14;0.04)	-0.08 (-0.17;0.01)	0.02 (-0.10;0.13)	0.10 (-0.04;0.24)	0.09 (-0.06;0.24)	-0.03 (-0.16;0.09)
2-4	78	-0.07 (-0.23;0.08)	0.05 (-0.15;0.24)	0.03 (-0.18;0.23)	0.07 (-0.09;0.23)	0.04 (-0.12;0.20)	-0.01 (-0.21;0.19)	-0.06 (-0.30;0.19)	-0.16 (-0.43;0.11)	-0.11 (-0.32;0.11)
>4	23	**-0.33 (-0.62;-0.03)**	**-0.52 (-0.88;-0.15)**	**-0.87 (-1.26;-0.47)**	**-0.35 (-0.65;-0.05)**	-0.22 (-0.52;0.08)	**-0.38 (-0.76;-0.002)**	-0.34 (-0.81;0.15)	-0.14 (-0.64;0.37)	-0.09 (-0.50;0.33)
Non-CAA related	123	-0.05 (-0.17;0.08)	0.08 (-0.08;0.23)	0.08 (-0.09;0.24)	0.001 (-0.13;0.13)	-0.11 (-0.23;0.01)	0.002 (-0.16;0.16)	0.02 (-0.16;0.20)	-0.05 (-0.23;0.14)	0.04 (-0.14;0.21)
Single	73	0.002 (-0.16;0.16)	0.09 (-0.11;0.29)	0.12 (-0.09;0.33)	0.09 (-0.07;0.25)	-0.05 (-0.20;0.11)	-0.05 (-0.25;0.15)	-0.001 (-0.23;0.22)	-0.15 (-0.40;0.10)	0.10 (-0.13;0.32)
2-4	29	0.01 (-0.25;0.27)	0.21 (-0.11;0.53)	0.19 (-0.16;0.53)	0.0004 (-0.26;0.26)	-0.23 (-0.48;0.03)	0.31 (-0.02;0.63)	0.14 (-0.23;0.51)	0.12 (-0.29;0.53)	0.26 (-0.10;0.63)
>4	21	-0.31 (-0.63;0.01)	-0.18 (-0.57;0.22)	-0.28 (-0.71;0.14)	**-0.35 (-0.66;-0.03)**	-0.19 (-0.50;0.12)	-0.23 (-0.63;0.17)	-0.06 (-0.51;0.39)	-0.11 (-0.61;0.39)	**-0.61 (-1.05;-0.17)**

Values represent mean differences in Z-score of various cognitive tests in those with microbleeds compared to those without microbleeds.
Values are adjusted for age, sex, education level, baseline cognitive tests score, and time between baseline and follow-up visit.
Nr. = number of participants.

Table 3. *Cerebral microbleed count per topographic brain region and decline in specific cognitive domains*

	G-factor	Executive functions	Information processing	Memory	Motor Speed
No microbleeds	Reference	Reference	Reference	Reference	Reference
Frontal microbleeds	-0.06 (-0.07;-0.05)	-0.06 (-0.07;-0.04)	-0.04 (-0.06;-0.03)	-0.03 (-0.05;-0.01)	-0.04 (-0.06;-0.02)
Temporal microbleeds	-0.03 (-0.04;-0.01)	-0.03 (-0.04;-0.02)	-0.01 (-0.02;0.005)	-0.04 (-0.05;-0.02)	-0.01 (-0.03;0.01)
Parietal microbleeds	-0.02 (-0.04;-0.01)	-0.03 (-0.04;-0.01)	-0.02 (-0.03;-0.004)	-0.01 (-0.03;0.01)	-0.01 (-0.03;0.02)
Occipital microbleeds	-0.003 (-0.02;0.01)	-0.002 (-0.02;0.02)	-0.02 (-0.03;-0.001)	-0.04 (-0.06;-0.02)	-0.05 (-0.07;-0.02)
Infratentorial microbleeds	-0.02 (-0.04;0.002)	-0.02 (-0.04;0.002)	-0.01 (-0.03;0.01)	-0.03 (-0.06;-0.01)	-0.002 (-0.03;0.03)
Deep microbleeds	-0.02 (-0.03;-0.002)	-0.03 (-0.05;-0.01)	-0.02 (-0.03;0.001)	-0.004 (-0.03;0.02)	-0.0002 (-0.03;0.03)

Values represent differences in Z-score for decline in specific cognitive domains for microbleed count per topographic brain region.
Values are adjusted for age, sex, and education level.

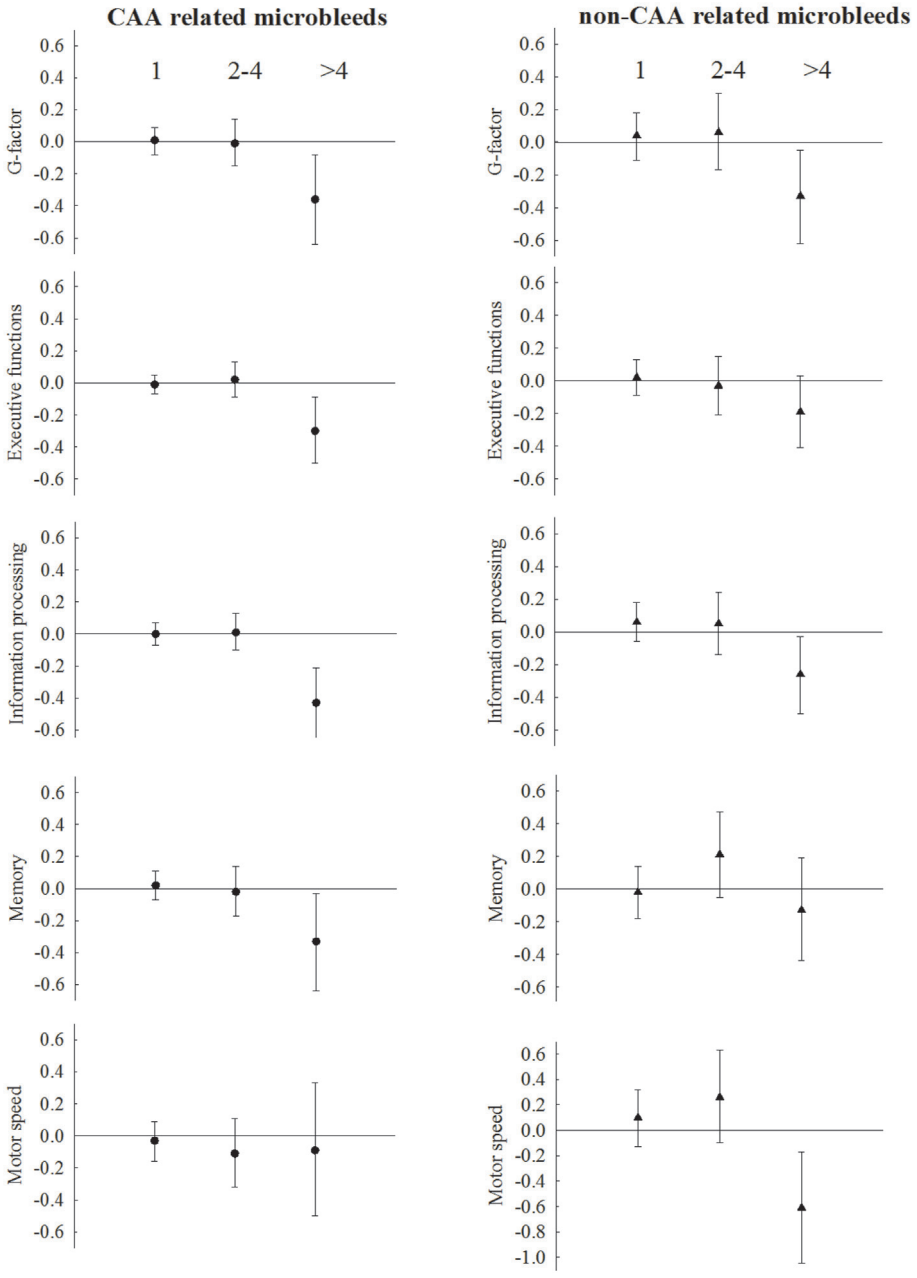

Figure. *Cerebral microbleeds and decline in specific cognitive domains*
The y-axis represents age, sex, and education adjusted Z-scores for decline in specific cognitive domains for categories of CAA related and non-CAA related microbleed count (x-axis), compared with a reference group without cerebral microbleeds. Error bars represent 95% confidence intervals.

Table 4. *Cerebral microbleeds and the risk of dementia*

	Dementia		Alzheimer's dementia	
	n/N	HR (95% CI)	n/N	HR (95% CI)
No microbleeds	39/3911	Reference	28/3911	Reference
Any microbleeds	33/930	2.02 (1.25-3.24)	25/930	2.10 (1.21-3.64)
CAA-related	21/648	1.81 (1.05-3.11)	17/648	2.00 (1.08-3.71)
Non-CAA related	12/282	2.39 (1.23-4.61)	8/282	2.15 (0.97-4.78)

Values represent age, sex, and education adjusted hazard ratios (HR) (95% confidence interval [CI]) for incident dementia in participants with microbleeds compared to those without microbleeds. n/N= number of dementia cases/total number of participants per strata. Complete case analysis.

Discussion

In this population-based study of middle-aged and elderly people we found that a high microbleed count was a determinant of cognitive decline. Also, presence of microbleeds was associated with an increased risk of dementia.

Presence of multiple microbleeds affected cognition in all domains in our population-based study. Previous cross-sectional studies in healthy adults already demonstrated that microbleeds, especially in large numbers, are related to lower MMSE scores, worse information processing, and worse executive functioning.[8-11] The only study that investigated the relationship between microbleeds and cognitive decline longitudinally was performed in patients with the genetic small vessel disease, CADASIL.[22] That study found similar results for decline in global cognition, executive function, and memory, but did not investigate this separately for different microbleed locations. Cross-sectional studies in patients with or at increased risk of cerebrovascular disease reported inconsistent results on the associations of microbleeds with worse performance on global cognition, tests for executive function, tests for memory function, and tests for psychomotor speed.[12-15] In addition, in memory clinic populations microbleeds associated with worse scores on MMSE (as measure for global cognition) and several cognitive domains with exception of language skills.[23,24] although the majority of studies were unable to demonstrate any association.[25-28]

Mechanisms by which microbleeds influence cognitive function remain speculative, and may be either causal or non-causal.[29] Microbleeds located strategically in the brain may cause focal damage to neurological tracts leading to impairment in specific cognitive domains.[14] On the other hand, microbleeds may represent a proxy measure of cerebral vascular pathology at large, and their presence may influence cognition indirectly. The latter hypothesis is supported by our findings as we found associations with multiple microbleeds in widespread

areas, rather than with single or multiple microbleeds clustered in a specific brain region. In addition, microbleeds in non-strategic topographic brain regions associated with impairments in executive functioning, information processing, and memory. Also, these associations were attenuated after adjusting for white matter lesions and lacunes, indicating that these lesions have a shared effect on cognition. Indeed, previous evidence also suggest that these lesions often co-exist, share risk factors, and even that their presence indicates a single pathological continuum.[30-35] Microbleeds may less likely be a sole causal determinant of cognitive deterioration but rather a downstream product of both severe vascular and neurodegenerative pathology.

CAA related microbleeds were associated with decline in distinct cognitive domains when compared with microbleeds in other locations. The association of CAA related microbleeds with memory might partly be explained by the fact that multiple lobar microbleeds had a predilection for the temporal lobes in our study.[36] In turn, non-CAA related microbleeds could strategically affect infratentorial and deep hemispheric brain regions (including basal ganglia and the internal capsule) to influence motor function. However, we did not observe these associations when looking at deep or infratentorial microbleeds separately. It should be noted that participants with non-CAA related microbleeds had more microbleeds and more often mixed microbleeds (i.e., microbleeds in lobar and non-lobar brain regions). Hence, microbleed count per topographic brain regions may be more informative than the categorizations per presumed underlying vasculopathy in assessing cognitive deterioration.

Microbleeds are found in 18-32% of patients with Alzheimer's disease,[37] with most patients exhibiting a predominance for cortical-subcortical microbleeds.[26] In the general population, we found that microbleeds related to an increased risk of dementia, including Alzheimer's dementia. We found strong associations for non-CAA related microbleeds. Our study underscores the role of vascular pathology in the pathogenesis of dementia, including Alzheimer's dementia. The question remains how vascular pathology interacts with amyloid pathology to cause clinical cognitive deterioration and dementia. In principle, the relationship could move in two directions: either vascular amyloid deposition adversely affects reactivity of cerebral microvasculature causing loss of function with ischemic and hemorrhagic damage, or hypertensive damage to small vessels leads to disturbances in amyloid clearance, increasing the amyloid deposits in vessel walls.[37] Accumulating evidence suggests that vascular damage may be of particular importance in the initiation of neurodegenerative disease whereas the influence of β-amyloid becomes more prominent in the clinical disease stage.[38,39]

Strengths of our study include the longitudinal population-based design with large sample size, the use of an extensive neuropsychological test battery, and the virtually complete screening for incident dementia. Some limitations of our study also have to be mentioned. First, we applied multiple statistical tests in our study, increasing the change of type I errors. However, correcting for multiple testing seems inappropriate since cognitive tests/domains were not independent from one another, and microbleeds in different locations are correlated. Second, selection bias may have influenced our results, as healthier people without subjective memory complains were more likely to receive follow-up cognitive testing. This would most likely have biased our results towards the null. Third, the microbleed number rated may not reflect the true biological number since microbleed detection strongly depends on technical imaging methods used. Fourth, the small number of incident dementia cases in our relatively young cohort hampered our ability to control for all potential confounders, and residual confounding may have affected our results. Fifth, we focused on selective cognitive domains (i.e., executive functioning, information processing speed, memory, motor speed), and lacked data to investigate other cognitive domains such as visuospatial processing or visuoperception.

In conclusion, microbleeds are associated with cognitive decline and dementia in the general population. A high microbleed count may represent a proxy for diffuse vascular and neurodegenerative brain damage, which predisposes to progressive cognitive deterioration.

