DETERMINANTS OF CEREBRAL INFARCTION AND INTRACEREBRAL HEMORRHAGE

The Rotterdam Study

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Determinanten van herseninfarct en hersenbloeding
De Rotterdam Studie

Proefschrift

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van Schie MC*, Wieberdink RG*, Koudstaal PJ, Witteman JCM, Hofman A, Breteler MMB, de Maat MPM, Leebeek FWG. Genetic determinants of von Willebrand factor plasma levels and the risk of stroke: the Rotterdam Study. *J Thromb Haemost*; in press.

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Wieberdink RG, Ho L, Ikram MK, Koudstaal PJ, Hofman A, de Jong PTVM, Vingerling JR, Breteler MMB. Age-related macular degeneration and the risk of stroke: the Rotterdam Study. *Stroke*. 2011; 42:2138-2142.

Chapter 5.1
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Chapter 5.3
Wieberdink RG, Koudstaal PJ, Hofman A, Breteler MMB, Ikram MA. Insulin resistance and the risk of stroke and stroke subtypes in nondiabetic elderly: the Rotterdam Study. *Submitted*.

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CHAPTER 1

GENERAL INTRODUCTION

Despite recent improvements in primary prevention, stroke remains a frequent disorder in the elderly with 40,000 people, of whom 85% older than 55 years, in the Netherlands being hospitalized for stroke in 2009.[1] Moreover, despite advances in patient care, treatment of acute stroke and secondary prevention, stroke remains one of the leading causes of death and disability.[2, 3] Given that our population is rapidly aging, it is inevitable that the burden of stroke will increase even further in the near future.[4] Therefore, stroke is of major concern for public health and additional targets for primary prevention are urgently needed.

The main goal for primary prevention is to interfere with the causal pathways leading to stroke. Apart from insight in the pathophysiologic mechanisms, this requires identification of preferably modifiable risk factors. Furthermore, identification of non-causal risk indicators may aid in the discrimination between people who will get the disease and people who will not get the disease.

Most previous epidemiologic studies have investigated determinants of any stroke. However, strokes are extremely heterogeneous. Roughly, they can be classified as either of ischemic (cerebral infarction) or hemorrhagic (intracerebral hemorrhage), each subgroup with its specific causes and prognosis. Cerebral infarctions comprise 85% to 90% of strokes and are caused by an arterial occlusion due to a thrombus or an embolus, by an occlusion of a small lenticulostriate artery, or by a severe hemodynamic disorder. Intracerebral hemorrhages account for the remainder 10% to 15% of strokes and result from the rupture of an artery or arterial malformation.[5] Distinction between these two subtypes requires brain imaging, which has become widely available and easily accessible nowadays. Although cerebral infarction and intracerebral hemorrhage produce virtually identical symptoms, the difference in etiology suggests that both subtypes may have rather distinct risk factor profiles. Therefore, successful primary prevention may benefit from the identification of subtype-specific risk indicators.

The aims of the work presented in this thesis were twofold. First of all, given the recent advances in primary prevention, I investigated whether stroke incidence rates and the prevalence of stroke risk factors have changed in the Netherlands since 1990. I describe these trends in chapter 2. The second aim was to identify novel determinants of stroke with a particular focus on the subtypes cerebral infarction and intracerebral hemorrhage (chapters 3, 4 and 5).

In chapter 3, I describe the relationship between the plasma glycoprotein von Willebrand factor and stroke and cerebral infarction. Von Willebrand factor is a marker of endothelial dysfunction, but it also has an essential role in hemostasis.[6, 7] Therefore, von Willebrand factor may be a risk factor for stroke. I first investigated the association between plasma von Willebrand factor levels and stroke (chapter 3.1). Subsequently, I studied the contribution of genetic determinants of von Willebrand factor levels to stroke risk (chapter 3.2).

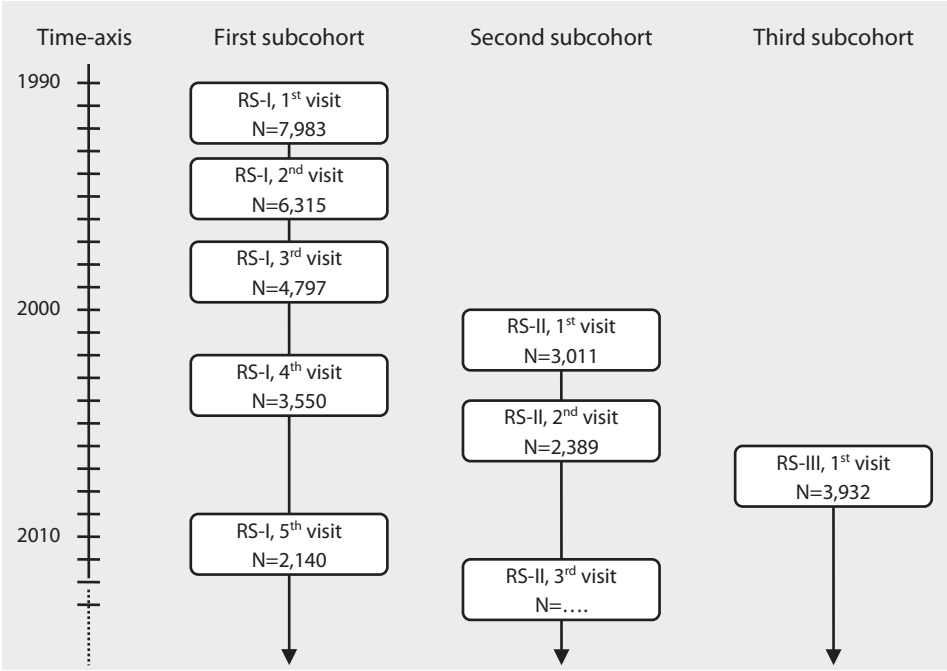
The cerebral circulation is difficult to assess in vivo, whereas the retinal vasculature, which shares many morphologic and physiologic properties with the cerebral vasculature, is easily accessible. Therefore, the retinal microvasculature is increasingly used as a model to study pathologic changes in the cerebral vasculature.[8, 9] Chapter 4 focuses on putative retinal determinants of cerebral infarction and intracerebral hemorrhage. In chapters 4.1, 4.2 and 4.3, I describe the associations of retinal vascular calibers, retinopathy signs, and age-related macular degeneration with risk of cerebral infarction and intracerebral hemorrhage.

I also determined the associations between several metabolic factors and stroke risk. I assessed the relationship between serum lipid fractions and risk of intracerebral hemorrhage and presence of cerebral microbleeds (chapter 5.1). In chapter 5.2, I describe the association between liver enzyme levels and risk of intracerebral hemorrhage. In chapter 5.3, I report my findings regarding the relationship between insulin resistance and risk of any stroke, cerebral infarction, and intracerebral hemorrhage.

Finally, in chapter 6, I discuss the main findings and methodological issues concerning the studies described in this thesis. Next, I discuss potential implications for clinical practice along with some suggestions for future research.

The research described in this thesis is embedded in the Rotterdam Study, a large prospective cohort study among community-dwelling middle-aged and elderly people living in the Ommoord district of Rotterdam in the Netherlands. The Rotterdam Study aims to study the causes and consequences of chronic and disabling diseases that are frequent in the elderly, including stroke.[10] The cohort started in 1990 and included 7,983 participants aged 55 years or older (Rotterdam Study I). In the year 2000, the cohort was expanded with 3,011 people who had reached the age of 55 or had moved into the district since the start of the study (Rotterdam Study II). In 2006, the cohort was further expanded with 3,932 participants aged 45–54 years (Rotterdam Study III). At baseline and during regular follow-up visits, participants undergo an extensive test battery including questionnaires, physical and ophthalmologic examinations and blood sampling. Furthermore, from baseline up to the present day, all participants are continuously monitored for the occurrence of stroke and other major diseases. The studies described in this thesis are based on data obtained from the first and second Rotterdam Study subcohorts (**Figure**).

Figure. Schematic overview of the Rotterdam Study population



Abbreviations: RS-I = Rotterdam Study I; RS-II = Rotterdam Study II; RS-III = Rotterdam Study III.

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CHAPTER 2

STROKE INCIDENCE

Chapter 2

Trends in stroke incidence rates and stroke risk factors in Rotterdam, the Netherlands from 1990 to 2008

ABSTRACT

Background and purpose – Stroke incidence rates have decreased in developed countries over the past 40 years, but trends vary across populations. We investigated whether age-and-sex-specific stroke incidence rates and associated risk factors as well as preventive medication use have changed in the Netherlands during the last two decades.

Methods – The study was part of the Rotterdam Study, a large population-based cohort study among elderly people. Participants were 10,994 men and women aged 55–94 years who were stroke-free at baseline. Trends were calculated by comparing the 1990 subcohort (N=7,516; baseline 1990–1993) with the 2000 subcohort (N=2,883; baseline 2000–2001). Poisson regression was used to calculate incidence rates and incidence rate ratios in age-and-sex-specific strata. We further compared the prevalence of stroke risk factors and preventive medication use in the two subcohorts.