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Supplementary Table 1. *Cerebral microbleeds and decline in specific cognitive domains*

	G-factor	Executive functions	Information processing	Memory	Motor Speed
			Model I		
CAA related	-0.02 (-0.09;0.05)	-0.03(-0.10;0.04)	-0.03 (-0.10;0.04)	-0.02 (-0.11;0.08)	-0.05 (-0.16;0.06)
Single microbleeds	0.0003 (-0.08;0.08)	-0.02 (-0.08;0.05)	-0.001 (-0.07;0.06)	0.01 (-0.08;0.10)	-0.03 (-0.16;0.09)
2-4 microbleeds	-0.01 (-0.15;0.13)	0.02 (-0.09;0.13)	0.01 (-0.11;0.12)	-0.02 (-0.17;0.14)	-0.11 (-0.32;0.11)
>4 microbleeds	**-0.37 (-0.65;-0.09)**	**-0.31 (-0.51;-0.11)**	**-0.44 (-0.65;-0.22)**	**-0.34 (-0.64;-0.03)**	-0.09 (-0.50;0.33)
Non-CAA related	-0.02 (-0.13;0.10)	-0.03 (-0.14;0.08)	0.01 (-0.11;0.13)	0.02 (-0.13;0.17)	0.03 (-0.15;0.20)
Single microbleeds	0.03 (-0.11;0.18)	0.02 (-0.09;0.12)	0.06 (-0.06;0.17)	-0.02 (-0.18;0.14)	0.10 (-0.13;0.32)
2-4 microbleeds	0.06 (-0.17;0.29)	-0.03 (-0.21;0.14)	0.05 (-0.14;0.24)	0.21 (-0.05;0.47)	0.26 (-0.10;0.63)
>4 microbleeds	**-0.34 (-0.62;-0.05)**	-0.20 (-0.41;0.02)	**-0.26 (-0.50;-0.03)**	-0.13 (-0.44;0.19)	**-0.61 (-1.05;-0.17)**
			Model II		
CAA related	-0.02 (-0.09;0.05)	-0.03 (-0.09;0.04)	-0.02 (-0.09;0.05)	-0.02 (-0.11;0.08)	-0.05 (-0.16;0.06)
Single microbleeds	0.002 (-0.08;0.08)	-0.01 (-0.07;0.05)	0.001 (-0.07;0.07)	0.01 (-0.08;0.10)	-0.03 (-0.16;0.09)
2-4 microbleeds	0.002 (-0.14;0.14)	0.03 (-0.08;0.13)	0.02 (-0.09;0.14)	-0.02 (-0.18;0.14)	-0.10 (-0.32;0.12)
>4 microbleeds	**-0.35 (-0.62;-0.07)**	**-0.29 (-0.49;-0.09)**	**-0.41 (-0.63;-0.19)**	**-0.34 (-0.65;-0.04)**	-0.07 (-0.49;0.35)
Non-CAA related	-0.003 (-0.12;0.11)	-0.02 (-0.13;0.09)	0.02 (-0.09;0.14)	0.02 (-0.14;0.17)	0.04 (-0.14;0.22)
Single microbleeds	0.04 (-0.11;0.18)	0.02 (-0.09;0.13)	0.06 (-0.06;0.18)	-0.02 (-0.18;0.14)	0.10 (-0.12;0.32)
2-4 microbleeds	0.08 (-0.15;0.32)	-0.02 (-0.19;0.16)	0.07 (-0.12;0.26)	0.21 (-0.05;0.46)	0.29 (-0.08;0.65)
>4 microbleeds	**-0.29 (-0.57;-0.002)**	-0.16 (-0.37;0.06)	-0.22 (-0.45;0.02)	-0.14 (-0.45;0.18)	**-0.56 (-1.00;-0.11)**
			Model III		
CAA related	-0.02 (-0.09;0.05)	-0.03 (-0.09;0.04)	-0.01 (-0.09;0.06)	-0.02 (-0.12;0.08)	-0.05 (-0.16;0.06)
Single microbleeds	-0.01 (-0.09;0.07)	-0.02 (-0.08;0.04)	0.01 (-0.06;0.07)	0.01 (-0.08;0.10)	-0.04 (-0.16;0.09)
2-4 microbleeds	-0.01 (-0.16;0.13)	0.02 (-0.08;0.13)	0.03 (-0.09;0.14)	-0.03 (-0.19;0.13)	-0.10 (-0.33;0.12)
>4 microbleeds	-0.29 (-0.57;0.002)	**-0.24 (-0.45;-0.03)**	**-0.40 (-0.62;-0.17)**	-0.28 (-0.60;0.04)	-0.01 (-0.43;0.42)

Non-CAA related	0.03 (-0.09;0.15)	0.02 (-0.10;0.13)	0.04 (-0.08;0.16)	0.05 (-0.11;0.21)	0.08 (-0.10;0.27)
Single microbleeds	0.04 (-0.11;0.18)	0.02 (-0.09;0.13)	0.05 (-0.07;0.17)	-0.01 (-0.17;0.15)	0.10 (-0.12;0.32)
2-4 microbleeds	0.10 (-0.14;0.33)	-0.01 (-0.18;0.17)	0.08 (-0.12;0.27)	0.23 (-0.03;0.49)	0.31 (-0.05;0.67)
>4 microbleeds	-0.13 (-0.45;0.20)	0.006 (-0.24;0.25)	-0.17 (-0.44;0.10)	-0.05 (-0.41;0.32)	-0.48 (-0.98;0.03)

Values represent differences in Z-score of various cognitive domains in those with microbleeds compared to those without microbleeds.
Model I: adjusted for age, sex, education level, baseline domain tests scores, and time between baseline and follow-up visit.
Model II: as Model I, additionally adjusted for APOE ε4 carriership and a propensity score of cardiovascular risk factors which included hypertension, total and HDL cholesterol, smoking status, diabetes mellitus, lipid-lowering and antithrombotic medication use.
Model III: as Model I, additionally for lacunes, intracranial volume, white matter lesion volume.
The following neuropsychological tests were included for G-factor: Stroop interference, LDST, WFT, delayed WLT, Perdue Pegboard; for executive functions: Stroop interference, LDST, WFT; for information processing: Stroop reading and naming, LDST; for memory: immediate and delayed WLT; for motor Speed: Perdue Pegboard.

4.4

Determinants, MRI-correlates, and prognosis of mild cognitive impairment

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Abstract

Background: Mild cognitive impairment (MCI) marks a transitional stage between healthy aging and dementia, but the understanding of MCI in the general population remains limited. We investigated determinants, MRI-correlates, and prognosis of MCI within the population-based Rotterdam Study.
Methods: Firstly, we studied age, *APOE* ε4 carriership, waist circumference, hypertension, diabetes mellitus, total and HDL-cholesterol levels, smoking, and stroke as potential determinants of MCI. Determinants were assessed cross-sectionally at baseline (2002–2005) and up to 7 years prior to baseline (1997–2001). Secondly, we compared volumetric, microstructural, and focal MRI-correlates in persons with and without MCI. Thirdly, we followed participants for incident dementia and mortality until 2012. Out of 4,198 participants, 417 had MCI, of whom 163 amnestic and 254 non-amnestic MCI.
Results: At baseline, older age, *APOE* ε4 carriership, lower total cholesterol levels, and stroke were associated with MCI. Additionally, lower HDL-cholesterol levels and smoking were related to MCI when assessed 7 years prior to baseline. Persons with MCI, particularly those with non-amnestic MCI, had larger white matter lesion volumes, worse microstructural integrity of normal-appearing white matter, and a higher prevalence of lacunes, compared to cognitively healthy participants. MCI was associated with an increased risk of dementia (hazard ratio [HR] 3.98, 95% confidence interval [CI] 2.97; 5.33), Alzheimer's disease (HR 4.03, 95% CI 2.92; 5.56), and mortality (HR 1.54, 95% CI 1.28; 1.85).
Conclusions: We found that several vascular risk factors and MRI-correlates of cerebrovascular disease were related to MCI in the general population. Participants with MCI had an increased risk of dementia, including Alzheimer's disease, and mortality.

Introduction

Although the etiology of dementia is largely unknown, it is well established that neuropathology related to dementia slowly accumulates over decades. Consequently, identifying persons at a higher risk of dementia could postpone or even prevent dementia by timely targeting modifiable risk factors.[1] In this light, mild cognitive impairment (MCI) has been identified as the transitional stage between normal aging and dementia.

Thus far, several studies have focused on identifying determinants, magnetic resonance imaging (MRI)-correlates, and prognosis of MCI. Various studies have established the role of amyloid pathology in MCI, but emerging evidence also implicates vascular factors as risk factors for MCI.[2-4] However, findings on determinants, MRI-correlates, and prognosis of MCI vary greatly due to differences in study populations, definitions of MCI, and determinants under investigation.[4-11] Studying MCI in the general population may strengthen previous findings on determinants and prognosis of MCI. More importantly, clinical studies may suffer from referral bias and reverse causality. In the general population, referral bias is less present and investigating determinants years before MCI could overcome the problem of reverse causality.

In the population-based Rotterdam Study, we investigated determinants, MRI-correlates, and prognosis of MCI.

Firstly, we focused on several vascular risk factors that were measured not only cross-sectionally but also up to 7 years prior to diagnosis of MCI. Secondly, we investigated the relation between MCI and volumetric, microstructural, and focal imaging markers. Thirdly, we followed participants over a period of 9 years to determine the risk of incident dementia, Alzheimer's disease, and mortality.

Methods

Setting and Study Population

The Rotterdam Study is a prospective population-based cohort that started in 1990. Inhabitants, aged 55 years and older, of Ommoord, a district of Rotterdam, the Netherlands were invited to participate in the study.[12] Out of 10,215 invited inhabitants, 7,983 (78%) agreed to participate. In 2000, this cohort was extended with 3,011 participants (67% of invitees) who had become 55 years of age or had moved into the district since the start of the study. Every 4 years, participants are re-invited to undergo home interviews and various examinations at the research center.[12]

Between 2002–2005, which was the fourth examination round of the original cohort and the second examination round of the extended cohort, an extensive neuropsychological test battery was implemented in the Rotterdam Study.[12] Given that extensive neuropsychological testing is required to assess MCI, 2002–2005 was set as baseline for MCI screening in our study. Of the 6,061 study participants that underwent examinations between 2002–2005, 192 participants were excluded because they were demented, 67 because they were not sufficiently screened for dementia, and another 250 participants because they did not answer the questions regarding subjective cognitive complaints. An additional 1,354 participants were excluded because they missed one or more cognitive test scores or had unreliable test scores. Eventually, 4,198 persons were eligible to participate in this study. Because MRI was implemented from 2005 onwards,[13] only a random subset of 697 out of 4,198 persons underwent MRI, which was on average 1.01 years (0.46 standard deviation (SD)) after MCI screening. Persons with cortical infarcts were excluded (n=15) as tissue loss and gliosis surrounding cortical infarcts may cause unreliable white matter lesion segmentations. Eventually, 682 participants were included in the analyses of MRI-correlates.

Determinants of MCI and Other Measurements

Determinants of MCI were selected based on biological plausibility and literature on established risk factors of dementia.[14-18] Educational level was assessed at study entry by interview and categorized into seven groups: primary education only or primary education with an unfinished higher education, lower vocational education, lower secondary education, intermediate vocational education, general secondary education, higher vocational education, and university. Since educational level was required for assessment of the MCI diagnosis, we imputed missing values for education (1.8%) based on age and gender. Information on *APOE* genotype was obtained using polymerase chain reaction on coded DNA samples without knowledge of MCI diagnosis. This method has been described in detail previously.[19,20] *APOE* ε4 carrier status was defined as carrier of one or two ε4 alleles. Waist circumference was measured in centimeters at the level midway between the lower rib margin and the iliac crest, with participants in standing position without heavy outer garments and with emptied pockets while breathing out gently. Blood pressure was measured in sitting position on the right arm and calculated as the average of two measurements using a random-zero sphygmomanometer. Hypertension was defined as a blood pressure ≥140/90 mmHg or use of blood pressure lowering medication, prescribed for the indication of hypertension. Diabetes mellitus was defined as a fasting serum glucose level ≥7.0 mmol/L, non-fasting serum glucose level ≥11.1 mmol/L, or use of anti-

diabetic medication. Serum glucose, total cholesterol, and HDL-cholesterol levels were acquired by an automated enzymatic procedure (Boehringer Mannheim System). Smoking habits were assessed by interview and categorized as current, former, and never smoking. At study entry, history of stroke was assessed using home interviews and confirmed by reviewing medical records. After entering the Rotterdam Study, participants were continuously followed-up for stroke through automatic linkage of general practitioner files with the study database. For potential strokes, additional information was collected from hospital, nursing home, and general practitioner records. An experienced neurologist adjudicated the strokes using standardized definitions, as described in detail previously.[21]

Apart from educational level and *APOE* ε4 carrier status, all measurements were assessed at each examination round of the Rotterdam Study. We used the measurements that were assessed at the baseline of this study (2002–2005), and the measurements that were assessed at the previous examination round, which was up to 7 years (mean 4.36 years, SD 0.55) prior to baseline (1997–2001).

Assessment of MCI

MCI was defined using the following criteria: 1) presence of subjective cognitive complaints, 2) presence of objective cognitive impairment and 3) absence of dementia.

Subjective cognitive complaints were evaluated by interview. This interview included three questions on memory (difficulty remembering, forgetting what one had planned to do, and difficulty finding words), and three questions on everyday functioning (difficulty managing finances, problems using a telephone, and difficulty getting dressed). Subjective cognitive complaints were scored positive when a subject answered "yes" to at least one of these questions. We assessed objective cognitive impairment using a cognitive test battery comprising letter-digit substitution task, Stroop test, verbal fluency test, and 15-word verbal learning test based on Rey's recall of words.[22] To obtain more robust measures, we constructed compound scores for various cognitive domains including memory function, information-processing speed, and executive function.[22,23] Briefly, compound score for memory was calculated as the average of Z-scores for the immediate and delayed recall of the 15-word verbal learning test. For information processing speed averaged Z-scores for the Stroop reading and Stroop color-naming sub- task and the letter-digit substitution task were used. Finally, executive function included Z-scores of the Stroop interference subtask, the letter-digit substitution task, and the verbal fluency test. We classified persons as cognitively impaired if they scored below 1.5 SD of the age and education

adjusted means of the study population. We subsequently classified the MCI subtypes amnestic and non-amnestic MCI. Amnestic MCI was defined as persons with MCI who had an impaired test score on memory function (irrespective of other domains). Non-amnestic MCI was defined as persons with MCI having normal memory function, but an impaired test score on executive function or information-processing speed.

Brain MRI and post-processing

We performed a multisequence MRI protocol on a 1.5-Tesla scanner (GE Healthcare). The sequences in the imaging protocol consisted of three high-resolution axial scans, i.e., a T1-weighted sequence (slice thickness 1.6 mm, zero-padded to 0.8), a proton density-weighted sequence (slice thickness 1.6 mm), and a fluid-attenuated inversion recovery (FLAIR) sequence (slice thickness 2.5 mm).[13] For cerebral microbleed detection, we used a custom-made accelerated three-dimensional T2*-weighted gradient-recalled echo (3D T2* GRE (slice thickness 1.6 mm, zero-padded to 0.8)).[24] For diffusion tensor imaging (DTI) scans, we used a 2D acquisition and EPI readout (slice thickness for DTI was 3.5 mm). Maximum b-value was 1000 s/mm^2 in 25 non-collinear directions (number of excitations (NEX)= 1) and one volume was acquired without diffusion weighting (b-value= 0 s/mm^2).

We used automated tissue segmentation, including conventional k-nearest-neighbor brain tissue classifier extended with white matter lesion (WML) segmentation,[25] to segment scans into gray matter volume, white matter volume, WML volume, cerebrospinal fluid, and background. Total brain volume was defined as the sum of total gray matter volume, white matter volume, and WML volume. Hippocampal volume was determined using an automated method, as described extensively before.[26]

The segmentation was brought to the DTI image space using boundary based registration performed on the white matter segmentation,[27] the b= 0 and T1-weighted images.

Diffusion data was pre-processed using a standardized processing pipeline.[28] In short, DTI data was corrected for subject motion and eddy currents by affine co-registration of the diffusion weighted volumes to the b= 0 volume, including correction of gradient vector directions. Diffusion tensors were estimated using a non-linear Levenberg Marquadt estimator, available in ExploreDTI.[29] Global fractional anisotropy (FA) and mean diffusivity (MD), measures of microstructural integrity, were computed from the estimated tensor images over the entire normal- appearing white matter in each subject. The final registration result of

each scan was checked visually for errors.[30,31] Partial volume effects and presence of multiple white matter fiber orientation within a voxel were thus minimized. Sixteen subjects had to be excluded from the white matter microstructural integrity analyses due to scanning artifacts or excessive motion. FA and MD were standardized to echo time (TE) values, because TE was not constant for all participants.

All scans were rated by 1 of 5 trained research-physicians to determine presence of microbleeds and lacunes of presumed vascular origin.[32] Microbleeds were rated as focal areas of signal loss, on 3D T2*Gradient Recalled Echo-weighted by MRI. Lacunes were rated on FLAIR, proton-density-weighted and T1-weighted sequences, and were defined as focal lesions ≥3 mm and <15 mm in size, with the same signal intensity as cerebrospinal fluid on all sequences and a hyperintense rim on the FLAIR (when located supratentorially).[33] Infarcts showing involvement of gray matter were classified as cortical infarcts.