Results – In the 1990 subcohort 467 strokes occurred during 45,428 person years; in the 2000 subcohort 115 strokes occurred in 18,356 person years. Comparing the subcohorts, incidence rates decreased by 34% in men, but remained unchanged in women. Blood pressure levels increased between 1990 and 2000, whereas the proportion of current smokers decreased in men, but not in women. There was a strong increase in medication use for treatment of stroke risk factors across all age categories in both sexes.

Conclusions – Our findings suggest that in Rotterdam between 1990 and 2008 stroke incidence rates have decreased in men but not in women.

INTRODUCTION

The burden of stroke is predicted to increase in developed countries due to increased life expectancy and population aging.[1] On the other hand, age-specific stroke incidence rates have decreased in high-income countries during the past 40 years.[2] Moreover, there are substantial geographic differences in stroke incidence rates and trends over time.[3] Currently it is not clear if stroke incidence rates are changing in the Netherlands.

Stroke is increasingly recognized as a disease that can be prevented.[4] Knowledge of modifiable risk factors and the availability of primary preventive medication has resulted in the development of evidence-based guidelines for the treatment of high-risk individuals.[5] Furthermore, public health campaigns were initiated to improve awareness of risk factors and to promote lifestyle changes on a population level. Whether these interventions resulted in a favorable risk factor profile of the general population and, in parallel, in a reduction in stroke events is unknown and requires investigation. Additionally, studies suggest that women may receive suboptimal treatment of cardiovascular risk factors because of lower perception of risk by treating physicians.[6] Therefore sex-specific changes in risk factor profiles and stroke incidence rates are of particular interest.

The aim of the present study was to describe temporal trends in stroke incidence rates in the Netherlands during the past 20 years, and to investigate changes in preventive medication use and the prevalence and severity of stroke risk factors. We particularly aimed to compare patterns between men and women. All analyses were based on data from the Rotterdam Study, a large prospective population-based cohort study among elderly people living in the city of Rotterdam in the Netherlands.

METHODS

Study population

The Rotterdam Study is an ongoing prospective population-based cohort study that focuses on causes and consequences of chronic and disabling diseases in the elderly.[7] The cohort started in 1990 and included 7,983 participants aged 55 years or older living in Ommoord, a district of the city of Rotterdam in the Netherlands (participation rate 78%). In 2000, the cohort was expanded with 3,011 people who had reached the age of 55 or had moved into the district since the start of the study (response rate 67%). The study was approved by the Medical Ethics Committee of the Erasmus University Medical Center and all participants gave written informed consent to participate in the study.

The present study included participants of the 1990 subcohort and the 2000 subcohort who were aged 55–94 at baseline (N=10,944). Participants who had had a stroke before baseline (N=361) and participants who had not signed consent for the collection of follow-up

data from general practitioners (N=184) were excluded. This resulted in 10,399 participants in the population for analysis (1990 subcohort, N=7,516; 2000 subcohort, N=2,883).

Stroke risk factors and preventive medication use

Trained research physicians visited all participants at home for standardized questionnaires about their health status, medical history and current medication use. Subsequently, participants visited the research center twice for physical examinations and blood sampling. Cigarette smoking behavior was classified as current, past or never. Blood pressure was measured twice at the right brachial artery with a random-zero sphygmomanometer after 5 minutes of rest while the subject was in a sitting position. Hypertension grade I was defined as a diastolic blood pressure of 90–99 mm Hg or a systolic blood pressure of 140–159 mm Hg. Hypertension grade II was defined as a diastolic or systolic blood pressure of ≥100/160 mm Hg, and/or use of blood pressure-lowering medication.[8] Total serum cholesterol, high-density lipoprotein (HDL-)cholesterol and serum glucose levels were measured using an automated enzymatic procedure. Nonfasting blood samples were obtained from the 1990 subcohort, fasting blood samples from the 2000 subcohort. Diabetes mellitus was defined as a fasting serum glucose level ≥7.0 mmol/L, a nonfasting or postload serum glucose level ≥11.1 mmol/L and/or the use of blood glucose-lowering drugs. Body mass index was calculated as weight (in kilograms) divided by the square of height (in meters). Atrial fibrillation was diagnosed when observed on ECG during the visit to the research center or when reported in medical records. History of myocardial infarction was considered positive when self-reported during the interview, observed on ECG during the center visit, and/or confirmed in medical records.

Assessment of stroke

Stroke was defined according to WHO criteria as a syndrome of rapidly developing clinical signs of focal (or global) disturbance of cerebral function, with symptoms lasting 24 hours or longer or leading to death, with no apparent cause other than of vascular origin.[9] History of stroke at baseline was assessed during the baseline interview and verified by reviewing medical records. After enrollment, participants were continuously monitored for incident stroke through automated linkage of the study database with files from general practitioners. Nursing home physicians' files and files from general practitioners of participants who moved out of the district were checked on a regular basis as well. Additional information was obtained from hospital records. Potential strokes were reviewed by research physicians, and verified by an experienced stroke neurologist (P.J.K.). Strokes were further classified as cerebral infarction or intracerebral hemorrhage based on neuroimaging reports. If neuroimaging was lacking, a stroke was classified as unspecified. Transient ischemic attacks or subarachnoid hemorrhages were not included. Participants were followed for incident stroke for a maximum of 7 years, or until death, or until they reached the age of 95, or, if lost to follow-

up, until their last health status update when they were known to be free of stroke, whichever came first. The follow-up of the 1990 subcohort was complete for 99.8% of potential person years; the follow-up of the 2000 subcohort was complete for 97.8% of potential person years.

Statistical analysis

We compared the prevalence of risk factors and preventive medication use between the 1990 and the 2000 subcohorts, in strata of men and women, and in 10-year age categories. Analysis of covariance was used to calculate age-adjusted *P* values for the differences between cohorts. We used Poisson regression to calculate age-adjusted incidence rates and rate ratios in 10 year age strata and in the total population aged 55–94. During the follow-up period, participants could move to a higher age category, i.e., we used a dynamic cohort population. Therefore, participants could contribute person years to subsequent age categories and could be included in the 'number at risk' in more than one age category. Stroke events were counted in the age category the participant belonged to at the moment the stroke occurred. We further assessed whether overall stroke incidence rate ratios differed between men and women by including a multiplicative interaction term (sex*subcohort) in the regression model and we calculated incidence rates and rate ratios in sex-specific strata.

RESULTS

The prevalence of stroke risk factors and preventive medication use in the 1990 and the 2000 subcohorts are presented in **Table 1** for men and in **Table 2** for women. The prevalence of grade II hypertension increased with age and was higher in women than in men within both cohorts. Comparing the 1990 with the 2000 subcohort, we observed an enormous increase in grade II hypertension over time, due to an increase of both systolic and diastolic blood pressure. Blood pressure-lowering medication use had not increased over time, except for men aged 85–94. The prevalence of diabetes mellitus and blood glucose-lowering medication use had increased only in the youngest age groups (men and women aged 55–64) but remained stable in the other strata. Serum HDL-cholesterol levels were higher in women than in men in all age categories. HDL-cholesterol levels did not vary with age and did not differ over time. The 2000 subcohort more often used lipid-lowering medication than the 1990 subcohort. However, in both subcohorts, lipid-lowering medication use was more common in the younger than in the older age groups and more common in men than in women. Body mass index increased over time and was higher in women than in men. The proportion of current cigarette smokers decreased in men, but remained more or less stable in women. In the 1990 subcohort, cigarette smoking was much more frequent in men than in women, but in the 2000 subcohort the proportion of smokers among men and women was almost the same. Men more often had a history of myocardial infarction than women. From 1990 to 2000, the