Assessment of Dementia

Participants were screened for dementia at baseline and at follow-up examinations using a three-step protocol. Screening was done using the Mini-Mental State Examination (MMSE) and the Geriatric Mental Schedule (GMS) organic level.[34,35] Screen-positives (MMSE <26 or GMS organic level >0) subsequently underwent an examination and informant interview with the Cambridge Examination for Mental Disorders in the Elderly (CAMDEX).[36] During this interview, more information on functional status and cognitive performance was collected. Participants who were suspected of having dementia underwent extra neuropsychological testing if necessary. Additionally, for persons not visiting the research center, the total cohort was continuously monitored for dementia through computerized linkage of the study database and digitized medical records from general practitioners and the Regional Institute for Outpatient Mental Health Care. When information on neuroimaging was required and available, it was used for decision making on the diagnosis. In the end, a consensus panel, led by a neurologist, decided on the final diagnosis in accordance with standard criteria for dementia (Diagnostic and Statistical Manual of Mental Disorders, Third Edition, Revised (DSM-III-R)) and Alzheimer's Disease (National Institute of Neurological and Communicative Diseases and Stroke/ Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA)).[37,38] Follow-up for incident dementia was complete until January 1st, 2012.

Assessment of Mortality

Deaths were continuously reported through automatic linkage with general practitioner files. In addition, municipal health records were checked bimonthly for information on vital status. Information about cause and circumstances of death was obtained from general practitioner and hospital records.[39] Follow-up for mortality was complete until January 1st, 2012.

Statistical Analysis

Firstly, we examined whether risk factors of dementia were related to MCI using multivariate logistic regression models adjusted for age and gender cross-sectionally. Since vascular risk factors often correlate,[14] we estimated the independent effect of each risk factor by including all risk factors into the same model. Age was included per 5 year increase into the model and waist circumference, total and HDL-cholesterol levels were included per SD increase into the model. Persons with missing values were excluded from these analyses. We investigated whether excluded persons had different characteristics than persons who were included in the analysis using Univariate Analysis of Variance, adjusting for age and gender where appropriate. The same models as in the cross-sectional analysis were used to examine the relation with risk factors assessed up to 7 years prior to MCI diagnosis.

Secondly, we used linear and logistic regression to investigate the relation of MCI with volumetric markers (i.e., total brain volume, hippocampal volume, WML volume), microstructural integrity markers (i.e., mean FA, MD) and focal markers (i.e., cerebral microbleeds, lacunes) of brain pathology on MRI cross-sectionally. Hippocampal volume was studied as the mean of the left and right hippocampal volume. WML was log-transformed due to the skewed distribution. Volumetric and microstructural measures were modeled continuously. Microbleeds and lacunes were dichotomized into present versus absent. These analyses were adjusted for age and gender (model I), and additionally for *APOE* ε4 carriership, waist circumference, hypertension, diabetes mellitus, total and HDL-cholesterol levels, and smoking (model II). In model II we investigated whether irrespective of the presence of vascular risk factors, persons with MCI had more volumetric, microstructural, and focal changes in the brain compared to cognitively healthy participants. Analyses of volumetric and microstructural integrity measures were also adjusted for intracranial volume. In addition we performed a sensitivity analysis for the imaging correlates, excluding participants who became demented in the period between MCI screening and MRI scanning (n=12). Thirdly, we used Cox proportional hazards to study the association

between MCI and risk of dementia, Alzheimer's disease, and mortality longitudinally. These models were adjusted for the same determinants as described in model II but with addition of prevalent stroke and educational level.

All analyses were repeated investigating the amnestic and non-amnestic MCI subtypes separately. Analyses were performed using statistical software package SPSS 20.0, using an α-value of 0.05.

Results

Characteristics of the study population are presented in Table 1. Out of 4,198 participants, 417 (9.9%) had MCI. Of these, 163 had amnestic MCI and 254 had non-amnestic MCI. Missing values for determinants of MCI occurred in 268 participants (6.4%) in 2002–2005 (baseline) and in 615 participants (14.6%) in 1997–2001. Participants excluded from the baseline analyses were more often female, suffered more from hypertension, and had a larger waist circumference than participants included in the analyses. Participants excluded due to missing data in 1997–2001 were also more often female and more often hypertensive but had lower cholesterol levels than participants included in our analyses (Supplementary Table 1).

At baseline, older age (odds ratio (OR) per 5 year increase in age 1.20, 95% confidence interval (CI) 1.11; 1.29), *APOE* ε4 carriership (OR 1.26, 95% CI 1.00; 1.59), lower total cholesterol levels (OR 0.87, 95% CI 0.78; 0.98), and stroke (OR 2.12, 95% CI 1.40; 3.19) were independently related to MCI (Table 2). Male gender and *APOE* ε4 carriership were only related to amnestic MCI, whereas older age and lower total cholesterol levels were only related to non-amnestic MCI.

Older age (OR per 5 year increase in age 1.18, 95% CI 1.09; 1.28), *APOE* ε4 carriership (OR 1.35, 95% CI 1.06; 1.72), lower HDL-cholesterol levels (OR 0.86, 95% CI 0.75; 0.98), current smoking (OR 1.49, 95% CI 1.06; 2.09), and prevalent stroke (OR 2.50, 95% CI 1.48; 4.23) were related to MCI when assessed up to 7 years (mean 4.36 years, SD 0.55) prior to MCI diagnosis (Table 3). *APOE* ε4 carriership and former and current smoking were related to amnestic MCI, whereas older age and lower HDL-cholesterol levels were related to non-amnestic MCI.

Out of 682 participants with MRI scanning, 49 screened positive for MCI. Participants with MCI, particularly those with non-amnestic MCI, had larger WML volumes compared to cognitively healthy participants (mean difference in log-transformed WML volume: 0.36, 95% CI 0.05; 0.68). Persons with non-

Table 1. *Characteristics of the study population*

	Examinations at baseline 2002-2005		Examinations before baseline 1997-2001	
	No MCI N=3781	MCI N=417	No MCI* N=3730	MCI* N=401
Age, years	71.5 (7.1)	73.5 (7.5)	67.1 (7.0)	68.9 (7.4)
Females	58.2%	52.0%	58.1%	51.1%
APOE-ε4 carrier	26.2%	29.7%	26.2%	30.8%
Educational level				
Primary education	16.8%	24.8%	16.4%	23.8%
Lower vocational education	20.2%	22.4%	20.3%	22.5%
Lower secondary education	17.5%	9.2%	17.6%	9.4%
Intermediate vocational education	26.7%	28.2%	26.8%	28.6%
General secondary education	4.6%	1.9%	4.7%	2.0%
Higher vocational education	12.7%	11.4%	12.7%	11.6%
University	1.6%	1.9%	1.6%	2.0%
Waist circumference, cm	93.4 (11.8)	94.6 (12.4)	93.0 (11.5)	94.1 (11.9)
Hypertension	80.6%	83.0%	65.2%	70.9%
Diabetes mellitus	14.1%	19.2%	8.3%	9.6%
Cholesterol, mmol/L	5.65 (1.00)	5.43 (0.96)	5.84 (0.97)	5.75 (0.90)
HDL-cholesterol, mmol/L	1.46 (0.40)	1.41 (0.40)	1.40 (0.39)	1.32 (0.36)
Smoking				
Former	55.1%	55.6%	50.4%	50.6%
Current	15.1%	17.5%	19.0%	23.4%
Stroke	3.4%	8.2%	2.0%	5.0%
MRI imaging markers¶				
Total brain volume, mL	923.9 (89.6)	906.3 (119.9)	NA	NA
Hippocampal volume, mL	3.0 (0.3)	3.0 (0.4)	NA	NA
White matter lesions, mL	3.5 (2.0-6.5)	4.5 (2.6-12.4)	NA	NA
Fractional anisotropy	0.35 (0.02)	0.34 (0.02)	NA	NA
Mean diffusivity, 10^{-3} mm^2/sec	0.77 (0.05)	0.79 (0.05)	NA	NA
Cerebral microbleeds	20.9%	28.6%	NA	NA
Lacunes	5.7%	16.3%	NA	NA

**MCI as assessed at baseline (2002-2005)*

¶MR imaging was performed in a randomly selected subset (n=682).

Continuous variables are presented as means (standard deviations) and categorical variables as percentages. White matter lesions are presented as median (interquartile range).

Abbreviations: MCI=mild cognitive impairment, N=number of participants, APOE=apolipoprotein E, HDL=high-density lipoprotein.

amnestic MCI also had worse microstructural integrity of normal-appearing white matter after adjustments for cardiovascular risk factors (mean difference in mean FA: -0.007, 95% CI -0.014; -0.001, mean difference in mean MD: 0.013, 95% CI 0.001; 0.024) (Table 4, model II). As for focal markers of vascular brain pathology, microbleeds were more frequent in persons with MCI, yet this association was not significant. Lacunes, however, were more frequent in participants with MCI, again particularly in those with non-amnestic MCI (age and gender adjusted OR 3.16, 95% CI 0.98; 10.19) (Table 4). MRI scanning was performed on average 1.01 years (SD 0.46) after MCI screening. During this time-interval, 12 of 682 participants who underwent MRI were diagnosed with dementia. Out of these, 6 were initially screened as having MCI. Excluding participants with dementia at time of MRI scanning did not change our results (data not shown).

During 24,934 person-years of follow-up, 215 participants developed incident dementia, of whom 177 had Alzheimer's disease. During 29,096 person-years of follow-up, 827 persons died. Participants with MCI had an increased risk of dementia (age and gender adjusted hazard ratio (HR) 3.98, 95% CI 2.97; 5.33) (Table 5). The risk of dementia was especially increased in persons with amnestic MCI (HR 6.89, 95% CI 4.74; 10.01), but was also increased in persons with non-amnestic MCI (HR 2.65, 95% CI 1.79; 3.92). Results were similar for Alzheimer's disease. We found that participants with MCI also had an increased risk of mortality (HR 1.54, 95% CI 1.28; 1.85) (Table 5). These results did not change across MCI-subtypes and additional adjustments did not change our results.

Table 2. *Associations between risk factors of dementia and MCI at baseline (cross-sectional)*

	MCI	Amnestic MCI	Non-amnestic MCI
	Odds ratio (95% CI) n/N 389/3541	**Odds ratio (95% CI) n/N 154/3541**	**Odds ratio (95% CI) n/N 235/3541**
Age, per 5 years	1.20 (1.11;1.29)	1.04 (0.92;1.17)	1.30 (1.19;1.43)
Females	0.91 (0.70;1.17)	0.66 (0.45;0.98)	1.11 (0.80;1.54)
APOE-ε4 carrier	1.26 (1.00;1.59)	1.43 (1.01;2.02)	1.17 (0.86;1.58)
Waist circumference, per SD	1.04 (0.92;1.18)	1.03 (0.84;1.25)	1.05 (0.90;1.24)
Hypertension	0.94 (0.70;1.25)	1.03 (0.66;1.59)	0.88 (0.61;1.28)
Diabetes mellitus	1.24 (0.93;1.65)	1.44 (0.94;2.20)	1.11 (0.77;1.59)
Cholesterol, per SD	0.87 (0.78;0.98)	0.94 (0.79;1.11)	0.84 (0.73;0.97)
HDL-cholesterol, per SD	0.94 (0.83;1.06)	1.02 (0.84;1.23)	0.88 (0.75;1.04)
Smoking			
Former	0.96 (0.74;1.25)	1.08 (0.71;1.66)	0.90 (0.65;1.25)
Current	1.21 (0.86;1.70)	1.37 (0.81;2.31)	1.13 (0.73;1.74)
Stroke	2.12 (1.40;3.19)	2.68 (1.51;4.76)	1.78 (1.04;3.03)

Values represent odds ratios and 95% confidence intervals, adjusted for all other risk factors.
Abbreviations: MCI=mild cognitive impairment, CI=confidence interval, n=number of cases, N=number of controls, APOE=apolipoprotein E, SD=standard deviation, HDL=high-density lipoprotein.

Table 3. *Associations between risk factors of dementia, assessed 7 years prior, and MCI*

	MCI	Amnestic MCI	Non-amnestic MCI
	Odds ratio (95% CI) n/N 348/3235	**Odds ratio (95% CI) n/N 140/3235**	**Odds ratio (95% CI) n/N 208/3235**
Age, per 5 years	1.18 (1.11;1.29)	1.08 (0.95;1.23)	1.25 (1.13;1.38)
Females	0.93 (0.71;1.21)	0.75 (0.50;1.11)	1.08 (0.77;1.51)
APOE-ε4 carrier	1.35 (1.06;1.72)	1.54 (1.08;2.22)	1.23 (0.90;1.69)
Waist circumference, per SD	1.02 (0.90;1.16)	1.02 (0.84;1.24)	1.03 (0.87;1.21)
Hypertension	1.17 (0.90;1.52)	1.00 (0.68;1.45)	1.33 (0.94;1.87)
Diabetes mellitus	1.05 (0.72;1.54)	0.87 (0.46;1.62)	1.16 (0.73;1.84)
Total cholesterol, per SD	0.95 (0.85;1.07)	0.94 (0.78;1.12)	0.96 (0.83;1.11)
HDL-cholesterol, per SD	0.86 (0.75;0.98)	0.95 (0.78;1.16)	0.80 (0.67;0.95)
Smoking			
Former	1.12 (0.84;1.49)	1.66 (1.01;2.73)	0.92 (0.65;1.31)
Current	1.49 (1.06;2.09)	2.45 (1.41;4.24)	1.12 (0.73;1.73)
Stroke	2.50 (1.48;4.23)	2.91 (1.40;6.06)	2.22 (1.14;4.33)

Values represent odds ratios and 95% confidence intervals, adjusted for all other risk factors and additionally for time between measurements and MCI diagnosis.
Abbreviations: MCI=mild cognitive impairment, CI=confidence interval, n=number of cases, N=number of controls, APOE=apolipoprotein E, SD=standard deviation, HDL=high-density lipoprotein.

Table 4. *Association between MCI and MRI markers of brain pathology (cross-sectional)*

	Volumetric measures Mean difference (95% CI)			Microstructural integrity measures Mean difference (95% CI)		Focal measures Odds ratio (95% CI)	
Model I	**Total brain**	**Hippocampus**	**WML**	**FA**	**MD**	**Microbleeds**	**Lacunes**
No MCI	Reference	Reference	Reference	Reference	Reference	Reference	Reference
MCI	-6.66 (-15.61;2.28)	0.01 (-0.07;0.08)	0.34 (0.11;0.58)	-0.003 (-0.008;0.001)	0.007 (-0.001;0.016)	1.42 (0.73-2.75)	2.68 (1.11-6.45)
Amnestic	-8.37 (-21.16;4.42)	0.05 (-0.06;0.15)	0.32 (-0.02;0.65)	-0.001 (-0.007;0.006)	0.003 (-0.009;0.016)	1.55 (0.61-3.95)	2.31 (0.69-7.68)
Non-amnestic	-5.04 (-17.10;7.01)	-0.03 (-0.13;0.07)	0.36 (0.05;0.68)	-0.006 (-0.012;0.001)	0.010 (-0.001;0.022)	1.35 (0.55-3.31)	3.16 (0.98-10.19)
Model II	**Total brain**	**Hippocampus**	**WML**	**FA**	**MD**	**Microbleeds**	**Lacunes**
No MCI	Reference	Reference	Reference	Reference	Reference	Reference	Reference
MCI	-6.37 (-15.31;2.57)	-0.01 (-0.08;0.07)	0.35 (0.11;0.58)	-0.004 (-0.009;0.001)	0.007 (-0.001;0.016)	1.51 (0.77-2.98)	2.55 (0.99-6.54)
Amnestic	-6.78 (-19.57;6.01)	0.04 (-0.07;0.15)	0.21 (-0.13;0.55)	-0.000 (-0.007;0.007)	0.001 (-0.012;0.013)	1.51 (0.58-3.97)	1.77 (0.49-6.36)
Non-amnestic	-5.77 (-17.86;6.32)	-0.03 (-0.13;0.07)	0.46 (0.14;0.78)	-0.007 (-0.014;-0.001)	0.013 (0.001;0.024)	1.55 (0.62-3.89)	3.83 (1.08 13.61)

Model I: adjusted for age and sex.

Model II: adjusted for age, sex, apolipoprotein E ε4 carriership, waist circumference, hypertension, diabetes mellitus, total and high-density lipoprotein cholesterol, and smoking. Model II was a complete case analysis.