Table 1. Prevalence of stroke risk factors and preventive medication use in men

Age category	55-64 years			65-74 years			75-84 years			85-94 years		
Subcohort	1990	2000		1990	2000		1990	2000		1990	2000	
No.	N=1,144	N=822	*P* value*	N=1,125	N=277	*P* value*	N=557	N=130	*P* value*	N=122	N=22	*P* value*
Age, years	60.4 (2.8)	59.8 (2.5)	<0.001	69.5 (2.9)	69.5 (2.8)	0.99	79.1 (2.8)	79.0 (3.0)	0.68	88.3 (2.4)	87.7 (2.5)	0.35
SBP, mm Hg	135 (21)	141 (20)	<0.001	140 (21)	148 (23)	<0.001	142 (23)	153 (22)	<0.001	144 (26)	145 (24)	0.96
DBP, mm Hg	76 (11)	82 (11)	<0.001	75 (12)	80 (11)	<0.001	72 (12)	77 (12)	<0.001	71 (13)	73 (11)	0.78
Hypertension, %												
Grade I	20.1	26.2	<0.001	23.9	26.5	0.37	24.9	26.1	0.79	29.6	31.6	0.89
Grade II	25.5	30.9	<0.001	33.4	41.5	0.01	34.3	51.3	<0.001	28.6	47.4	0.12
Diabetes mellitus, %	7.2	10.7	0.01	11.4	13.8	0.28	13.9	17.1	0.36	18.8	14.3	0.71
HDL-cholesterol, mmol/L	1.2 (0.3)	1.2 (0.3)	0.70	1.2 (0.3)	1.2 (0.3)	0.30	1.2 (0.3)	1.3 (0.3)	0.09	1.3 (0.3)	1.3 (0.4)	0.32
Cigarette smoking, %												
Current	29.0	23.7	0.01	23.4	16.3	0.01	20.8	10.1	0.01	20.2	4.5	0.08
Past	55.8	55.1	0.96	63.5	70.7	0.03	56.9	72.1	<0.001	39.5	81.8	<0.001
Myocardial infarction, %	8.0	3.3	<0.001	13.1	10.8	0.30	13.5	9.2	0.19	8.6	13.6	0.41
Atrial fibrillation, %	1.6	1.1	0.40	6.1	5.4	0.67	12.9	8.5	0.17	16.9	22.7	0.30
Body mass index, kg/m^2	25.8 (2.9)	27.2 (3.4)	<0.001	25.7 (2.9)	26.7 (3.2)	<0.001	25.2 (3.2)	25.9 (3.0)	0.04	24.3 (3.6)	25.3 (2.9)	0.26
Preventive medication use, %												
Blood pressure-lowering	22.2	22.5	0.64	30.4	30.7	0.93	36.3	36.9	0.89	38.8	54.5	0.19
Lipid-lowering	3.4	12.7	<0.001	2.7	16.6	<0.001	0.5	9.2	<0.001	0.8	4.5	0.20
Antithrombotic	3.9	12.2	<0.001	9.6	30.0	<0.001	8.1	32.3	<0.001	9.9	40.9	<0.001
Glucose-lowering	2.5	4.0	0.04	5.7	7.2	0.34	5.2	10.0	0.04	4.1	9.1	0.27

Abbreviations: BMI = body mass index; DBP = diastolic blood pressure; SBP = systolic blood pressure; WHR = waist-to-hip-ratio.
Values are means (SD) or percentages.
**P* values are adjusted for age.

Table 2. Prevalence of stroke risk factors and preventive medication use in women

Age category	55-64 years			65-74 years			75-84 years			85-94 years		
Subcohort	1990	2000		1990	2000		1990	2000		1990	2000	
No.	N=1,537	N=1,059	*P* value*	N=1,500	N=282	*P* value*	N=1,055	N=229	*P* value*	N=476	N=62	*P* value*
Age, years	60.2 (2.8)	60.0 (2.5)	0.01	70.0 (2.8)	69.8 (3.0)	0.38	79.5 (2.9)	79.2 (2.7)	0.10	88.5 (2.6)	87.6 (2.0)	0.01
SBP, mm Hg	131 (21)	137 (19)	<0.001	142 (21)	149 (23)	<0.001	148 (23)	159 (23)	<0.001	148 (24)	163 (24)	<0.001
DBP, mm Hg	74 (11)	78 (10)	<0.001	73 (11)	78 (11)	<0.001	73 (12)	78 (11)	<0.001	71 (14)	78 (10)	0.01
Hypertension, %												
Grade I	15.5	24.2	<0.001	22.9	23.0	0.97	23.0	23.4	0.98	23.5	15.9	0.22
Grade II	26.4	28.1	0.20	40.3	48.0	0.02	50.5	63.4	<0.001	51.7	72.7	0.01
Diabetes mellitus, %	4.8	7.7	<0.001	10.4	11.8	0.50	16.5	14.0	0.42	17.9	13.1	0.28
HDL-cholesterol, mmol/L	1.5 (0.4)	1.5 (0.4)	<0.001	1.4 (0.4)	1.4 (0.4)	0.39	1.4 (0.4)	1.4 (0.4)	0.12	1.4 (0.4)	1.5 (0.3)	0.03
Cigarette smoking, %												
Current	25.8	24.5	0.01	18.3	17.1	0.56	10.4	9.2	0.49	3.3	6.6	0.32
Past	32.2	37.5	<0.001	28.6	43.1	<0.001	22.3	38.4	<0.001	15.6	32.8	<0.001
Myocardial infarction, %	1.2	1.1	<0.001	3.6	2.8	0.56	5.9	3.1	0.11	5.0	9.7	0.12
Atrial fibrillation, %	0.8	0.9	0.40	4.0	2.5	0.22	8.5	10.5	0.30	17.1	6.5	0.03
Body mass index, kg/m^2	26.3 (4.0)	27.4 (4.6)	<0.001	27.1 (4.1)	27.6 (4.6)	0.04	26.9 (4.2)	27.8 (4.4)	0.01	26.4 (4.2)	26.5 (4.1)	0.88
Preventive medication use, %												
Blood pressure-lowering	21.7	22.5	0.64	35.2	36.5	0.61	46.6	47.2	0.85	53.8	41.9	0.08
Lipid-lowering	2.6	10.5	<0.001	3.1	13.8	<0.001	0.9	12.7	<0.001	0.4	1.6	0.29
Antithrombotic	1.3	5.3	<0.001	3.7	14.2	<0.001	5.4	26.2	<0.001	5.5	40.3	<0.001
Glucose-lowering	1.7	2.9	0.03	4.9	6.0	0.43	8.1	7.4	0.76	6.5	11.3	0.21

Abbreviations: BMI = body mass index; DBP = diastolic blood pressure; SBP = systolic blood pressure; WHR = waist-to-hip-ratio.
Values are means (SD) or percentages.
**P* values are adjusted for age.

prevalence of myocardial infarction had decreased in men aged 55–64, but was unchanged in other age and sex strata. The presence of atrial fibrillation increased with age but did not change over time. Antithrombotic medication use increased with age and increased enormously over time both in men and women.

The **Figure** shows age-and-sex-specific Kaplan-Meier curves for the stroke-free survival in the 1990 versus the 2000 subcohort. Age-adjusted stroke incidence rates and incidence rate ratios are shown in **Table 3**. In the total population including both men and women, we observed a nonsignificant decline in age-adjusted stroke incidence rates. However, after stratification for sex, we found that the change in incidence rate ratios was different in men and women (*P* interaction=0.04). In men, stroke incidence rates were unchanged in the 55–64 year age category, but decreased in the 65–74, 75–84 and 85–94 year age categories. In all men (55–94), the overall stroke incidence rate had decreased by 34%. In women, age-specific stroke incidence rates showed a variable pattern. Incidence rates decreased in women aged 55–64, increased in women aged 65–74, and were stable in women aged 75–84 and 85–94. In all women (55–94), stroke incidence rates had not changed over time.

DISCUSSION

We investigated trends in stroke incidence rates in Rotterdam in the Netherlands from 1990 to 2008 by comparing two subcohorts of the large population-based Rotterdam Study. We also investigated changes in stroke risk factors and preventive medication use. We found that stroke incidence rates decreased by 34% in men, but remained unchanged in women. In men, but not in women, the proportion of current cigarette smokers had decreased. However, both in men and women, blood pressure levels had increased, whereas blood pressure-lowering medication use was unchanged. Body mass index also increased in men and women. The use of lipid-lowering medication and antithrombotic medication had increased enormously both in men and women.

This study has several strengths, including the prospective and population-based design and the large number of participants. Furthermore, we compared two subcohorts with the same study design and the same inclusion criteria at two different time periods (1990–1998 versus 2000–2008). Thorough stroke monitoring procedures and the nearly complete follow-up allowed us to include virtually all stroke events, even in participants who had not been referred to a neurologist or admitted to a hospital, for example people living in nursing homes or participants who had a fatal stroke. An advantage of this approach was that the trends we observed have not been influenced by changes in referral or admission patterns. However, a disadvantage was that neuroimaging had not been performed in all stroke patients. Because the 2000 subcohort had more widely access to brain CT or MRI than the 1990 subcohort, a larger proportion of events in the 2000 subcohort could be classified as cerebral infarction

Figure. Age-and-sex-specific Kaplan-Meier curves for the stroke-free-survival of the 1990 versus the 2000 subcohort