Analyses involving volumetric or microstructural integrity measures were additionally adjusted for intracranial volume.

Volumetric measures were expressed in milliliter (mL), FA has no unit, and MD is expressed in 10^{-3} mm^2/sec. Focal measures were expressed as present versus absent.

Abbreviations: MCI= mild cognitive impairment, MRI=magnetic resonance imaging, CI=confidence interval, WML= white matter lesions volume, FA= fractional anisotropy, MD= mean diffusivity.

Table 5. *Associations between MCI and risk of dementia, Alzheimer's disease, and mortality (longitudinal)*

	Dementia		Alzheimer's disease		Mortality	
	n/N	**Hazard ratio (95% CI)**	**n/N**	**Hazard ratio (95% CI)**	**n/N**	**Hazard ratio (95% CI)**
Model I						
No MCI	149/3781	Reference	122/3,781	Reference	695/3,781	Reference
MCI	66/417	3.98 (2.97;5.33)	55/417	4.03 (2.92;5.56)	132/417	1.54 (1.28;1.85)
Amnestic MCI	35/163	6.89 (4.74;10.01)	29/163	7.21 (4.77;10.89)	50/163	1.74 (1.30;2.31)
Non-amnestic MCI	31/254	2.65 (1.79;3.92)	26/254	2.69 (1.75;4.12)	82/254	1.44 (1.14;1.81)
Model II						
No MCI	140/3541	Reference	114/3,541	Reference	653/3,541	Reference
MCI	59/389	3.70 (2.70;5.05)	49/389	3.75 (2.66;5.30)	123/389	1.48 (1.22;1.80)
Amnestic MCI	33/154	6.76 (4.55;10.03)	27/154	7.20 (4.64;11.18)	46/154	1.58 (1.17;2.14)
Non-amnestic MCI	26/235	2.30 (1.50;3.54)	22/235	2.38 (1.49;3.80)	77/235	1.41 (1.12;1.80)

Model I: adjusted for age and sex.

Model II: adjusted for age, sex, apolipoprotein E ε4 carriership, waist circumference, hypertension, diabetes mellitus, total and high-density lipoprotein cholesterol, smoking, stroke, and educational level.

Values represent hazard ratios and 95% confidence intervals.

Abbreviations: n=number of cases, N=number of persons at risk, CI=confidence interval, MCI=mild cognitive impairment.

Discussion

We found that in the general population, older age, *APOE* ε4 carriership, lower total cholesterol levels, and prevalent stroke were associated with MCI. Lower HDL-cholesterol levels and current smoking were only related to MCI when assessed up to 7 years prior to MCI screening. Compared to cognitively healthy participants, participants with MCI had larger WML volumes, worse microstructural integrity of normal-appearing white matter, and a higher frequency of lacunes. MCI was associated with an increased risk of dementia, Alzheimer's disease, and mortality.

Major strengths of our study are its population-based setting, large sample size, and extensive data collection. Some limitations of our study need to be considered. Firstly, the extensive neuropsychological test battery required for the MCI diagnosis was implemented in 2002–2005 (baseline), and therefore we were not able to assess MCI status on the examination rounds prior to baseline. Thus, it is possible that some persons may already have had MCI at the previous examination round. Secondly, the cross-sectional setting in the analyses of risk factors prevented inferring causality. However, the extensive data collection enabled us to investigate determinants both cross-sectionally at baseline and 7 years prior to baseline, overcoming reverse causality in our study. Thirdly, we did not measure visuospatial ability, and could therefore not include this component in our diagnostic criteria for MCI. Finally, MRI scanning was performed on average 1.01 years (SD 0.46) later than the initial screening for MCI, and misclassification of participants may be present. Nonetheless, 90% of our study participants underwent MRI within 1.5 years of MCI screening, and if present this non-differential misclassification would have led to an underestimation of the true association. Also, we repeated the analyses after excluding incident dementia cases and found that results did not change materially. We found that some determinants of MCI differed over time. Lower HDL-cholesterol levels and current smoking were only related to MCI when assessed up to 7 years prior to MCI screening. There is a possibility that persons with a declining cognitive ability change their daily habits, including dietary and smoking habits, which could result in reverse causality in cross-sectional analysis. Another explanation is that these associations indeed differ over time, as has been shown for several risk factors for dementia.[2,40]

In line with previous clinical and population-based studies, we found that people with MCI had larger WML volumes, worse microstructural integrity of normal-appearing white matter, and a higher prevalence of lacunes compared to cognitively healthy participants.[23,41-43] As regional measurements of DTI were

not available in our study, we examined DTI measures averaged over the entire normal appearing white matter. For future investigations, however, it would be interesting to study regional differences in FA and MD. MCI was not associated with total brain volume, hippocampal volume, and cerebral microbleeds. These imaging markers have been implicated in persons with MCI before,[44-49] but relatively small sample size hampered our ability to investigate these associations more thoroughly. Also, smaller total brain volume, hippocampal volume, and microbleeds may mark more downstream neuropathology and as such would be a better marker for clinical dementia rather than the transitional stage of MCI.[50] Participants with MCI had an increased risk of dementia and an increased risk of mortality, independently of several risk factors of dementia. Because of this poorer prognosis, our findings underline the importance of identifying persons with MCI.

It is hypothesized that different subtypes of dementia are preceded by different subtypes of MCI. Amnestic MCI is supposed to especially increase the risk of Alzheimer's disease, whereas non-amnestic MCI more likely increases the risk of vascular dementia and other dementia subtypes, such as Lewy body dementia and frontotemporal dementia.[51,52] This would suggest that determinants might also differ per subtype of MCI. However, our findings propose that this distinction is not as unambiguous. On the one hand, we found that there are indeed some differences in determinants for amnestic and non-amnestic subtypes; e.g., *APOE* ε4 carriership and smoking were related to amnestic MCI only and MRI-correlates of vascular damage, such as larger WML load, altered DTI measures, and lacunes, were more strongly related to non-amnestic MCI. On the other hand, we found that persons with MCI who converted to dementia, most often converted to Alzheimer's disease, regardless of the MCI subtype. Moreover, stroke was related to both subtypes of MCI. Our results therefore suggest that accumulating vascular damage plays a role in both amnestic and non-amnestic MCI. This is consistent with the fact that vascular disease not only plays an important role in vascular dementia, but also in Alzheimer's disease.[2-4,6,14,53,54] Therefore, we propose that timely targeting modifiable vascular risk factors might contribute to the prevention of MCI and dementia. Nonetheless, it should be kept in mind that the cross-sectional setting of our study in the analyses of risk factors prevents us from drawing any conclusions regarding causality.

We found that persons with amnestic MCI had a larger risk of dementia than persons with non-amnestic MCI. This difference might be a consequence of the definitions of the MCI subtypes. Study participants with amnestic MCI may have experienced difficulties on other cognitive domains besides memory alone, while participants with non-amnestic MCI per definition did not experience any

memory problems. Hence, persons with amnestic MCI may have been cognitively more impaired than persons with non-amnestic MCI.

In conclusion, in our population-based study we found that several vascular risk factors and MRI-correlates of cerebrovascular disease were associated with MCI. Persons with MCI had an increased risk of dementia, Alzheimer's disease, and mortality.

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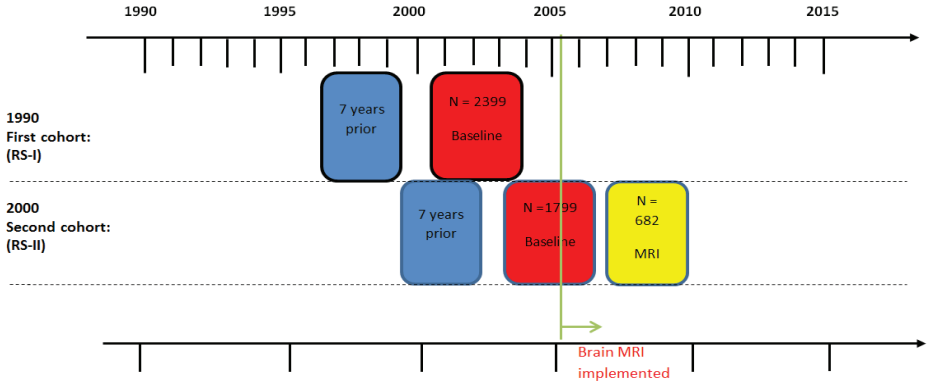
Supplementary Table 1. *Characteristics of the included and excluded participants*

	Examinations at baseline 2002-2005		Examinations before baseline 1997-2001	
	Included in analysis N=3930	Excluded from analysis N=268	Included in analysis N=3583	Excluded from analysis N=615
Age, years	71.8 (7.2)	71.2 (7.2)	67.5 (7.0)	67.3 (7.0)
Females	57.0%	66.0% *	56.3%	64.4% *
APOE-ε4 carrier	26.5%	26.3%	26.9%	23.9%
Waist circumference, cm	93.5 (11.8)	94.5 (12.8)*	93.0 (12.3)	93.1 (11.5)
Hypertension	80.4%	86.9% *	64.9%	72.1% *
Diabetes mellitus	14.4%	17.9%	8.6%	7.5%
Cholesterol, mmol/L	5.62 (0.99)	5.79 (1.03)	5.84 (0.96)	5.76 (1.00) *
HDL-cholesterol, mmol/L	1.45 (0.40)	1.45 (0.40)	1.40 (0.39)	1.33 (0.36)
Smoking				
Former	55.5%	50.0%	51.2%	44.8%
Current	15.2%	18.3%	19.0%	22.7%
Stroke	3.9%	3.0%	2.3%	1.6%

**Significantly different (P<0.05) between included participants and excluded participants, after sex and age adjustment – if applicable. Participants excluded from the analysis missed at least one value of the determinants mentioned in the table.*



Supplementary Figure. *Assessment of determinants, MCI and MRI examination.*
In red: baseline measurement of determinants and MCI assessed in 2002-2005. In blue: measurement of determinants assessed in the examination round 7 years prior to baseline. In yellow: a random subset of 682 persons with MCI screening at baseline and brain MRI examination performed on average 1.01 years after baseline (2005 onwards). Abbreviations: MCI= mild cognitive impairment, MRI=magnetic resonance imaging, RS = Rotterdam Study.

CHAPTER 5

General Discussion

General Discussion

With the studies presented in this thesis I aimed to add new insight into the etiology and clinical implications of cerebral microbleeds. My goal was to further elucidate their role as a marker of both cerebrovascular and neurodegenerative disease, and to clarify to what extent microbleeds can help explain the overlap between the two pathologies. In this chapter, I will first focus on the main findings reported in this thesis and interpret these findings in the context of current literature. Next, I will discuss methodological challenges encountered. This chapter will conclude with a review of clinical implications and directions for future research.

5.1 Main Findings

5.1.1 Cerebral Microbleeds: an Etiological Marker of Vascular Brain Disease

The first histopathology report that correlated the presence of cerebral microbleeds on MRI to accumulating blood products in the vicinity of pathologically altered blood vessels dates from 1999.[1] Changes to the cerebral microvasculature were typically described as cerebral small vessel pathology in the context of arteriosclerotic vasculopathy and cerebral amyloid angiopathy (CAA).[1-4] With the use of susceptibility weighted MR imaging it became possible to visualize the consequences of these types of vasculopathy *in vivo* through the detection of microbleeds. Ever since, microbleeds have been associated with numerous cardiovascular risk factors and *APOE* ε4 genotype in various populations.[5-12] The distinct distribution of specific risk factors in people with lobar and non-lobar microbleeds, and the predominant posterior clustering of lobar cerebral microbleeds[13-15] gave rise to the notion that microbleeds in specific brain regions reflect different subtypes of small vessel disease. CAA is thought to be the prevailing pathology underlying lobar microbleeds, whereas non-lobar microbleeds more likely reflect hypertensive arteriopathy.[1,3,9] It should be noted, however, that the different types of vasculopathy interact resulting in mixed brain pathology (i.e., CAA and arteriosclerosis), as I shall discuss in more detail later.

Other radiographic markers of cerebral small vessel disease are white matter lesions and lacunes.[16] These markers are typically considered ischemic markers and often found in patients with arteriosclerotic changes in cerebral microvasculature.[17] However, ischemic changes to cerebral small vessels have also increasingly been recognized in CAA,[18-20] a pathology characteristically known for its bleeding tendency. The co-existence of ischemic and hemorrhagic

lesions intriguingly suggests a common pathological pathway for the phenotypically different lesions. In a longitudinal study, we showed that microbleeds and ischemic markers of cerebral small vessel disease progress together, suggesting that microbleed presence marks a stage in which vasculopathy is still very active.

If microbleeds indeed mark ongoing pathological changes to blood vessels, the question arises whether their presence also indicates the presence of more widespread microscopic changes to brain parenchyma which cannot be visualized on conventional imaging. When studying white matter microstructural integrity using diffusion tensor imaging, we indeed found that people with microbleeds had diffuse poorer microstructural integrity of brain white matter. This finding, which has been replicated by others,[21,22] indicates that the focal lesions depicted as microbleeds represent only the tip of the iceberg regarding the true underlying vasculopathy. Interestingly, we found that even a single microbleed heralds the presence of microscopically diffuse brain pathology. The significance of a single microbleed has so far been uncertain. Although the presence of a single microbleed may not have direct clinical relevance, current evidence does suggest a potential pathophysiological significance as people with a single microbleed proved more likely to progress to multiple microbleeds and have diffuse microscopically white matter brain pathology.

The study described in chapter 2.3 zooms in on the group of people with lobar microbleeds and describes the relationship between these bleeds and cerebrovascular reactivity. Lobar microbleeds are a diagnostic hallmark of CAA.[23] Accumulating evidence suggests that reduced cerebrovascular reactivity is another phenotypic trait of CAA.[24-26] Since strictly lobar microbleeds in asymptomatic individuals are potential markers of CAA, we aimed to determine whether in these people vascular reactivity to visual stimulation would also be impaired. For this purpose, we invited a sample of participants from the Rotterdam Study with lobar microbleeds and age-matched controls without microbleeds to undergo functional brain MRI with a visual stimulation paradigm. We found no difference in amplitude and time to peak response on BOLD-fMRI between people with and without microbleeds, suggesting that cerebrovascular reactivity was not impaired in asymptomatic individuals with lobar microbleeds. This either advocates that CAA pathology in these people was too subtle to cause any measurable reduction in vascular reactivity, or, provocatively, that lobar microbleeds reflect vascular pathology other than CAA. The former hypothesis may be more robust given the large bulk of evidence correlating lobar microbleeds with amyloid pathology, also in the general population. In addition, more direct evidence has become available, which showed that asymptomatic individuals

with lobar microbleeds had higher retention of Pittsburgh Compound B ligand, relative to those without microbleeds.[27] Nonetheless, population-based studies that use ligands that bind vascular β-amyloid specifically, and not both vascular and parenchymal β-amyloid, are warranted to further elucidate our understanding of the true pathology underlying lobar microbleeds in this particular population.

Since small vessel disease is considered a systemic disorder,[28] pathology in one end organ may provide information on coexistent or future damage in another end organ. We therefore investigated the association between kidney function and structural markers of cerebral small vessel disease, both ischemic and hemorrhagic. Kidney vessels are of particular interest in this regard due to hemodynamic and anatomical similarities with cerebral small vessels.[29] Previous studies have indeed shown that systemic subclinical vascular disease is consistent with subclinical cerebral vascular disease.[30-32] Instead of focusing on a single marker of renal function, as most studies have done, we aimed to investigate multiple markers of renal function (i.e., serum creatinine and cystatin C based glomerular filtration rate and urinary albumin-to-creatinine ratio). Distinct markers may provide more specific insight into the pathological pathways linking kidney and cerebral small vessel disease. We found that of all renal markers, albumin-creatinine ratio showed strongest associations with cerebral small vessel disease. This finding points towards endothelial dysfunction as important contributor to small vessel disease through the entire body. The association with microbleeds was only present in those with worst kidney function (i.e., quartile with highest urinary albumin-to-creatinine ratio). This endorses microbleeds as a more downstream marker of, more severe, small vessel pathology.