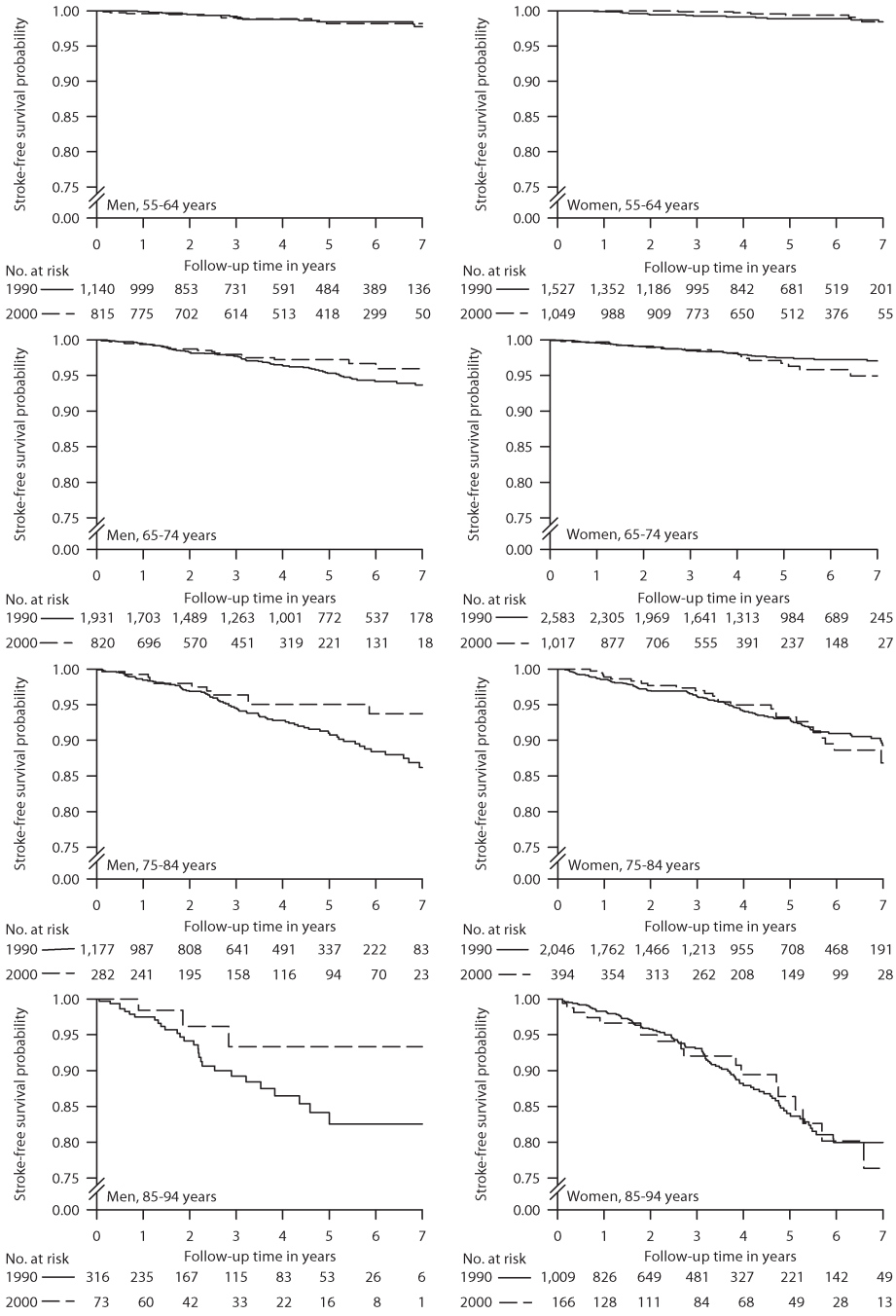

Table 3. Stroke incidence rates and rate ratios

	1990 Subcohort				2000 Subcohort				
	At risk N*	Events N	P yrs	IR/1,000 P yrs †	At risk N*	Events N	P yrs	IR/1,000 P yrs †	IRR (95% CI)†
Total population									
55-64 years	2,667	28	11,289.9	2.65	1,864	19	8,689.9	2.33	0.88 (0.49-1.58)
65-74 years	4,514	120	18,261.6	5.77	1,837	39	6,263.1	6.43	1.11 (0.77-1.61)
75-84 years	3,223	196	11,845.6	16.44	676	36	2,628.7	13.58	0.83 (0.58-1.18)
85-94 years	1,325	123	4,031.1	27.71	239	21	774.8	25.85	0.93 (0.59-1.48)
All ages	7,516	467	45,428.2	7.70	2,883	115	18,356.5	6.85	0.89 (0.72-1.10)
Men									
55-64 years	1,140	14	4,755.1	3.32	815	11	3,826.5	3.30	1.00 (0.45-2.20)
65-74 years	1,931	72	7,883.0	8.47	820	18	2,824.2	6.46	0.76 (0.45-1.29)
75-84 years	1,177	79	4,133.5	18.90	282	11	1,024.6	10.62	0.56 (0.30-1.06)
85-94 years	316	28	845.0	28.05	73	3	217.0	12.95	0.46 (0.14-1.53)
All ages	2,948	193	17,616.6	9.01	1,251	43	7,892.2	5.95	0.66 (0.47-0.92)
Women									
55-64 years	1,527	14	6,534.8	2.05	1,049	8	4,863.4	1.58	0.77 (0.32-1.84)
65-74years	2,583	48	10,378.6	3.70	1,017	21	3,439.0	6.34	1.71 (1.01-2.90)
75-84years	2,046	117	7,712.1	15.10	394	25	1,604.1	15.47	1.02 (0.66-1.58)
85-94 years	1,009	95	3,186.1	27.61	166	18	557.8	30.89	1.12 (0.68-1.85)
All ages	4,568	274	27,811.6	6.79	1,632	72	10,464.3	7.38	1.09 (0.83-1.41)

Abbreviations: IR = incidence rate; IRR = incidence rate ratio; P yrs = person years.
*Persons could contribute person years to more than one age category.
†Incidence rates and incidence rate ratios are adjusted for age and sex.

or intracerebral hemorrhage and a smaller proportion remained unspecified as compared to the 1990 subcohort. As a consequence, we could not compare incidence rates of the stroke subtypes cerebral infarction and intracerebral hemorrhage.

Many previous studies have reported stroke incidence rates and temporal trends. Incidence rates are, however, often hard to compare across studies, because of differences in study design and case ascertainment methods. Trends should be comparable if within-study case ascertainment methods have not changed over time. When we compare our findings to results of other studies covering the same time period, most studies reported either declining[10–16] or stable stroke incidence rates.[17–19] None of the studies, except one, reported divergent trends for men and women.[10] Associated changes in risk factors were usually favorable, contradictory to the increase in blood pressure levels we observed.[13, 15] However, the Auckland Regional Community Stroke Study from New Zealand also reported a decline in men but not in women, despite an increase in blood pressure levels, diabetes mellitus and body mass index, but a reduction in smoking. These finding were very similar to the results of our study, although they did not report on medication use for the treatment of stroke risk factors.[10]

Previous studies have shown that guidelines for the prevention of cardiovascular disease are less strictly applied to women than men, mainly because the perceived risk is lower than the calculated risk.[6] Our findings suggest that this may also be the case in our population. Nevertheless, the decline in stroke incidence rates in men seems somewhat paradoxical to the increase in risk factors we observed, especially the increase in blood pressure levels. Based on the increase in blood pressure levels alone, one would have expected an increase in stroke incidence rates. However, a plausible explanation is that all participants in whom hypertension was diagnosed during the baseline visit were advised to visit their general practitioner for blood pressure surveillance and treatment if indicated. Therefore, blood pressure-lowering drugs will have been described to a substantial number of participants after the baseline examinations, which may have resulted in lower population blood pressure levels during follow-up. Because thresholds for initiating blood pressure treatment decreased in the past 20 years, the beneficial effect of blood pressure-lowering treatment will have been greater to the 2000 subcohort.[8] However, we also observed favorable trends, particularly the reduction in male cigarette smokers, which might have had a beneficial effect on stroke risk. The more widespread prescription of preventive medication such as lipid-lowering drugs and antithrombotic agents may have contributed to the decreased stroke incidence as well.

In conclusion, our study suggests that in Rotterdam in the Netherlands between 1990 and 2008, stroke incidence rates have decreased in men but not in women. The findings support the notion that preventive strategies have been implemented quite successfully, at least in men, but also stress the importance of recognition and treatment of stroke risk factors in women.

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CHAPTER 3

VON WILLEBRAND FACTOR

Chapter 3.1

High von Willebrand factor levels increase the risk of stroke

ABSTRACT

Background and purpose – Many studies have investigated the role of plasma von Willebrand factor level in coronary heart disease, but few have investigated its role in stroke. The aim of this study was to determine if von Willebrand factor levels are associated with the risk of stroke.

Methods – The study was part of the Rotterdam Study, a large population-based cohort study among people aged 55 years or older. We included 6,250 participants who were free from stroke at baseline (1997 to 2001) and for whom blood samples were available. Follow-up for incident stroke was complete up to January 1, 2005. Data were analyzed with Cox proportional hazards models adjusted for age and sex and additionally with models adjusted for other potential confounders including ABO blood group. A subgroup analysis was performed in participants without atrial fibrillation. Effect modification by sex was tested on a multiplicative scale and on an additive scale.

Results – During an average follow-up time of 5.0 years, 290 first-ever strokes occurred, of which 197 were classified as cerebral infarction. The risk of stroke increased with increasing von Willebrand factor levels (age- and sex-adjusted hazard ratios per SD increase in von Willebrand factor level: 1.12 [95% CI, 1.01 to 1.25] for stroke, 1.13 [95% CI, 0.99 to 1.29] for cerebral infarction). Adjustments for additional confounders slightly attenuated the association. The association was also present in people without atrial fibrillation and did not differ between sexes.

Conclusion – High von Willebrand factor levels are associated with stroke risk in the general population.