In the last study of Chapter 2, we investigated genetic risk scores of serum lipids to establish a potential genetic basis of the previously reported inverse association of serum lipid levels with clinical and subclinical intracerebral hemorrhages. High serum cholesterol levels are typically associated with a higher risk of ischemic brain disease. Paradoxically, studies also report that high serum cholesterol lowers the risk of subclinical and clinical hemorrhagic brain disease.[5,12,33] The mechanism by which lipids influence the pathogenesis of hemorrhagic brain disease remains unknown, although it has been suggested that low lipid levels affect the integrity of vessel walls negatively.[34,35] We showed that a high genetic risk score of LDL associated with a higher risk of intracerebral hemorrhages and higher prevalence of multiple lobar microbleeds. Thus, our study does not support findings from previous observational studies proposing a paradoxical association of serum lipid fractions with ischemic stroke and intracerebral hemorrhages. The discrepancy between our findings and that of

previous studies may partly be explained by the fact that serum lipid fractions in those studies were typically measured only once, whereas lipid genes provide a more reliable lifetime exposure risk to elevated serum lipids levels. Also, due to competing risk of ischemic brain or heart disease and death, fewer people with elevated serum lipid levels may have been at risk for intracerebral hemorrhages. Our results suggest that high LDL cholesterol is amongst the modifiable risk factors for intracerebral hemorrhages, and in contrast to what has been suggested by others,[36-38] that individuals with hypercholesterolemia may benefit from lipid-lowering treatment.

5.1.2 Use of Pharmacological Drugs with Bleeding Potential in People with Cerebral Microbleeds

Anticoagulant drug use increases the risk of intracerebral hemorrhages seven-fold,[39] and associates with a 60% mortality rate.[40] Since microbleeds are thought to reflect an underlying bleeding-prone arteriopathy, it remains debatable whether it is desirable to give antithrombotic therapy to people with microbleeds. The debate is complicated by the fact that microbleeds are not only presumed precursors of intracerebral hemorrhages but also increase the risk of ischemic stroke.[41] In the population-based Rotterdam Study, we previously showed that antithrombotic drug use correlated with microbleed presence.[42] Similar findings were reported in a systematic review conducted in patients with intracerebral hemorrhages and ischemic stroke.[43] It should be noted that results from observation epidemiological studies are partly confounded by the indication for which the drugs were prescribed. Confounding by indication poses a problem as microbleed presence relates to cardiovascular disease and antithrombotic drugs are prescribed particularly in those with or at increased risk of cardiovascular disease. Confounding may be reduced by investigating the associations longitudinally, adjusting for cardiovascular risk factors, and adjusting for the indication of antithrombotic drug use. In our study, we tackled confounding by indication through all of the above and by studying international normalized ratio (INR). The indication by which antithrombotic medication is prescribed should influence INR levels to a lesser degree. We showed that the use of oral anticoagulant drug associates with an increased risk of developing new microbleeds. More importantly, we found proof that a higher maximum INR, and fluctuations of INR during the initiation period of anticoagulant use were both associated with higher prevalence of microbleeds. Although cross-sectional clinical studies mostly support the hypothesis that lobar microbleeds play an important role in antiplatelet-associated intracerebral hemorrhages,[44,45] we report contradictory findings. We found that anticoagulant drug use associated most consistently with deep and infratentorial microbleeds. This suggests that

bleeding risk in people with microbleeds who use anticoagulant drugs varies across populations, and factors other than type of underlying vasculopathy may be of more value when predicting bleeding risk (i.e., dose and duration of anticoagulant drug use, and/or microbleeds count).[46]

Another drug that is prescribed widely and is feared for its potential adverse (intracranial and extracranial) bleeding events are the selective serotonin reuptake inhibitors (SSRIs).[47] SSRIs block the reuptake of serotonin by platelets and decrease serotonin platelet concentration, potentially impairing platelet aggregation and prolonging bleeding times.[48,49] In our cross-sectional study, we found no association between SRRI use and microbleeds. However, in a longitudinal study we did observe an increased risk of incident microbleeds in SSRI users compared to non-users. This finding was in line with results from a meta-analysis that showed an increased risk of intracerebral hemorrhages in those using SSRIs.[50] It should be noted that in our study non-SSRI antidepressants associated with incident microbleeds in the same magnitude as SSRIs, suggesting that another pathophysiological pathway might also be at play. Despite adjusting for depressive symptoms at baseline, we cannot rule out that the association is confounded by (severity of) depressive disorder. Also, since we cannot pinpoint the exact time that microbleeds occur, there is a possibility that incident microbleeds may have developed before the initiation of antidepressant drug use during follow-up. As such, we should consider reverse causality by depression or antidepressants.

5.1.3 Clinical Outcomes of Cerebral Microbleeds

Despite our increased understanding of microbleed pathology, their clinical implications remain largely unknown. This is especially true for the general population in which people present with – sometimes multiple - microbleeds without any obvious neurological symptoms. The relative ease with which microbleeds can be detected on T2*-GRE MRI[51] has triggered a growing interest in their use as marker of poor outcome in persons with vascular brain disease. In this thesis, I investigated microbleeds as a determinant of mortality, stroke, cognitive decline, and dementia. I found that microbleed presence associated with poorer survival, as people with microbleeds had a 2-fold increased risk of cardiovascular mortality compared to those without microbleeds. This association was not explained by other cardiovascular risk factors or imaging markers of vascular brain disease. We showed a clear dose-response association with poorer survival in those with multiple microbleeds. Very similar results were found in a memory clinic,[52] and in the PROSPER study,[53] a population with high burden of cardiovascular disease, although both studies lacked generalizability. It has

been speculated that the increased risk of mortality is mainly due to an increased occurrence of stroke mortality, in particular hemorrhagic stroke.[54] Unfortunately, the number of stroke fatalities in our study was too small to detect any significant differences. Still, we did observe a strong association between non-lobar microbleeds and non-cardiovascular death, suggesting that (a high number of non-lobar) microbleeds not only signify changes in cerebral vessels but also reflect systemic vascular changes, which indicates poorer health.

In Chapter 4.2, I further explored the temporal association of microbleeds with (fatal and non-fatal) stroke, and found that microbleeds associated with an increased risk of recurrent and first-ever stroke, both ischemic and hemorrhagic. These results are in line with findings from previous clinical studies that reported that microbleed presence increases the risk of stroke recurrence in patients with a history of ischemic stroke, transient ischemic attacks, and primary intracerebral hemorrhage in context of CAA.[41,55,56] Our results confirm that the increased risk is not confined to people with prior strokes, and can be extrapolated to people from the general population. A second intriguing finding in our study was the correlation in anatomical location between cerebral microbleeds and intracerebral hemorrhage. The majority of the intracerebral hemorrhage cases developed a symptomatic bleeding in the same anatomical location as that of a pre-existing microbleed. Although these anatomical correlations were studied in only 6 cases and merely served descriptive purposes, this finding implies that microbleeds may be of value in pinpointing focal areas in the brain with more active vasculopathy.

In the final two subsections of Chapter 4 I correlated microbleeds with MCI, and investigated whether microbleed presence was a determinant for cognitive decline and dementia. Since microbleed pathology underlies both vascular and amyloid burden it makes microbleeds a particularly interesting marker to study pathological mechanisms in cognitive deterioration. Our studies revealed that people with MCI had more white matter lesions and lacunes, and more microbleeds, when compared with people without MCI, though the correlations with microbleeds were non-significant. These findings are in line with other clinical studies.[57-59] Since white matter lesions and lacunes associated more robustly with MCI it was suggested that microbleeds mark more downstream neuropathology and as such would be a better marker for dementia than the transitional clinical stage of MCI.[60] In longitudinal studies, we found that a high number of microbleeds marked decline in all cognitive domains. In addition, microbleed presence increased the risk of dementia, including Alzheimer's dementia.

Depending on the population under investigation, microbleeds have been associated with impairments in different cognitive domains, yet longitudinal

studies are scarce.[61,62] In patients with Alzheimer's disease, microbleeds did not affect the rate of cognitive decline, though it should be noted that only MMSE - a crude global measure of cognition – was used to determine cognitive deterioration in this particular study.[54] Our study is the first to report on the relationship between microbleeds and dementia in the general population, and strengthens the notion that microbleeds may indeed link vascular and neurodegenerative pathology in (Alzheimer's) dementia. Vascular and amyloid pathology often co-exist and a majority of the autopsied brains of demented patients show high burden of arteriosclerosis and amyloid pathology.[63] The question then remains how vascular and amyloid pathology interact to cause significant cerebral tissue loss, and how microbleeds fit into this mechanism. On the one hand, vascular amyloid could adversely affect reactivity of cerebral microvasculature causing vessels to lose their hemodynamic function. On the other hand, hypertensive damage to small vessels may lead to disturbances in amyloid clearance, increasing amyloid deposits in vessel walls. Both pathways may facilitate a focal inflammatory response with leakage of blood plasma into brain parenchyma. The leakage of blood products has direct toxic effects on neurons and neuronal dysfunction may eventually lead to cell death.[64]

To date, studies that have investigated the clinical implications of microbleeds have focused either on the relationship of microbleeds with stroke or microbleeds with dementia. Given the numerous common risk factors of both brain diseases it may be suggested that at least a part of their pathogenesis is shared. Microbleeds may possibly mark this common pathophysiological pathway, especially in an early asymptomatic stage, and serve as a more general marker of (neurodegenerative) brain disease. For illustration purposes, I have shown results on the association of microbleeds with first-ever stroke and dementia combined in Table 1. Persons were free of cognitive impairment at baseline. These results suggest that the presence of multiple microbleeds mark an

Table 1. *Microbleeds and the risk of overt cerebrovascular disease*

	Hazard Ratios (95% Confidence Intervals)*					
	Cases/ nr at risk	Stroke	Cases/ nr at risk	Dementia	Cases/ nr at risk	Stroke or Dementia
No microbleeds	56/4790	Reference	39/4889	Reference	84/4663	Reference
Any microbleeds	29/4790	1.64 (1.03-2.61)	33/4889	2.00 (1.24-3.21)	51/4663	1.73 (1.21-2.48)
Single microbleed	6/4790	0.64 (0.27-1.48)	15/4889	1.75 (0.96-3.20)	15/4663	0.96 (0.55-1.68)
Multiple microbleeds	23/4790	2.96 (1.77-4.96)	18/4889	2.27 (1.28 -4.04)	36/4663	2.69 (1.78-4.04)

**Adjusted for age and sex*

advanced stage of brain disease or signify an increased risk of acute cerebral dysfunction.

5.2 Methodological Considerations

5.2.1 Study Design

The longitudinal population-based Rotterdam Study was designed to investigate occurrence, determinates, and consequences of chronic diseases in an aging population.[65] The implementation of brain MRI in 2005 made it one of the few large studies able to investigate subclinical brain disease on a population level.[66] Results described in this thesis should be interpreted in light of methodological advantages and disadvantages of population-based cohort studies.

All individuals aged 45 years and over living in Ommoord, a suburb of Rotterdam, the Netherlands, were invited to participate in the Rotterdam Study, which limited the possibility of selection bias due to non-random sampling. Still, selection bias may have influenced the results of studies in which we used serial MRI, as people who attended follow-up visits for brain MRI were on average younger and healthier than those who did not participate at either baseline or follow up.[10] A well-known advantage of population-based studies over clinical studies is the high external validity. Yet, the vast majority of participants in the Rotterdam Study are of Caucasian descent. Although ethnic differences may not have been of interest to most hypotheses tested in the Rotterdam Study, it should be noted that (clinical and subclinical) intracerebral bleeding risk differs across ethnicities,[67] and some findings described in this thesis may not directly translate to non-Caucasian populations. Furthermore, cohort studies can help clarify the role of a single exposure in multiple outcomes, which is a special point of interest in studies trying to comprehend the multifactorial nature of cerebral microbleeds. In the Rotterdam Study, information on exposure and outcome status was collected periodically in all participants, using standardized techniques. Although microbleeds were detected on state-of-the art MRI sequences, the ratings were done visually and misclassification may have occurred. Since microbleed raters were blinded to all clinical data it is assumed that potential misclassification should be non-differential.

5.2.2 Imaging Parameters

Brain MRI has a prominent role in detecting subclinical brain pathology non-invasively. In the Rotterdam Study, a standardized imaging protocol has been in place since the implementation of MRI in 2005.[66] The choice to refrain from

regularly changing hardware and software allowed us to study pathological brain changes over time. The automatic segmentation of brain tissue and white matter lesions was done using approaches that were specifically developed for MRI data acquired in the Rotterdam Study, with a similarity index comparable to a good interrater reliability if segmentation was performed manually by two raters.[68] Optimization of the T2*-weighted GRE sequence enhanced cerebral microbleed detection on our 1.5-Tesla MRI scanner.[69] Ratings of microbleeds and lacunes were done by trained research-physicians with good understanding of human brain anatomy. All raters were trained 1 on 1 by an experienced neuroradiologist to get acquainted with markers of subclinical brain pathology. To qualify as a rater, research-physicians had to pass an MRI training set which included scans of participants with and without brain pathology (i.e., microbleeds, infarcts, white matter lesions, and incidental findings).

Since the focus of this thesis was on cerebral microbleeds, I will briefly review the methodical challenges of detecting microbleeds on MRI. The detection of microbleeds strongly depends on scanner characteristics and sequence parameters. Magnetic field strength, echo time, slice thickness, slice gap, spatial resolution, and post-processing techniques largely determine the accuracy and ease of microbleed detection.[70] Microbleeds typically appear as small black dots on susceptibility weighted images due to paramagnetic effects of hemosiderin when brought into the magnetic field of an MRI scanner.[51] Hemosiderin causes macroscopic inhomogeneities in the magnetic field surrounding microbleeds leading to distortions of the local MRI signal. Since the distortion affects a larger area surrounding microbleeds, the microbleeds appear larger than their actual size in histopathological coupes. This so called blooming effect enhances the visual detection of microbleeds on MRI.[51] The visual detection can be enhanced further by prolonging the susceptibility effect through extended echo times.[70] This has been done in the Rotterdam Study and has increased our detection rate of microbleeds considerably.[69] It has been mentioned, however, that a longer echo time increases uncertainties regarding the cause of the susceptibility effect as the quality of images reduces.[71] Mineralization of calcium and iron in the brain may cause similar distortions as hemosiderin and are considered well known mimics of microbleeds.[72] A higher echo time may thus make it more difficult to distinguish microbleeds from their mimics. Another way to improve microbleed detection is by using MRI with higher field strength. Although we used a 1.5-Tesla MRI scanner, the prolonged echo time partly reduced the need to upbeat the field strength in our study. On the one hand, our study may still benefit from higher field strengths as this will reduce background noise on MR images and lead to more accurate ratings in areas with high susceptibility artefacts (i.e., near the skull base or air-tissues). On the other hand, introducing

a higher field strength would result in less comparable serial MRIs in the Rotterdam Study.

The use of standardized visual ratings scales has improved the inter- and intra-rater reliability of microbleed detection.[73,74] Automatic detection is, however, preferable since visual ratings are very time consuming. To date, fully automatic detection of microbleeds remains challenging. A major problem lies in the high false positive lesions detected, i.e., differentiating between microbleeds and its mimics. Semi-automated processes have been proposed with relatively reliable detection once manual corrections are incorporated.[75,76] Although these tools may allow for high reproducibility, corrections are still labor intensive.

5.2.3 Categorization of Microbleeds

Microbleeds are traditionally categorized according to their presumed underlying etiology into lobar (CAA related pathology) and deep or infratentorial (arteriosclerosis) microbleeds. It should be noted, however, that mainly indirect evidence supports this claim in the general population. Microbleeds are considered multifactorial lesions since both environmental and genetic factors contribute to their development. Given that different types of vasculopathy often interact to cause overt or clinical relevant cerebrovascular disease, it is debatable whether the current classification system truly reflects this multifactorial nature of microbleeds. Also, the division into lobar versus non-lobar microbleeds does not accurately reflect the microbleed lesion burden in persons. Most people with microbleeds have a single strictly lobar microbleed, whereas people with deep or infratentorial microbleeds often have higher numbers of microbleeds due to the concomitant presence of lobar microbleeds. Thus, with the current classification we should acknowledge that associations found for deep or infratentorial microbleeds and not in lobar microbleeds may be driven by the difference in lesion load rather than underlying pathology.

In some studies included in this thesis we used an alternative classification, dividing microbleeds according to the revised Boston criteria for CAA into microbleeds in locations suggestive of CAA (lobar with or without cerebellar microbleeds) and other brain regions (strictly cerebellar, deep or infratentorial with or without lobar microbleeds). An important point of criticism when using this classification is that the neurological diseases under study (intracerebral hemorrhages, cognitive impairment) were partly used in the clinical criteria for CAA on which we based our categorization of microbleeds. Another point to note is that by using this categorization, cerebellar microbleeds were analyzed alongside lobar microbleeds, assuming that CAA plays a role in cerebellar

microbleeds. However, recently, a histopathology study was not able to show a higher frequency of cerebellar microbleeds in CAA compared with non-CAA brains.[77] On the contrary, when CAA brains were compared with pure and mixed vascular dementia brains without CAA, the latter group had significantly higher mean values of cerebellar microbleeds, suggesting that vascular risk factors play an important role in their etiology.[77] In the Rotterdam Study, we also found that vascular risk factors correlated strongly with cerebellar microbleeds, though the lack of an association between *APOE* ε4 and cerebellar microbleeds may have been due to the small numbers of individuals with cerebellar microbleeds.
As illustrated by these examples, classifying microbleeds according to their presumed underlying etiologies leaves us with some uncertainties. Future studies may benefit from a more basic classification by studying microbleeds per anatomical brain regions, as we preliminary sought to do in Chapters 2.3 and 4.3. More ideally, the effect of each single microbleed in distinct topographic brain regions (i.e., lobes, deep hemispheric, brainstem, cerebellum) should be studied. Clustering of microbleeds within persons has to be dealt with. The advantage of such a subdivision is that it takes in to account lesion load, and the anatomical region may provide better understanding of functional defects in the presence of microbleeds.

5.2.4 Confounding Factors

To control for potential confounding bias we often used multivariable models adjusting for cardiovascular risk factors. These corrections were also necessary to establish the additive value of microbleeds over other markers of cardiovascular disease (including imaging markers of cerebral small vessel disease). It should be noted that if microbleeds mark the presence of vascular pathology, and traditional cardiovascular risk factors are determinants of these pathologies, microbleeds might be considered an intermediate factor. Multivariable adjustments in our studies would thus have led to an overcorrection. Mediation analysis may help us to understand what proportion of the association between vascular risk factors and stroke or dementia (direct effect) would go via microbleeds (indirect effect). In this thesis, I did not statistically test if microbleeds should be considered a mediator. This because some of the assumptions for mediation analysis may not hold in our setting.[78] Relatively newly developed techniques enable mediation analysis in studies with censored data even if these assumptions are violated. Future studies may want to use these techniques to quantify the direct and indirect effects of vascular risk factors on stroke and dementia through microbleeds.[78,79]

A second consideration in our study of pharmacological therapies is confounding

by indication. The lack of randomization in observational epidemiological allows for a disproportional distribution of (known and unknown) prognostic factors that influences treatment decisions. In our study, we applied traditional methods (i.e., restriction, stratification, and multivariable adjustments) to control for known prognostic factors in order to reduce potential confounding by indication. Despite the fact that potential residual confounding cannot be ruled out, previous methodological studies have shown that with a careful set-up of study design and data-analysis, results from observational pharmacological studies coincide with those of randomized clinical trials.[80,81] Careful set-up of study design includes a cautious selection of start of follow-up (preferably time of estimated exposure/treatment rather than regular intervals in which treatment data is collected). In addition, bias is reduced by adjusting for potential confounders both at baseline and during follow-up, and by stratifying on indication and duration of drug use. Regarding our pharmacological studies, a potential criticism could be the choice of start of follow-up, which was based on the first MRI rather then initiation of treatment. Also, the lack of data on potential confounders during follow-up may have led to suboptimal adjustment.

Clinical trials have yet to publish their results on bleeding complications in antithrombotic drugs users with microbleeds. Should their results conflict with previous reports then we might explain the differences by suggesting confounding from non-randomization in observational studies or a suboptimal study design. In addition, we should consider post-randomization confounding and selection bias due to non-random dropout of participants during follow-up in clinical trials.[81]

Finally, given the positive relationship of microbleeds with multiple disease outcomes we should consider the potential of competing risk. Competing risk is presumed present when an individual is at risk of more than one mutually exclusive outcome, and the occurrence of one outcome prevents any other outcome from happening. In our study of mortality, microbleeds were strong determinants of all-cause, cardiovascular, and non-cardiovascular mortality. Thus, competing risk of death may have affected the hazard of incident stroke and dementia, as individuals with microbleeds may have died before either of the two diseases became manifest. Figure 1 shows the cumulative probabilities of stroke and dementia before and after adjusting for the competing risk of death. The relatively large difference in the cumulative incidences, especially for incident stroke, suggests that there is indeed competing risk of death. Note that the estimated cumulative incidence of stroke and dementia is lower when competing risks are taken into account. In order to get the event of interest (either dementia or stroke) in a specific time-interval, persons have to be alive during that time interval and experience the event. If we do not take into account

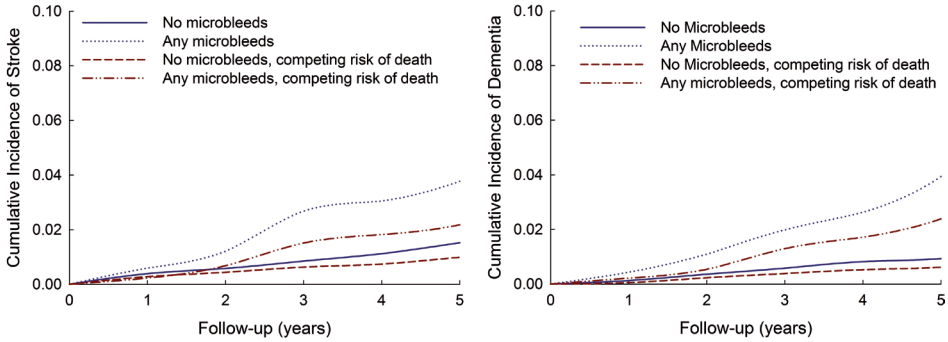


Figure 1. *Cumulative incidence of stroke (left graph) and dementia (right graph) in people with and without microbleeds. The solid and dotted lines represent cumulative incidence for stroke or dementia in persons with any and no microbleeds without adjusting for competing risk, and the dashed lines show the cumulative incidence after taking into account competing risk of death.*

competing risk, persons who die are eliminated from the risk set. When competing risk of death is taken into account, the overall survival is reduced and thus the incidence of the event of interest is reduced too, although the hazard ratio does not necessarily have to decrease as well.[82]

5.3 Clinical Implications and Future Research

5.3.1 Clinical Implications

Although cerebral microbleeds have been considered clinically silent lesions in the past, their association with stroke and cognitive impairment has led to a reassessment of their clinical relevance.

I have shown that microbleed presence marks diffuse and active vasculopathy in the brain, even if just a single microbleed is present. Although the presence of a single microbleed will not cause overt neurological deficit, clinicians should be aware that their presence is merely a tip of the iceberg regarding the underlying pathology. Also, microbleed presence does not only mark progression of bleeding tendencies in the brain but also progression of ischemic brain pathology. The simultaneous progression of both hemorrhagic and ischemic lesions may complicate future therapeutic decision-making. The difficulty is clearly illustrated by the paradoxical findings of previous studies that showed that high serum cholesterol increased the risk of ischemic brain lesions but reduced the risk of hemorrhagic brain lesions, raising the question whether the widespread use of statins should be constrained to people with a strict indication. Our study found no genetic basis for such a contradictory association, and is consistent with evidence suggesting the (bleeding) safety of statin use in people with

elevated lipid levels. Notwithstanding, clinicians should be informed that observational studies show that people with microbleeds who use drugs that prolong bleeding times (i.e., antithrombotic drugs, SSRIs) are at increased risk of developing new microbleeds (or even symptomatic hemorrhages). Although to date, no definite evidence from ongoing clinical trials and cost-benefit studies are available to generally discourage use of antithrombotic drugs and SSRIs in people with microbleeds, clinicians should carefully consider individual patient history when taking therapeutic decisions.

The most clinically relevant finding in our population-based study is that microbleeds were found to be determinants of neurological diseases such as stroke, cognitive decline, and dementia. The increased risk of stroke was not confined to people with a history of ischemic stroke or intracerebral hemorrhage. Also, as suggested by cross-sectional studies, a high number of microbleeds affected cognitive function and indicated an increased risk of cognitive deterioration and dementia in the general population. These findings emphasize the role of microbleeds as an early imaging marker of future overt cerebrovascular disease. Since microbleeds themselves represent vascular and neurodegenerative brain damage, effective preventive strategies should preferably target modifiable risk factors of microbleeds before their onset on MRI.

5.3.2 Recommendations for Future Research

The number of studies investigating microbleeds has increased drastically in the last decade, and our knowledge on microbleed etiology and clinical implications has improved considerably. Nonetheless, future studies still have to replicate findings and fill in some gaps in knowledge.

Firstly, it should be noted that in the general population cerebral microbleeds are thought to reflect different types of small vessel vasculopathy primarily based on indirect evidence. The correlations of vascular risk factors with deep or infratentorial microbleeds suggest underlying arteriosclerosis. The predilection of lobar microbleeds for posterior brain regions and association with *APOE* ε4 mostly suggest underlying amyloid angiopathy. Although histopathological evidence is hard to collect in the general population, the use of Pittsburg Compound B PET scans with radioactive ligands that bind vascular amyloid exclusively, may provide more direct evidence for the accumulation of β-amyloid in the cerebral vessels of people with lobar microbleeds. Furthermore, despite the exponential growth in CAA research,[83] our current etiological knowledge on the disease is strikingly restricted. Aside from *APOE* ε4, no specific risk factors, and more importantly modifiable risk factors, have been identified for CAA. Given that *APOE* ε4 is a risk factor of amyloid pathology both in patients with

Alzheimer's dementia and persons without cognitive impairments,[84,85] it may be suggested that in the presence of *APOE* ε4, still unidentified factors accelerate neurodegeneration. Future studies investigating CAA or their presumed markers (i.e., lobar microbleeds) should therefore focus on non-traditional (vascular) risk factors (e.g., inflammation markers) and address their interaction with *APOE* ε4 genotype.

Secondly, it should be noted that microbleeds, which are thought to be downstream markers of cerebral small vessel disease, often co-occur with other lesions such as white matter lesions and lacunes. Their co-existence makes it extremely difficult to disentangle the individual effects of these markers on clinical cerebrovascular disease. For risk stratification of stroke and dementia it may be more useful to assess the entire burden of small vessel disease. Therefore, I would suggest that future studies calculate validated lesion burden scores that quantify the overall burden of vascular brain disease incorporating all imaging markers of cerebral small vessel disease.

Thirdly, we anticipate results from ongoing clinical trials investigating hemorrhagic complications in patients with microbleeds treated with antithrombotic drugs. In the future, these trials should not only focus on populations with atrial fibrillation or ischemic stroke but also expand to seemingly asymptomatic populations with extensive ischemic small vessel disease. The increased use of susceptibility weighted imaging in routine clinical practice has improved the detection of microbleeds in people on antithrombotic treatment and ischemic small vessel disease, leaving physicians wondering whether or not to start, continue, or discontinue treatment. Clinical evidence and subsequent recommendations are necessary to guide clinicians uniformly through these therapeutic decisions.

Finally, future studies should shed more light on factors that promote progression of vasculopathy, since clinically relevant disease in our population-based setting was confined to people with multiple microbleeds. In addition, little is known about the prognostic value of microbleeds in symptomatic patients. Future research may want to investigate whether people with microbleeds who develop stroke or dementia suffer a more severe disease course or respond differently to therapy than those without microbleeds. This evidence may provide the necessary basis for individualized treatment.

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CHAPTER 6

SUMMARY

English summary

With increasing life expectancy, age-related disease such as stroke and dementia are becoming more prevalent. Stroke is the second leading cause of death worldwide and dementia is a major cause of disability and dependency. The therapeutic options for stroke are limited, and, to this date, no treatment options are available to halt the process of cognitive deterioration in dementia. Implementing preventive strategies for both diseases is therefore crucial. To achieve this, a comprehensive understanding of disease etiology is necessary and early markers of disease have to be identified. Advances in neuroimaging have made it possible to detect lesions that mark subclinical neurodegenerative and cerebrovascular disease on MRI. This thesis focuses on etiological pre-clinical imaging markers of vascular brain disease, with a particular interest in cerebral microbleeds. The aim of this thesis was to investigate whether microbleeds represent active and diffuse cerebral vasculopathy. In addition, I aimed to determine what the clinical implications of microbleeds are in the general population. All studies were imbedded in the Rotterdam Study, a population-based cohort that uses neuroimaging to investigate occurrences, determinants, and consequences of chronic disease in an aging population.

Cerebral microbleeds as a marker of vascular brain disease

Chapter 2 covers studies regarding cerebral microbleed etiology and pathophysiology. In Chapter 2.1, I investigated the concomitant progression of microbleeds and ischemic markers of vascular brain disease (i.e., white matter lesions and lacunes). I found that the development of new microbleeds associated with the progression of ischemic vascular brain lesions. This finding suggests that microbleeds may serve as a marker of active vasculopathy. Moreover, the concurrent progression of hemorrhagic and ischemic vascular brain lesions supports the hypothesis of a common pathophysiologic pathway for these lesions.
In Chapter 2.2, I used diffusion tensor imaging techniques to study whether microbleeds, like white matter lesions and lacunes, associate with more diffuse, microscopic damage to white matter. Microbleeds were indeed related to poorer microstructural integrity of brain white matter. This was true even if just a single microbleed was present, suggesting that microbleeds represent merely the tip of the iceberg regarding the true underlying vasculopathy.
In Chapter 2.3, I zoomed into the participants with strictly lobar microbleeds. Lobar microbleeds are potential markers of cerebral amyloid angiopathy (CAA). In patients with CAA, it has been shown that cerebrovascular reactivity is impaired. We used BOLD-fMRI with a visual stimulation paradigm to investigate whether people with lobar microbleeds in the general population also had reduced vascular reactivity. We found no differences in BOLD-fMRI amplitude and time to peak response in people with and without lobar microbleeds, suggesting that in asymptomatic individuals, lobar microbleeds may either reflect less advanced CAA pathology insufficient to cause functional vascular

impairment, or reflect vascular pathology other than CAA.
The link between renal and cerebral small vessel disease is discussed in Chapter 2.4. I showed that a high albumin-to-creatinine ratio was associated with both hemorrhagic (cerebral microbleeds) and ischemic (white matter lesions, lacunes) cerebral lesions. This finding emphasizes that small vessel disease is a systemic disorder, and that common etiological factors link small vessel disease in different end organs.
Aside from modifiable determinants, I also investigated non-modifiable determinants of microbleeds. In Chapter 2.5 I computed genetic risk scores for lipid fractions, and investigated whether a higher genetic load for high serum lipid levels was a determinant of clinical and subclinical intracerebral hemorrhage risk. I found that a higher genetic risk score for total and LDL-cholesterol was associated with an increased risk of clinical intracerebral hemorrhages and a higher prevalence of lobar microbleeds. I found no genetic basis for the previous reported associations that proclaimed an increased risk of intracerebral hemorrhages in the presence of low serum cholesterol.