INTRODUCTION

The plasma glycoprotein von Willebrand factor has an essential role in hemostasis because it promotes platelet adhesion and aggregation at sites of vascular injury and acts as a carrier protein for Factor VIII.[1] Von Willebrand factor is almost exclusively synthesized, stored, and secreted by endothelial cells.[2] The release of von Willebrand factor is increased when endothelial cells are activated or damaged.[3] Therefore, plasma von Willebrand factor level is considered a marker of endothelial dysfunction, a condition that predisposes to atherosclerosis and thrombosis.[4] Because of its direct role in hemostasis, and its indirect role as a marker of endothelial dysfunction, von Willebrand factor is a potential risk indicator for cerebrovascular disease.

Although many investigators have studied the relationship between plasma von Willebrand factor levels and coronary heart disease,[5] little is known about the association between von Willebrand factor levels and stroke. Several case-control studies did report an association with stroke, but von Willebrand factor levels were measured after the stroke was diagnosed. Therefore, it remains debatable whether high levels are a cause or a consequence of stroke.[6–10]

Results from longitudinal studies are limited. Thus far, only one longitudinal study of a stroke-free cohort with a sufficiently large number of incident stroke cases has investigated the relationship between von Willebrand factor levels and stroke in the general population.[11] Because the population of this study was relatively young (45 to 64 years old at baseline), it needs to be investigated if these findings also apply to elderly people, who are at the highest risk of stroke.

We investigated whether plasma von Willebrand factor levels are associated with the risk of stroke and its subtype cerebral infarction in a large population-based cohort study among elderly people. We further examined if the association was different in men and women and whether it could be attributed to the effect of atrial fibrillation on von Willebrand factor levels.

METHODS

Source population

This study is part of the Rotterdam Study, an ongoing prospective population-based cohort study, which started in 1990.[12] Initially, 7,983 persons (of 10,215 invitees) who were aged 55 years or older and living in Ommoord, a district in the city of Rotterdam in the Netherlands, were enrolled in the cohort. In the year 2000, the cohort was expanded with 3,011 persons (of 4,472 invitees) who had reached the age of 55 years or moved into the study district since the start of the study. Baseline examinations consisted of an interview at home and two visits

to the research center for physical examination and blood sampling. These examinations were repeated every 3 to 4 years. All participants were continuously monitored for disease.

For the present study, we included participants of the third examination cycle of the original cohort (baseline 1997 to 1999) and participants of the first examination cycle of the extension of the cohort (baseline 2000 to 2001). The study was approved by the Medical Ethics Committee of the Erasmus University Medical Center in Rotterdam. Written informed consent was obtained from all participants.

Assessment of stroke

History of stroke at baseline was assessed during the baseline interview and verified by reviewing medical records (N=419). After enrollment in the Rotterdam Study, participants were continuously monitored for incident strokes through automated linkage of the study database with files from general practitioners. Nursing home physicians' files and files from general practitioners of participants who moved out of the district were scrutinized as well. Additional information was obtained from hospital records. Potential strokes were reviewed by research physicians and verified by an experienced stroke neurologist (P.J.K.). Subarachnoid hemorrhages were excluded.

Strokes were further classified as cerebral infarction or intracerebral hemorrhage based on the following criteria: cerebral infarction was diagnosed if a CT or MRI scan carried out within four weeks after the event ruled out other diagnoses or if indirect evidence (neurological deficit limited to one limb or completely recovered within 72 hours or atrial fibrillation in the absence of anticoagulant therapy) indicated that the stroke was of an ischemic nature; intracerebral hemorrhage was diagnosed if a relevant hemorrhage was shown on CT or MRI scan or if the person lost consciousness permanently or died within hours after the onset of focal signs. If a stroke did not match any of these criteria, it was classified as unspecified.

Participants were followed from baseline to stroke, death, last health status update when they were known to be free of stroke, or January 1, 2005, whichever came first. For the analysis of cerebral infarction, we censored participants who were diagnosed with intracerebral hemorrhage or unspecified stroke at the date of the event. Follow-up was complete up to January 1, 2005, for 98.6% of potential person years.[13]

Blood sampling procedure and plasma von Willebrand factor measurement

Fasting venous blood samples were taken at the research center and collected in citrated tubes. Samples were stored at -80°C. Von Willebrand factor antigen was determined with an in-house enzyme-linked immunosorbent assay using polyclonal rabbit antihuman von Willebrand factor antibodies (DakoCytomation, Glostrop, Denmark) for catching and tagging.

The intra-assay coefficient of variation was 5.8% and the interassay coefficient of variation was 7.8%.

Baseline measurements

Smoking behavior and current medication use were assessed during the interview at home. Clinical measurements were obtained during 2 visits to the research center. Blood pressure was calculated as the mean of 2 measurements with the random-zero sphygmomanometer at the right brachial artery as the subject was in a sitting position. Hypertension was defined as a diastolic blood pressure ≥90 mm Hg and/or a systolic blood pressure ≥140 mm Hg and/or the use of blood pressure-lowering medication indicated for the treatment of high blood pressure (grade I hypertension or higher according to World Health Organization criteria).[14] Total cholesterol and high-density lipoprotein (HDL-)cholesterol were measured with an automated enzymatic procedure. Diabetes mellitus was defined as the use of serum glucose-lowering medication and/or a fasting serum glucose level ≥7.0 mmol/L. The waist-to-hip ratio was calculated by dividing the waist circumference (in centimeters) by the hip circumference (in centimeters). Body mass index was calculated as weight (in kilograms) divided by the square of height (in meters). History of myocardial infarction was determined during the baseline interview and verified in medical records. History of coronary heart disease was positive if the participant underwent a revascularization procedure or fulfilled the criteria of myocardial infarction. Prevalent and incident atrial fibrillation were ascertained using the following methods: (1) an electrocardiogram was recorded during baseline visits and follow-up rounds; and (2) information was obtained from general practitioners' files, hospital records, and the national registration system of hospital discharge diagnoses.[15] The presence of peripheral arterial disease was evaluated by measuring the systolic blood pressure level of the posterior tibial artery at both legs using a Doppler probe and a random-zero sphygmomanometer with the subject in a supine position. The ratio of the systolic blood pressure at the ankle to the systolic blood pressure at the brachial artery was calculated for each leg. Peripheral arterial disease was considered present if the ankle-brachial index was <0.9 in at least one leg.[16] Blood group antigen phenotypes were reconstructed by haplotype analysis of four single nucleotide polymorphisms, rs687289, rs507666, rs8176704, and rs8176749, which collectively serve as tagging single nucleotide polymorphisms for the O, A1, A2, and B allele.[17]

Population for analysis

A total of 8,517 persons were free from stroke at baseline and eligible to participate. Of these, 2,267 persons were not included in the analyses because they did not visit the research center (N=1,869) or because blood draw or storage failed (N=398). In total, 6,250 participants were included in the analyses.

Statistical analysis

Von Willebrand factor levels were truncated at the mean±4 standard deviations (SD) to remove outliers. Age- and sex-adjusted mean values (SD) or percentages of stroke risk factors across von Willebrand factor quartiles were computed by analysis of covariance. We used Cox proportional hazards regression to determine hazard ratios with 95% confidence intervals for the association between plasma von Willebrand factor levels and stroke. Only first-ever strokes were included in the analyses. Hazard ratios were expressed per standard deviation increase in von Willebrand factor level and in strata of von Willebrand factor quartile (relative to the lowest quartile). The linear trend across quartiles was tested by including the quartile categories as a continuous variable in the models. All hazard ratios were adjusted for age and sex (Model 1) and additionally for other putative confounders (systolic blood pressure, diabetes mellitus, total serum cholesterol level, serum HDL-cholesterol level, lipid-lowering medication use, current cigarette smoking, waist-to-hip ratio, atrial fibrillation, coronary heart disease, peripheral arterial disease, and antithrombotic medication use; Model 2) and ABO blood group (Model 3). Missing values in covariates were imputed by using a linear regression model based on age and sex.

Subsequently, we analyzed if the association between von Willebrand factor and stroke was different in men and women. Interaction, or effect modification, is usually determined by entering a product term in the regression model. Because the Cox proportional hazards model is a multiplicative model, adding a product term results in a measure of interaction as a departure from multiplicity. However, the preferred approach to examine biological interaction rather than statistical interaction is to estimate interaction as a departure from additivity.[18] In this study, we examined both types of interaction. We tested for interaction on a multiplicative scale by adding a product term to the model, and we tested for biological interaction or effect measure modification on an additive scale by estimating the relative excess risk due to interaction and its 95% confidence interval.[19–21]

Finally, we performed a subgroup analysis among participants who were free from atrial fibrillation at baseline (N=5,959). Participants who developed atrial fibrillation during follow-up were censored at the date of onset of atrial fibrillation.

RESULTS

Baseline characteristics of the study population are shown in **Table 1**. At baseline, the mean age was 69.1 years, and 57.2% of the participants were female. **Table 2** describes the baseline characteristics across quartiles of the von Willebrand factor distribution. Von Willebrand factor levels rose with age. ABO blood group was strongly related to von Willebrand factor level. Diabetes mellitus and cardiovascular disease were more prevalent in participants with higher von Willebrand factor levels.