Use of pharmacological drugs with bleeding potential in people with cerebral microbleeds

In Chapter 3 I discuss the association of coumarin anticoagulants and selective serotonin reuptake inhibitors (SSRIs) with cerebral microbleeds. Both drugs are widely used and known for their adverse bleeding events. Compared to no-use, the use of coumarin anticoagulants was associated with a higher prevalence of microbleeds (Chapter 3.1). Additionally, within the group of coumarin users, I found that those with a high variability of INR during the initiation of treatment had a higher prevalence of deep or infratentorial microbleeds. Next, in Chapter 3.2 and 3.3 I studied the association of SSRIs with microbleeds respectively in crosssectional and longitudinal setting. Cross-sectional results revealed no association between SSRIs use and microbleeds. In our longitudinal study, however, I did observe a positive association. Compared to no use, the use of both SSRIs and non-SSRIs was associated with an increased risk of developing new microbleeds.

Clinical outcomes of cerebral microbleeds

Chapter 4 of this thesis was dedicated to studies investigating the clinical implications of cerebral microbleeds. First, I studied whether the presence of microbleeds was an indicator of poor general health by investigating whether people with microbleeds were at increased risk of mortality (Chapter 4.1). I found that, compared to people without microbleeds, people with microbleeds in deep or infratentorial brain regions, where at an increased risk of all-cause and cardiovascular-related mortality. These associations were independent of other cardiovascular risk factors. Thus, microbleeds likely mark severe underlying pathology associate with poorer survival.

It has been suggested that this increased risk of mortality is particularly due to increased risk of (hemorrhagic) stroke. Thus, in Chapter 4.2, I examined the association between microbleeds and (fatal and non-fatal) stroke. Our results showed that, in the general population, people with microbleeds were at increased risk of developing (first-ever and recurrent) ischemic stroke and intracerebral hemorrhages. Those with lobar microbleeds were particularly at risk of developing intracerebral hemorrhage, whereas those with at least one non-lobar microbleed were at risk of developing both ischemic stroke and intracerebral hemorrhage.
The relationship between microbleeds and cognitive deterioration is discussed in the last part of Chapter 4. First, I investigated whether microbleeds were determinants of cognitive decline and dementia in the general population (Chapter 4.4). Afterwards, I studied the role of microbleeds and ischemic markers of small vessel disease in MCI (Chapter 4.3). MCI is considered a transitional stage between healthy aging and dementia. I found strong correlations of MCI with lacunes and white matter lesions. Although strong effect estimates were also found for microbleeds, these results were non-significant. In the follow-up study I found that a high load of microbleeds indicated decline of cognitive function in various domains. Furthermore, we found that microbleeds were associated with an increased risk of dementia, including Alzheimer's dementia, in the general population.

In Chapter 5, I reviewed the main findings of this thesis and discuss how they fit the context of existing literature. The methodological challenges I faced are also discussed. Finally, I provide some recommendations for future studies. There are three main conclusions that may be derived from this thesis. First, cerebral microbleeds mark the presence of severe, active, and diffuse underlying vasculopathy. Second, microbleeds indicate the presence of bleeding-prone vasculopathy and as such, caution is warranted when using medication with high bleeding risk. Third, the presence of microbleeds on brain MRI yields clinical relevance, as their presence is associated with overall poorer survival and an increased risk of stroke and dementia.

Dutch summary

Beroertes en dementie zijn belangrijke gevolgen van vasculaire hersenschade. De toenemende vergrijzing heeft er toe geleid dat het aantal mensen dat een beroerte krijgt of lijdt aan dementie sterk toeneemt. Wereldwijd zijn beroertes de op een na belangrijkste reden van overlijden. Daarnaast is dementie een van de belangrijke redenen van invaliditeit en afhankelijkheid. Beide aandoeningen leggen een aanzienlijke belasting op de gezondheidszorg. De behandelingsmogelijkheden voor beroertes zijn beperkt en voor dementie is de behandeling slechts symptomatisch. Het is daarom van belang om het ziekteproces van beroertes en dementie al in een vroeg stadium te herkennen. Het herkennen van hersenschade in een vroeg stadium kan er toe leiden dat risicofactoren effectiever behandeld kunnen worden, nog voordat er irreversibel schade ontstaat. Middels MRI kan hersenschade al in een vroeg stadium worden opgespoord, zonder dat hier invasieve ingrepen aan te pas komen. In dit proefschrift onderzoek ik MRI markers van vroege vasculaire hersenschade en richt ik mij daarbij in het bijzonder op de zogenaamde cerebrale microbloedingen. De belangrijkste doelen van de gepresenteerde onderzoeken zijn (1) om te achterhalen of microbloedingen een uiting zijn van progressieve en wijdverspreide vasculaire hersenschade en (2) om te achterhalen welke klinische gevolgen samenhangen met de aanwezigheid van microbloedingen. De onderzoeken zijn uitgevoerd in de Rotterdam Studie. De Rotterdam Studie is een langlopend cohort dat onderzoek doet naar het voorkomen, risicofactoren en gevolgen van chronische ziekten in de algemene bevolking.

Microbloedingen in de hersenen: een marker van vasculaire hersenschade

Hoofdstuk 2 van dit proefschrift beschrijft studies aangaande de etiologie en pathofysiologie van cerebrale microbloedingen. Hoofdstuk 2.1 beschrijft de bevindingen van de studie naar het samen voorkomen van ischemische en hemorragische laesies in de hersenen. Uit mijn onderzoek concludeer ik dat het ontstaan van nieuwe microbloedingen gepaard gaat met het ontstaan van nieuwe wittestofafwijkingen en lacunaire infarcten. Deze bevindingen suggereren dat de aanwezigheid van microbloedingen duidt op vasculaire hersenschade dat progressief van aard is en dat er gedeelde risicofactoren zijn voor hemorragische en ischemische laesies in de hersenen. Gezien de bevinding dat microbloedingen gepaard gaan met macroscopisch zichtbare ischemische laesies richtte ik mij in het volgende hoofdstuk, Hoofdstuk 2.2, op de vraag of microbloedingen ook geassocieerd zijn met ischemische microstructurele laesies. Ik vond inderdaad dat de aanwezigheid van microbloedingen slechts het topje van de ijsberg is en dat de onderliggende microstructurele schade aan de wittestof van het brein veel uitgebreider is.
In het daaropvolgende hoofdstuk, Hoofdstuk 2.3, richtte ik mij op de personen met lobaire microbloedingen. De huidige literatuur schrijft het ontstaan van deze microbloedingen toe aan de aanwezigheid van cerebrale amyloid pathologie.

Van patiënten met klinische klachten passend bij cerebrale amyloid pathologie weten wij dat de vasculaire reactiviteit in de hersenen is verlaagd. Ik onderzocht of dat ook het geval was voor personen met lobaire microbloedingen zonder klinische klachten. Daartoe ondergingen deelnemers met en zonder lobaire microbloedingen een functionele MRI. Er werd geen verschil gevonden tussen de twee groepen met betrekking tot hun fMRI parameters. Deze bevinding suggereert mogelijk dat asymptomatische personen met lobaire microbloedingen minder ernstige amyloid pathologie hebben of dat de microbloedingen toch door een pathologie anders dan amyloid wordt veroorzaakt.
In Hoofdstuk 2.4 onderzocht ik het gezamenlijk voorkomen van schade aan de kleine bloedvaten in de nieren en hersenen. De bloedvaten van beide organen delen veel fysiologische eigenschappen. Het idee is daarom dat schade aan bloedvaten van een orgaan informatie geeft over schade in het ander orgaan. Ik observeerde dat personen die schade hadden aan de kleine bloedvaten van de nieren, zoals bepaald werd door de aanwezigheid van een laag albumine-creatinine ratio in de urine, ook vaker schade hadden aan de bloedvaten van de hersenen. Deze hersenschade was zowel ischemisch (aanwezigheden van wittestofafwijkingen en lacunaire infarcten) als hemorragisch (cerebrale microbloedingen) van aard.
Tot slot, onderzocht ik in Hoofdstuk 2.5 of de paradoxale relatie tussen een laag serum cholesterol en het risico op hersenbloedingen een genetische basis had. Ik vond dat een hoge genetische gevoeligheid voor een hoog serum totaal en LDL-cholesterol gepaard ging met een verhoogd risico op symptomatische hersenbloedingen en met een hogere prevalentie van lobaire microbloedingen. In tegenstelling tot andere studies, wijzen mijn bevinden erop dat een hoog serum (LDL-) cholesterol behandeld moet worden om het risico op beroertes en microbloedingen te verlagen.

Geneesmiddelen met een verhoogde bloedingsrisico en cerebrale microbloedingen

In Hoofdstuk 3 onderzocht ik de associatie tussen geneesmiddelen die het bloedingsrisico verhogen en cerebrale microbloedingen. De geneesmiddelen die wij onderzochten waren orale bloedverdunners (coumarines) en selectieve serotonine-heropnameremmers (SSRIs). Ik vond dat personen die coumarines gebruikten vaker microbloedingen hadden dan personen die deze medicijnen niet gebruikten (Hoofdstuk 3.1). Binnen de groep van gebruikers, vond ik dat personen die slecht gereguleerd waren gedurende de beginfase van hun behandeling vaker microbloedingen hadden dan zij die wel goed gereguleerd waren. In de daaropvolgende hoofdstukken beoordeelde ik de relatie tussen SSRIs en microbloedingen in een cross-sectionele analyse (Hoofdstuk 3.2) en longitudinale analyse (Hoofdstuk 3.3). In de cross-sectionele studie vond ik geen associatie tussen SSRIs gebruik en microbloedingen. Wel vond ik in onze longitudinale studie dat SSRIs gebruik het ontstaan van nieuwe microbloedingen vergroot.

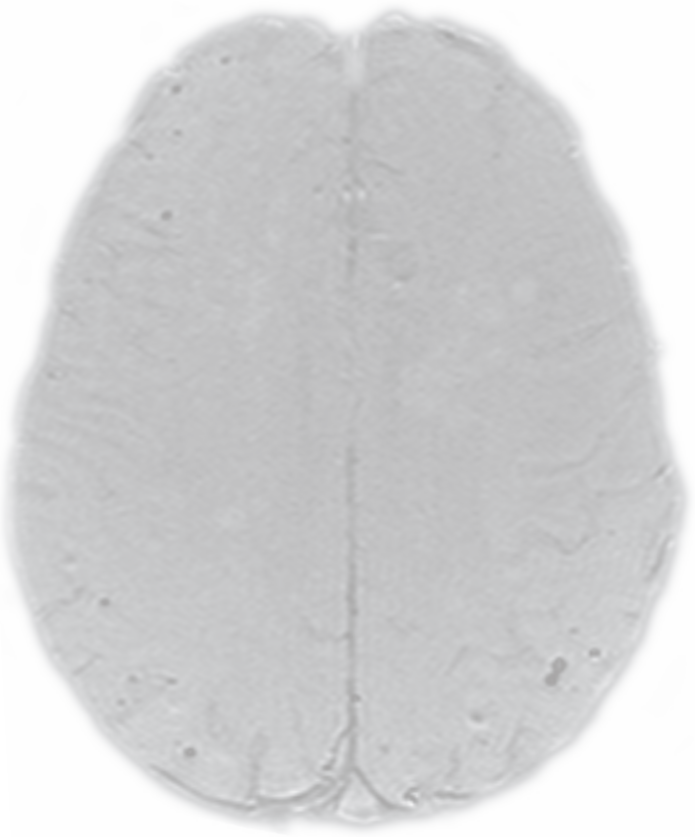
Cerebrale microbloedingen en klinische uitkomsten

Hoofdstuk 4 van dit proefschrift was gewijd aan studies die de relatie onderzochten tussen microbloedingen en klinische uitkomsten, te weten mortaliteit, beroertes, cognitieve achteruitgang en dementie. In hoofdstuk 4.1 toonde ik aan dat personen met microbloedingen een verhoogde kans hadden om te komen overlijden in vergelijking tot personen zonder microbloedingen. Het verhoogde risico werd vooral geconstateerd in personen met tenminste één microbloeding in het diepe gedeelte van de grote hersenen, in de hersenstam of de kleine hersenen. Deze associatie was ook aanwezig na correctie voor cardiovasculaire risicofactoren. In Hoofdstuk 4.2, onderzocht ik het risico op (fatale en niet-fatale) beroertes. Ik vond dat personen met microbloedingen vaker een beroerte kregen dan personen zonder microbloedingen. Dit verhoogde risico gold zowel voor symptomatische hersenbloedingen als herseninfarcten. Personen met lobaire microbloedingen hadden een verhoogd risico op hersenbloedingen terwijl personen met tenminste één microbloeding in een non-lobaire regio daarnaast ook een verhoogd risico hadden op herseninfarcten. De laatste twee studies in Hoofdstuk 4 beschrijven hoe microbloedingen samenhangen met een achteruitgang in hersenfuncties.
In Hoofdstuk 4.2 toonde ik aan dat personen met multiple microbloedingen sneller achteruit gingen in hun cognitief functioneren dan personen zonder microbloedingen. Verder vond ik dat het hebben van microbloedingen, ongeacht de locatie of het aantal microbloedingen, gepaard ging met een verhoogd risico op dementie (Hoofdstuk 4.3). Tot slot vond ik dat personen met 'mild cognitive impairment' (MCI), een voorstadium van dementie, vaker wittestofafwijkingen, lacunes, en microbloedingen hadden op hun MRI scans ten opzichte van personen zonder MCI (Hoofdstuk 4.4). De associatie tussen MCI en microbloedingen was echter niet significant.

In Hoofdstuk 5 bediscussieer ik de belangrijkste bevinden van dit proefschrift en vergelijk daarbij onze resultaten met die van eerdere studies. De methodologische tekortkomingen en klinische implicaties worden ook besproken, alsmede mijn suggesties voor toekomstig onderzoek. Samenvattend zijn er drie belangrijke conclusies te trekken op basis van de bevinden genoemd in dit proefschrift. Allereerst, cerebrale microbloedingen duiden op progressieve en wijdverspreide onderliggende hersenschade. Ten tweede, omdat de aanwezigheid van microbloedingen duidt op fragiele en lekkende bloedvaten moet men voorzichtig zijn met het toedienen van geneesmiddelen die het bloedingsrisico verhogen. Tot slot, microbloedingen zijn van klinische betekenis, gezien personen met microbloedingen een verhoogde kans hebben te komen overlijden, een verhoogd risico hebben op het krijgen van een beroerte, sneller achteruit gaan in hun hersenfuncties en een verhoogd risico hebben op dementie in vergelijking tot personen zonder microbloedingen.

CHAPTER 7

APPENDICES



7.1

Acknowledgements

Acknowledgements

Alhamdulillah, after three-and-one-half-years of hard work the time has finally come to thank those who have shown their support throughout my entire PhD period. Although I am not able to mention all individuals who have inspired me over the years to grow as a person and a scientist, I hope you all know that I appreciate your support tremendously.

My first and foremost thanks go to Allah, subhana wa ta'ala, who has blessed me countlessly. Ya Allah, la bi shai'in min ni'mati Rabbi-na nukadhdhib.

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Meike, I am pretty sure that if I would google superwoman your picture would come up. I admire how you manage to do your clinical work and still stay on top of all the developments in your research group. I am definitely indebted to you for all the support and encouragement you have shown me during my PhD. I have enjoyed working with you, not just on the microbleeds, but also on the side projects like the Junior Med School. You are an extremely talented researcher, and I would take pride in becoming half the scientist you are.

A special word of thanks to the promotion committee, prof.dr. Hans Leebeek, prof.dr. Albert Hofman, prof.dr. Geert Jan Biessels, prof.dr. Bruno Stricker, prof.dr. Wiro Niessen, and dr. Anand Viswanathan, who devoted their valuable time to go over my dissertation.

Dear prof. Albert Hofman, my research journey started in 2007, when I participated in the Master of Science program in Clinical Epidemiology. It was

your invitation to take part in this master that triggered my interest in the field of epidemiology. Thank you for giving me the opportunity to be part of the incredible Rotterdam Study.

I extend my sincere appreciation to prof.dr. Moyses Szklo and dr. Anna Kottgen for the amazing research period I had abroad. Dear Moyses, though my period at Bloomberg School of Public health was relatively short I did learn much from the Hopkins epi family. Also, after 8 years of (on and off) epi research I have to say I agree with you: once you get afflicted with the epidemiology fever there is no way back. Dear Anna, I was so lucky to have you as a supervisor during my first scientific project. Thank you for your patience and your efforts to help improve my research skills.

I would like to thank all the co-authors for their constructive feedback on my papers. Your input has been greatly appreciated.

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at the hospital. Thanks for your trust in me and the nice conversations we shared. Liz, my microbleed buddy, I have definitely missed you since you moved to Boston. Thank you for always lightening the mood when necessary, you are a sunshine. Lotte and Renee, working with you girls on our MCI paper was a treat. Lotte, your fun and open personality make you so easy to talk to and confide in. Renee, I had much fun on our conference in Philadelphia, and during the trip you showed me again what a carrying person you are. I am excited to continue working with you in the clinic. Marcel, I could not have done much of the microbleed labeling if it were not for your programming skills, thanks very much. Marileen, we had so much fun in Londen and Glasgow. Also, I cannot thank you enough for all the times you helped me out when I was stuck on something. You are the best. Marius, I have learned so much from you during our joint projects. I do not think there is anything you cannot do. I am sure that with your work ethics and personality you are ready to conquer the world. Nikkie, working with you was always so easy. Thank you for the constructive and fun collaboration. Saira, thank you for always having your door open for me, I will miss you when you move to Toronto. Sanaz, thank you for always being so considerate. Collaborating with you on the kidney project was a delight. Sirwan, thank you for always offering your help whenever I needed it. Vincent, the neurogroup did not feel quit the same without you in it. Thank you for always including me in your lunch round even though I had to skip once or twice :P.

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My warmest thanks to my A-team, my family. Throughout my studies it was your support that has always motivated me to aim high and reach for the sky. They say behind every successful man stand a woman. Well, I believe that behind every successful woman stands a strong, wise, and supportive family. The contentment I feel after having spent time with you is priceless and cannot be matched by any piece of paper or degree.

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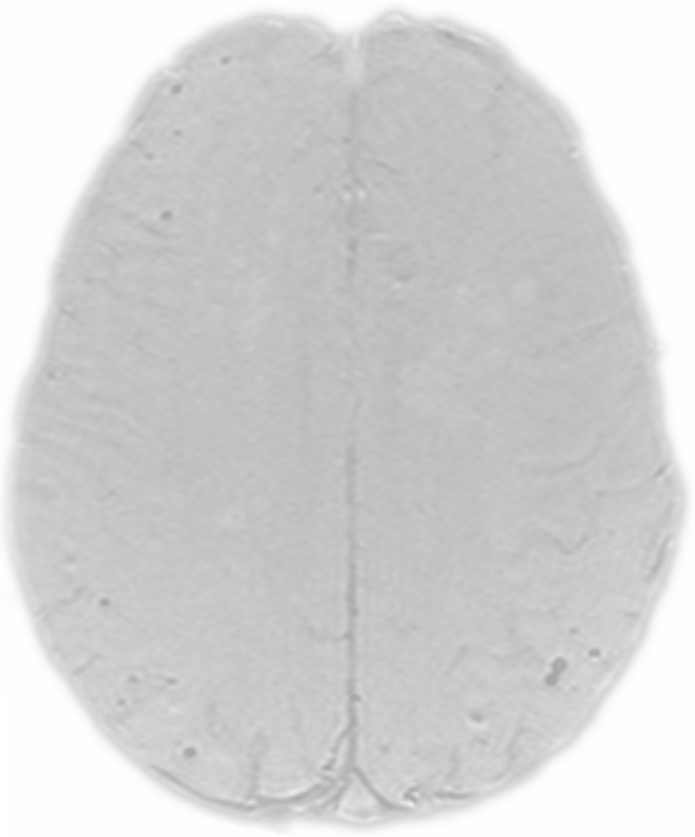
To my beloved parents, the *noor* of my life. There are not enough words to express my gratitude to you. I would not be where I am today if it were not for your love, your sacrifices, and your faith in me. My accomplishments are your accomplishments. I love you to infinity and beyond, jazakallahu khairan.

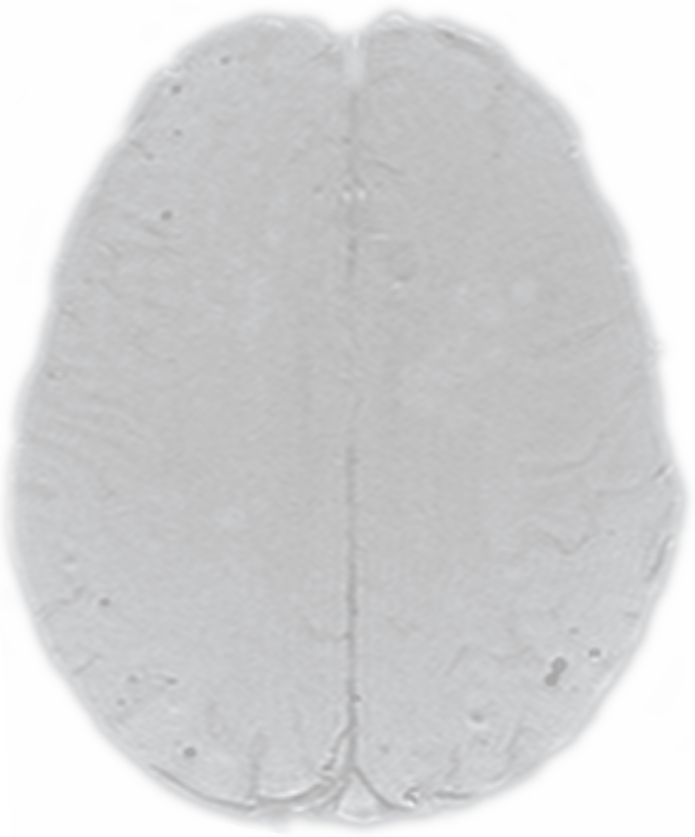


7.2

List of Publications

List of Publications

1. Akoudad S, Portegies ML, Koudstaal PJ, et al. Cerebral Microbleeds are Associated With an Increased Risk of Stroke: The Rotterdam Study. Circulation 2015. (Epub ahead of print)
2. Charidimou A, Linn J, Vernooij MW, et al. Cortical superficial siderosis: detection and clinical significance in cerebral amyloid angiopathy and related conditions. Brain : a journal of neurology 2015. (Epub ahead of print)
3. de Groot M, Ikram MA, Akoudad S, et al. Tract-specific white matter degeneration in aging: the Rotterdam Study. Alzheimer's & dementia : the journal of the Alzheimer's Association 2015;11:321-330.
4. Akoudad S, Sedaghat S, Hofman A, et al. Kidney function and cerebral small vessel disease in the general population. International journal of stroke : official journal of the International Stroke Society 2015;10:603-608.
5. Loehrer E, Ikram MA, Akoudad S, et al. Apolipoprotein E genotype influences spatial distribution of cerebral microbleeds. Neurobiology of aging 2014;35:899-905.
6. de Bruijn RF, Akoudad S, Cremers LG, et al. Determinants, MRI correlates, and prognosis of mild cognitive impairment: the Rotterdam Study. Journal of Alzheimer's disease : JAD 2014;42 Suppl 3:S239-249.
7. Akoudad S, Ikram MA, Koudstaal PJ, et al. Cerebral microbleeds are associated with the progression of ischemic vascular lesions. Cerebrovascular diseases 2014;37:382-388.
8. Akoudad S, Darweesh SK, Leening MJ, et al. Use of coumarin anticoagulants and cerebral microbleeds in the general population. Stroke; a journal of cerebral circulation 2014;45:3436-3439.
9. Aarts N, Akoudad S, Noordam R, et al. Inhibition of serotonin reuptake by antidepressants and cerebral microbleeds in the general population. Stroke; a journal of cerebral circulation 2014;45:1951-1957.
10. Lahousse L, Vernooij MW, Darweesh SK, et al. Chronic obstructive pulmonary disease and cerebral microbleeds. The Rotterdam Study. American journal of respiratory and critical care medicine 2013;188:783-788.
11. Darweesh SK, Leening MJ, Akoudad S, et al. Clopidogrel use is associated with an increased prevalence of cerebral microbleeds in a stroke-free population: the Rotterdam study. Journal of the American Heart Association 2013;2:e000359.
12. Akoudad S, Ikram MA, Koudstaal PJ, Hofman A, van der Lugt A, Vernooij MW. Cerebral microbleeds and the risk of mortality in the general population. European journal of epidemiology 2013;28:815-821.
13. Akoudad S, de Groot M, Koudstaal PJ, et al. Cerebral microbleeds are related to loss of white matter structural integrity. Neurology 2013;81:1930-1937.
14. Akoudad S, Szklo M, McAdams MA, et al. Correlates of kidney stone disease differ by race in a multi-ethnic middle-aged population: the ARIC study. Preventive medicine 2010;51:416-420.



7.3

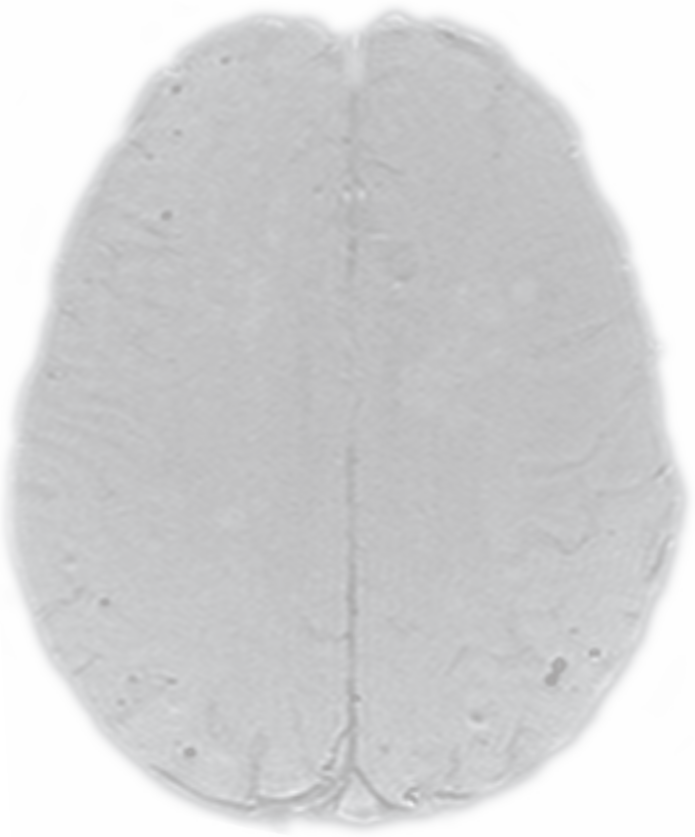
PhD Portofolio

PhD Portofolio

Name PhD candidate: Saloua Akoudad
Erasmus MC department: Epidemiology, Radiology, and Neurology
Research School: Netherlands Institute for Health Sciences (NIHES)
PhD period: January 2012 – July 2015
Promotors: prof.dr. A. van der Lugt, prof.dr. P.J. Koudstaal
Co-promotors: dr. M.W. Vernooij, dr. M.A. Ikram

1. PhD training	Year	Workload (ECTS)
General academic skills		
Master of Science in Clinical Epidemiology, NIHES	2007-2010	42.0
Biomedical English Writing and Communication	2013	4.0
National Institute of Health Ethics Course: Protecting Human Research Participants	2013	1.0
In depth courses		
Conceptual Foundation of Epidemiologic Study Design	2012	0.7
Causal Inference	2012	0.7
Repeated Measurements	2013	1.4
Conferences		
International Cerebral Amyloid Angiopathy Conference, Leiden, the Netherlands.	2012	1.0
NCHA Cognitive function research line meeting, Rotterdam, the Netherlands	2012	0.2
European Stroke Conference, London, UK	2013	1.0
Alzheimer's Association International Conference, Boston, USA	2013	1.0
American Academy of Neurology, Philadelphia, USA	2014	1.0
European Stroke Organisation, Glasgow, UK	2015	1.0
Oral Presentations		
Cerebral microbleeds and white matter microstructural integrity. *International Cerebral Amyloid Angiopathy Conference*	2012	0.6
Cerebral microbleeds and white matter microstructural integrity. *NCHA Cognitive function research line meeting*	2012	0.6
Cerebral microbleeds and the risk of mortality in the general population. *European Stroke Conference*	2013	0.6

Cerebral microbleeds are associated with loss of white matter microstructural integrity. *Alzheimer's Association International Conference*	2013	0.6
Cerebral microbleeds is associated with the progression of ischemic vascular brain lesions. *American Academy of Neurology*	2014	0.6
Use of coumarin anticoagulant drugs and cerebral microbleeds. *American Academy of Neurology*	2014	0.6
Microbleeds and the risk of stroke in the general population. *European Stroke Organisation*	2015	0.6
Genetic risk scores of lipids and intracerebral hemorrhages. *European Stroke Organisation*	2015	0.6
Poster Presentations		
Cerebral microbleeds are associated with poorer white matter microstructural integrity. *Alzheimer's Imaging Consortium, Boston, USA.*	2013	0.6
Cerebral microbleeds and the risk of mortality in the general population. *Alzheimer's Association International Conference, Boston, USA*	2013	0.6
Use of coumarin anticoagulant drugs and cerebral microbleeds. *American Academy of Neurology, Philadelphia, USA.*	2014	0.6
Coumarin anticoagulant use is associated with microbleeds presence in the general population. *International Cerebral Amyloid Angiopathy Conference, London, UK*	2014	0.6
2. Teaching activities		
Assisting NIHES practicals	2013	1.0
Supervising master students	2012-2013	7.0
Supervising Junior Med School students	2014	2.0
3. Other		
Peer Reviewer International Scientific Journals	2012-present	3.0
Assisting during Junior Med School classes	2013-2015	2.0



7.4

About the Author

About the Author

Saloua Akoudad, the daughter of Omar Akoudad and Zaïne El-Mourabit Akoudad, was born in Gouda the Netherlands, on September 24th 1986. After finishing high school at the Goudse Scholengemeenschap Leo Vroman in 2004 (curricula included mathematics, economics, history, and geology), and at ID College in 2005 (curricula included physics, chemistry, and biology) she started her training in medicine at the Erasmus MC University Medical Center Rotterdam. Upon invitation of the scientific director of the Netherlands Institute for Health Sciences, Saloua participated in the Master of Science program in Clinical Epidemiology during her third and fourth year of medical school. As part of this program, Saloua received a basic training in epidemiology and conducted research at the department of Epidemiology under the supervision of Prof.dr. J.C.M. Wittenman and dr. J.F. Felix. After having received a research scholarship Saloua attended Johns Hopkins Bloomberg School of Public Health to complete a six months research period and to participate in the annual Johns Hopkins School of Public Health summer program in Epidemiology and Biostatistics. Upon completion of the research master in 2010, Saloua was awarded the best scientific paper of that academic year.

Following a two-year clinical internship program, Saloua graduated medical school cum laude in January 2012. Starting from January 2012, she started working on her dissertation at the department of Epidemiology at the Erasmus MC University Medical Center Rotterdam. Her dissertation comprises studies that discuss the etiology and clinical relevance of cerebral microbleeds, and was conducted under the supervision of prof.dr. A. van der Lugt, prof.dr. P.J. Koudstaal, dr. M.W. Vernooij, and dr. M.A Ikram. During her PhD period, Saloua was awarded the Young Investigator Award twice: at the 2013 European Stroke Conference for her work on microbleeds and the risk of mortality, and at the 2015 European Stroke Organisation Conference for her work on microbleeds and the risk of stroke.

As of August 2015, Saloua will start her residency in Neurology at the Erasmus Medical Center Rotterdam (head of department, prof.dr. P.A.E. Sillevis Smitt).