Tabel 1. Baseline characteristics (N=6,250)

Age, years	69.1 (8.2)
Female sex, %	57.2
Systolic blood pressure, mm Hg	143.2 (21.3)
Diastolic blood pressure, mm Hg	76.8 (11.2)
Hypertension, %	62.5
Blood pressure-lowering medication use, %	23.6
Fasting serum glucose level, mmol/L	6.0 (1.6)
Diabetes mellitus, %	12.8
Current cigarette smoking, %	17.4
Serum total cholesterol level, mmol/L	5.81 (0.98)
Serum HDL-cholesterol level, mmol/L	1.39 (0.40)
Lipid-lowering medication use, %	12.8
Waist-to-hip ratio	0.92 (0.10)
Body mass index, kg/m^2	27.0 (4.0)
History of cardiovascular disease, %	26.7
Atrial fibrillation, %	4.6
Myocardial infarction, %	8.0
Coronary heart disease, %	10.5
Peripheral arterial disease, %	15.3
Antithrombotic medication use, %	19.1
ABO blood group, %	
O	45.6
A	42.3
B	8.8
AB	3.3
Plasma von Willebrand factor level, IU/mL	1.31 (0.54)

Values are means (SD) or percentages.

During 31,489 person years of follow-up (mean 5.0 years), 290 participants developed a stroke (153 women), of which 197 were classified as cerebral infarction, 28 as intracerebral hemorrhage, and 65 as unspecified. CT or MRI imaging reports were available for 72.4% of strokes and for 92.4% of cerebral infarctions. **Table 3** shows the association between plasma von Willebrand factor level and the risk of stroke and cerebral infarction. After adjustment for age and sex, higher von Willebrand factor levels were associated with an increased risk of stroke. Additional adjustment for multiple putative confounders, including ABO blood group, had only a minor effect on the association. Effect estimates for the association between von Willebrand factor level and cerebral infarction were of similar magnitude, although not statistically significant.

Sex was not a significant effect modifier of the association between von Willebrand factor and stroke either on a multiplicative scale (P=0.33) or on an additive scale (P=0.69), indicating that the association between von Willebrand factor and stroke was not different in men and women.

The association between plasma von Willebrand factor level and stroke in the subgroup of participants without atrial fibrillation was similar to the association we found in the total cohort. Age- and sex-adjusted hazard ratios per SD increase in von Willebrand factor level were 1.15 (95% CI, 1.03 to 1.18) for stroke and 1.15 (1.01 to 1.32) for cerebral infarction.

Tabel 2. Baseline characteristics across von Willebrand factor quartiles

Von Willebrand factor quartile	1	2	3	4	*P* value*
Range, IU/mL	0.24–0.92	0.92–1.20	1.20–1.59	1.60–3.64	
No.	1,563	1,556	1,557	1,554	
Age, years	66.7 (7.3)	68.0 (7.9)	69.7 (8.1)	71.9 (8.4)	<0.001
Female sex, %	59.2	56.9	55.8	56.9	0.26
Systolic blood pressure, mm Hg	142.9 (21.1)	143.6 (20.9)	144.0 (20.9)	142.2 (21.2)	0.10
Diastolic blood pressure, mm Hg	76.8 (11.1)	77.0 (11.0)	77.1 (11.0)	76.1 (11.1)	0.06
Hypertension, %	61.4	62.7	63.8	62.0	0.55
Blood pressure-lowering medication use, %	24.6	22.7	23.6	23.4	0.65
Serum glucose level, mmol/L	5.8 (1.6)	5.9 (1.6)	6.0 (1.5)	6.2 (1.6)	<0.001
Diabetes mellitus, %	11.2	10.9	13.3	15.9	<0.001
Current cigarette smoking, %	17.2	16.8	17.9	18.0	0.79
Serum total cholesterol level, mmol/L	5.85 (0.95)	5.81 (0.95)	5.81 (0.95)	5.77 (0.94)	0.15
Serum HDL-cholesterol level, mmol/L	1.41 (0.39)	1.41 (0.35)	1.39 (0.36)	1.36 (0.39)	<0.001
Lipid-lowering medication use, %	11.0	12.2	13.2	14.8	0.02
Waist-to-hip ratio	0.91 (0.08)	0.92 (0.08)	0.92 (0.08)	0.92 (0.08)	0.04
Body mass index, kg/m^2	26.5 (4.0)	26.6 (3.9)	27.2 (3.9)	27.5 (4.0)	<0.001
History of cardiovascular disease, %	25.7	25.0	26.1	29.8	0.01
Atrial fibrillation, %	3.9	3.9	4.2	6.5	<0.001
Myocardial infarction, %	7.0	7.2	7.7	10.2	0.01
Coronary heart disease, %	9.6	9.5	9.7	13.1	<0.001
Peripheral arterial disease, %	15.3	15.5	15.7	14.7	0.87
Antithrombotic medication use, %	18.5	17.7	18.8	21.4	0.05
ABO blood group, %					
O	70.7	52.3	37.0	22.2	<0.001
A	25.3	38.0	47.5	58.6	<0.001
B	3.3	6.8	12.0	13.2	<0.001
AB	0.8	3.0	3.6	5.9	<0.001

Values are means (SD) or percentages.
**P* values are adjusted for age and sex when applicable.

DISCUSSION

In the present study among people aged 55 years or older who were free from stroke at baseline, we found that plasma von Willebrand factor levels were associated with the risk of stroke. The association was only slightly attenuated after adjustment for multiple potential confounders and was similar in participants without atrial fibrillation. There was no evidence

Tabel 3. Association between plasma von Willebrand factor level and stroke

		Model 1*	Model 2†	Model 3‡
		HR (95% CI)	HR (95% CI)	HR (95% CI)
Any stroke (N=290)				
Per SD		1.12 (1.01–1.25)	1.11 (1.00–1.24)	1.12 (1.00–1.25)
Quartile 1	[0.24–0.92]	1.00 (ref)	1.00 (ref)	1.00 (ref)
Quartile 2	[0.92–1.20]	1.03 (0.70–1.51)	1.02 (0.69–1.49)	1.02 (0.70–1.50)
Quartile 3	[1.20–1.59]	1.29 (0.91–1.84)	1.26 (0.88–1.79)	1.27 (0.89–1.81)
Quartile 4	[1.59–3.64]	1.37 (0.97–1.93)	1.32 (0.93–1.87)	1.33 (0.93–1.91)
P trend		0.035	0.056	0.061
Cerebral infarction (N=197)				
Per SD		1.13 (0.99–1.29)	1.12 (0.98–1.27)	1.10 (0.95–1.26)
Quartile 1	[0.24–0.92]	1.00 (ref)	1.00 (ref)	1.00 (ref)
Quartile 2	[0.92–1.20]	1.00 (0.64–1.58)	0.99 (0.63–1.55)	0.96 (0.61–1.52)
Quartile 3	[1.20–1.59]	1.26 (0.83–1.93)	1.23 (0.80–1.87)	1.19 (0.77–1.82)
Quartile 4	[1.59–3.64]	1.38 (0.91–2.09)	1.32 (0.87–2.00)	1.25 (0.81–1.92)
P trend		0.066	0.108	0.194

*Model 1: adjusted for age and sex.
†Model 2: adjusted for age, sex, systolic blood pressure, diabetes mellitus, serum total cholesterol level, serum HDL-cholesterol level, lipid-lowering medication use, current cigarette smoking, waist-to-hip ratio, atrial fibrillation, coronary heart disease, peripheral arterial disease, and antithrombotic medication use.
‡Model 3: like Model 2, additionally adjusted for ABO blood group.

for effect modification by sex.

Before interpreting the results, some methodological issues need to be considered. The strengths of our study are its prospective and population-based design, the large number of participants, and the long and nearly complete follow-up (loss of potential person years only 1.4%). Thorough stroke monitoring procedures allowed us to include also patients with stroke who had not been referred to a neurologist. A disadvantage of this procedure was that neuroimaging had not been performed in 28% of patients with stroke. However, 92% of cerebral infarctions had been confirmed by CT or MRI. We used single imputation methods to replace missing values in covariates. Single imputation methods are considered to produce unbiased results but too much precision compared with multiple imputation methods.[22] However, because the overall number of missing values in our data set was small, we think the method we used did not have a strong influence on the results.

This is the first study that shows an association between von Willebrand factor levels and stroke in an elderly population independent from conventional cardiovascular risk factors and ABO blood group. Previous studies have investigated the relationship between von Willebrand factor levels and stroke. The majority of these studies used a case-control design in which von Willebrand factor levels were determined after the stroke was diagnosed.[6–10] Results

from these studies are likely to be biased by post-stroke changes in von Willebrand factor levels. Four longitudinal studies have reported on the association between von Willebrand factor and stroke in the general population.[11, 23–26] In a matched case-control study among people with atrial fibrillation nested within the Rotterdam Study, no association was found between von Willebrand factor levels and stroke.[23] The Caerphilly Study among middle-aged men also did not find an association between von Willebrand factor levels and stroke after a median follow-up period of 13 years.[24] The Edinburgh Artery Study found a modest, nonsignificant association when participants were followed for a maximum of 5 years,[25] but the association disappeared when the average follow-up period was extended to 17 years.[26] The lack of an association in the Caerphilly Study and the prolonged Edinburgh Artery Study might be explained by interindividual variability in von Willebrand factor levels in the long-term, resulting in dilution of the presumed effect. Furthermore, the results of these studies have to be interpreted carefully because they were small or had not excluded participants with prevalent stroke at baseline. Results of the Atherosclerosis Risk in Communities (ARIC) Study, the only prospective study of a cohort free of stroke at baseline with a large number of incident stroke cases, were very much in line with the results of our study.[11] Because ARIC Study participants were younger (45 to 64 years of age at baseline), our study is the first to provide information about von Willebrand factor level and stroke risk in the elderly population (aged 55 years or older).

ABO blood group is a strong determinant of plasma von Willebrand factor levels. Blood group A and B antigens, which are located on the surface of von Willebrand factor molecules, decrease von Willebrand factor clearance. As a result, von Willebrand factor levels are approximately 25% higher in individuals with blood group non-O than in individuals with blood group O.[27] Because several studies have linked ABO blood group to stroke risk,[28] blood group may be a confounder of the association between von Willebrand factor level and stroke. However, adjustment for ABO blood group did not alter the association between von Willebrand factor and stroke, suggesting that the association between von Willebrand factor and stroke is independent from ABO blood group.

We performed a subgroup analysis among participants without atrial fibrillation and found that the association between von Willebrand factor level and stroke was also present in this subgroup and of similar magnitude as the association we found in the total cohort. Unfortunately, we were not able to examine this association in participants with atrial fibrillation, because the number of stroke cases in the subpopulation with atrial fibrillation was small. However, our findings in participants without atrial fibrillation suggest that the association between von Willebrand factor and stroke is not principally driven by the presence of atrial fibrillation.

Recently, there has been increasing awareness of sex differences in stroke. Several studies have shown that the risk factor profile is different in male and female patients with stroke.

Men with stroke are more likely to have a history of heart disease, myocardial infarction, peripheral arterial disease, diabetes mellitus, and alcohol and tobacco use, whereas women with stroke are older at onset and more likely to have atrial fibrillation and hypertension.[29–34] Furthermore, it has been suggested that risk factors may have a different effect on stroke risk in men and women.[34] This motivated us to investigate if sex differences influenced the association between von Willebrand factor and stroke risk. However, we did not detect any evidence for the presence of effect modification by sex in our study.

The association we found between von Willebrand factor levels and the subtype cerebral infarction was of similar magnitude as the association we found with any stroke. However, probably due to the smaller number of events, the association was no longer statistically significant at the conventional α=0.05 level. Further studies and systematic reviews are required to establish the nature of the association with confidence.

To conclude, plasma von Willebrand factor levels are associated with risk of stroke in the general population, independent from cardiovascular risk factors and ABO blood group. The association is also present in people without atrial fibrillation and does not differ between men and women.

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Chapter 3.2

Genetic determinants of von Willebrand factor plasma levels and the risk of stroke

ABSTRACT

Background and purpose – High plasma von Willebrand factor levels are associated with an increased risk of stroke. Von Willebrand factor levels are strongly heritable. A meta-analysis of five large genome-wide association studies identified single nucleotide polymorphisms (SNPs) within eight genetic loci as determinants of von Willebrand factor levels. Whether these SNPs are associated with stroke risk is unknown. The aim of this study was to investigate the association between genetic determinants of plasma von Willebrand factor levels and risk of stroke.

Methods – The study was part of the Rotterdam Study, a large population-based cohort study among people aged 55 years or older. A total of 5,763 participants who were stroke-free at baseline and for whom DNA was available were included in the analysis. Von Willebrand factor levels were measured in 3,379 participants. In each of the eight previously identified loci, one top SNP was defined. First, we analyzed the associations between these eight top SNPs and the risk of stroke. Second, we constructed a genetic score based on these eight SNPs, and we investigated the association of the genetic score with plasma von Willebrand factor levels and with the risk of stroke.

Results – None of the eight top SNPs were individually associated with stroke risk. Higher values of the genetic score were associated with higher plasma von Willebrand factor levels, but they were not associated with an increased risk of stroke.

Conclusion – In this large population-based cohort study among elderly people, eight SNPs that strongly determine plasma von Willebrand factor levels were not associated with stroke risk, either individually, or combined in a genetic score.

INTRODUCTION

The large multimeric glycoprotein von Willebrand factor is involved in platelet adhesion and aggregation at sites of vascular injury and therefore has a central role in primary hemostasis.[1, 2] Plasma von Willebrand factor levels are influenced by non-genetic factors, such as hormones and inflammation, but are also strongly heritable with estimates of heritability ranging from 53–75%.[3, 4] Although variants within the von Willebrand factor gene have shown to be associated with plasma von Willebrand factor levels,[5, 6] the blood group gene is considered the strongest genetic determinant. Variants within the ABO blood group gene encode blood group A and B antigens, which increase plasma von Willebrand factor levels by at least 25%.[7] Previously, a meta-analysis of five genome-wide association studies conducted in the Cohort for Heart and Aging Research in Genomic Epidemiology (CHARGE) Consortium identified single nucleotide polymorphisms (SNPs) in eight different loci that were genome-wide significantly associated with plasma von Willebrand factor levels.[8] Apart from known SNPs located in the blood group gene and the von Willebrand factor gene, the meta-analysis also revealed unknown SNPs within new candidate genes, among which were the syntaxin-2 (STX2) gene and the syntaxin-binding-protein-5 (STXBP5) gene.

Because several studies have shown that high plasma von Willebrand factor levels are associated with an increased risk of stroke,[9–11] we hypothesized that genetic determinants of von Willebrand factor levels may be associated with risk of stroke as well.

Previous studies that investigated the relationship between genetic determinants of plasma von Willebrand factor levels, either located in the blood group gene or located in the von Willebrand factor gene, and cardiovascular disease reported inconsistent results.[6, 12–14] Possible explanations for the inconsistencies could be that these studies were underpowered and heterogeneous with respect to study endpoint, study population and study design. In addition, these studies primarily focused on coronary heart disease rather than stroke. Whether variants in the new candidate genes, as identified by the CHARGE meta-analysis, are associated with the risk of stroke has yet not been investigated.

The aim of the present study was to investigate if genetic determinants of plasma von Willebrand factor levels are associated with the risk of any stroke and cerebral infarction. We analyzed both the individual effects and the joint effects of genetic variants in association with stroke risk.

METHODS

Source population

The study was part of the Rotterdam Study, an ongoing prospective population-based cohort study, which started in 1990.[15] All inhabitants of Ommoord, a district of the city of

Rotterdam in the Netherland, who were 55 years of age or older were invited to participate, and 7,983 persons agreed (response rate 78%). Invitation into the study occurred in random order. Baseline examinations consisted of an interview at home and two visits to the research center for physical examination and blood sampling. The examinations were repeated every three to four years. All participants are continuously followed for a variety of diseases that are frequent in the elderly, including stroke.[15]

The study was approved by the Medical Ethics Committee of the Erasmus University Medical Center in Rotterdam. Written informed consent was obtained from all participants.

Assessment of stroke

Stroke was defined as rapidly developing clinical signs of focal (or global) disturbance of cerebral function with no apparent cause other than a vascular origin.[16] History of stroke at baseline was assessed during the baseline interview and verified by reviewing medical records. After enrollment in the Rotterdam study, participants were continuously monitored for stroke through automated linkage of the study database with files from general practitioners. Nursing home physicians' files and files from general practitioners of participants who moved out of the district were scrutinized as well. For reported strokes, additional information was obtained from hospital records. Potential strokes were reviewed by research physicians, and verified by an experienced stroke neurologist (P.J.K.).

Strokes were further classified as cerebral infarction or intracerebral hemorrhage based on neuroimaging results. If neuroimaging was lacking, a stroke was classified as unspecified. Subarachnoid hemorrhages were excluded. Follow-up was complete up to January 1, 2005 for 98.6% of potential person years.[17]

Blood sampling procedure, plasma von Willebrand factor measurement and genotyping

Fasting venous blood samples were taken at the research center. Samples were snap frozen in liquid nitrogen and stored at -80°C. Von Willebrand factor antigen levels were determined with an in-house enzyme-linked immunosorbent assay, using polyclonal rabbit antihuman von Willebrand factor antibodies (DakoCytomation, Glostrop, Denmark) for capturing and detecting. The intra-assay coefficient of variation was 5.2% and the interassay coefficient of variation was 6.3%. The assay used commercial reference plasma (Normal reference plasma, Precision Biologic, Kordia, Leiden, the Netherlands) which was standardized against the World Health Organization standard by the manufacturer and expressed in IU/mL.

Genomic DNA was extracted from whole blood samples according to standard methods. In the Rotterdam Study, genotyping was attempted with the Infinium HumanHap 550 K chip (Illumina, version 3) in persons with high-quality extracted DNA (N=6,449). From these 6,449, samples with low call rate (<97.5%, N=209), samples with excess autosomal

heterozygosity (>0.336, N=21), samples with sex-mismatch (N=36), or outliers identified by the identity-by-state (IBS) clustering analysis (>3 standard deviations from population mean, N=102 or IBS probabilities >97%, N=129) were excluded from the study population. Some participants met more than one exclusion criterion. In total, 5,974 samples with good quality genotyping data were available. Genotype data were used to impute the ~2.6 million autosomal SNPs using HapMap CEU (phase II, release 22, build 36) as the reference population. The set of genotyped input SNPs used for imputation was selected based on their highest quality genome-wide association data. We used a call rate >98%, a minor allele frequency >0.01; a Hardy-Weinberg *P* value >$1x10^{-6}$; and a test of differential missingness by the 'mishap' test in PLINK *P* value >$1x10^{-9}$. We used the Markov Chain Haplotyping (MaCH) package for imputation. Imputation and quality-control measures have been described in more detail previously.[8, 18–20]

Baseline measurements

Smoking behavior was assessed during the interview at home. Clinical measurements were obtained during the visits to the research center. Blood pressure was calculated as the mean of two measurements with the random-zero sphygmomanometer at the right brachial artery while the subject was in a sitting position. Hypertension was defined as a diastolic blood pressure of ≥90 mm Hg and/or a systolic blood pressure of ≥140 mm Hg and/or the use of blood pressure-lowering medication indicated for the treatment of high blood pressure (grade I hypertension or higher according to World Health Organization criteria).[21] Serum total cholesterol level was measured with an automated enzymatic procedure. Diabetes mellitus was defined as the use of serum glucose-lowering medication and/or a fasting serum glucose level ≥7.0 mmol/L. Body mass index was calculated as weight (in kilograms) divided by the square of height (in meters). History of myocardial infarction was determined during the baseline interview and verified in medical records. History of coronary heart disease was positive if the participant underwent a revascularization procedure, or fulfilled the criteria of myocardial infarction. Prevalent and incident atrial fibrillation were ascertained using the following methods: (1) An ECG was recorded during baseline visits and follow-up rounds; (2) information was obtained from general practitioners files, hospital records, and the national registration system of hospital discharge diagnoses.[22] The presence of peripheral arterial disease was evaluated by measuring the systolic blood pressure level of the posterior tibial artery at both legs using a Doppler probe and a random-zero sphygmomanometer with the subject in a supine position. The ratio of the systolic blood pressure at the ankle to the systolic blood pressure at the brachial artery was calculated for each leg. Peripheral arterial disease was considered present if the ankle-brachial index was <0.9 in at least one leg.[23] Blood group genotypes were reconstructed by haplotype analysis of four SNPs; rs687289, rs507666, rs8176704, and rs8176749, which collectively serve as tagging SNPs for the O, A1, A2 and B alleles.[24]

Haplotypes were inferred using the R package haplo.stats.[25, 26] The haplotype analysis calculates posterior probabilities for every possible haplotype (and for each possible diplotype). In this way, the blood group genotype of all participants could be estimated with a probability of more than 99%.

Population for analysis

From the 7,983 Rotterdam Study participants, we did not include in the study population participants who had had a stroke before baseline (N=261), participants who had refused written informed consent for the collection of follow-up data from general practitioners (N=193), and participants of whom we did not have DNA (N=1,784). This resulted in 5,763 participants in the population for analysis. Plasma von Willebrand factor levels were measured in blood collected during the third examination visit (1997 to 1999) and could be determined for 3,379 participants.

Statistical analysis

In the present study, we used eight SNPs, so called 'top SNPs' (rs9390459, rs2726953, rs687621, rs1063857, rs4981022, rs7978987, rs10133762, and rs868875). These SNPs were identified previously by the CHARGE meta-analysis as the variant within each von Willebrand factor-level-influencing genetic locus that most strongly associated with plasma von Willebrand factor level.[8]

First, we investigated if these eight top SNPs associated with plasma von Willebrand factor levels in our study population by means of linear regression. We used Cox proportional hazards regression to determine the associations between the individual SNPs and risk of any stroke and cerebral infarction. Hazard ratios were expressed per von Willebrand factor level increasing allele.

Second, we constructed two genetic scores.[27, 28] The first genetic score included the eight top SNPs as weighted sums of the number of von Willebrand factor level increasing alleles carried by an individual. Weighting was based on the effect estimates obtained from the CHARGE meta-analysis, which were presented as percentage change in von Willebrand factor level per allele. For instance, the genetic score for an individual who carries two von Willebrand factor level increasing alleles for the blood group SNP rs687621, and one von Willebrand factor level increasing allele for the von Willebrand factor SNP rs1063857 is (2 x 24.1%) + (1 x 6.0%) = 54.2. The second genetic score was created to investigate the association between the genetic determinants and stroke risk independent from ABO blood group. This second score was based on seven SNPs and did not include the blood group SNP rs687621. Analysis of covariance was used to determine age- and sex-adjusted mean von Willebrand factor levels per quartile of the genetic scores and linear regression was used to estimate the trend across quartiles. Regarding the second genetic score, analyses were performed separately in partic-

ipants with blood group O and in participants with a blood group different from O (non-O). We used Cox proportional hazards regression to determine hazard ratios and 95% confidence intervals for the associations between the two genetic scores and risk of any stroke and cerebral infarction. Hazard ratios for the genetic scores were expressed in quartiles (relative to the lowest quartile). The linear trend across quartiles was calculated by including the quartile categories as a continuous variable in the model. Associations of the second genetic score with risk of any stroke and cerebral infarction were stratified by blood group (O versus non-O). All hazard ratios were adjusted for age and sex, and additionally for potential confounders (current cigarette smoking, hypertension, diabetes mellitus, serum total cholesterol level, body mass index and history of cardiovascular disease).

We then investigated the contribution of ABO blood group to von Willebrand factor levels and stroke risk in more detail. We calculated age- and sex-adjusted mean von Willebrand factor levels for each blood group genotype using analysis of covariance. Next, we calculated mean von Willebrand factor levels per number of non-O blood group alleles (0, 1, or 2), and per number of von Willebrand factor level increasing blood group alleles (0, 1, or 2). Von Willebrand factor level increasing alleles were all non-O alleles except the A2 allele. In addition, we used Cox proportional hazards regression to determine association between the number of blood group non-O alleles and risk of stroke. We also determined the association between the number of von Willebrand factor level increasing blood group alleles and risk of stroke. For all analyses, we assumed an additive and independent genetic effect model. Analyses were performed using R, SPSS version 15 (SPSS Inc, Chicago, USA), Haploview, and the haplo.stats package.

Table 1. Baseline characteristics (N=5,763)

Age, years	68.0 (61.9-75.2)
Female sex, %	59.4
European ancestry, %	100
Current cigarette smoking, %	20.5
Hypertension, %	50.9
Diabetes mellitus, %	9.7
Serum total cholesterol level, mmol/L	6.5 (5.8-7.4)
Body mass index, kg/m^2	25.9 (23.8-28.4)
History of cardiovascular disease, %*	28.0
Von Willebrand factor level, IU/mL	1.25 (0.95-1.66)

Values are medians (interquartile range) or percentages.
* History of cardiovascular disease includes atrial fibrillation, coronary heart disease and peripheral arterial disease.

RESULTS

Baseline characteristics of the study population are presented in **Table 1**. During 58,371 person years of follow-up (mean, 10.1 years), 632 participants suffered a stroke which was classified as cerebral infarction in 348, as intracerebral hemorrhage in 44, and as unspecified

Table 2. Association between top SNPs and von Willebrand factor levels (N=3,379)

Region	Gene	SNP	VWF increasing allele frequency*		β (s.e.)†	*P* value
6q24	STXBP5	rs9390459	G/A	0.59	0.057 (0.02)	<0.001
8p21	SCARA5	rs2726953	T/C	0.31	0.034 (0.02)	0.04
9q34	ABO	rs687621	C/T	0.33	0.297 (0.02)	<0.001
12p13	VWF	rs1063857	C/T	0.35	0.055 (0.02)	<0.001
12q23	STAB2	rs4981022	T/C	0.68	0.044 (0.02)	<0.001
12q24.3	STX2	rs7978987	A/G	0.34	0.029 (0.02)	0.07
14q32	TC2N	rs10133762	T/G	0.45	0.008 (0.02)	0.58
19p13.2	CLEC4M	rs868875	A/G	0.75	0.047 (0.02)	0.04

Abbreviations: SNP = single nucleotide polymorphism; VWF = von Willebrand factor.
*The VWF level increasing allele is underlined.
†β indicates the change in VWF level (IU/mL) per VWF level increasing allele, adjusted for age and sex.

in 240. At baseline, the median age was 68.0 (61.9–75.2) years. Of the study participants, 59.4 % were women and 28.0% had a history of cardiovascular disease.

Associations between the eight top SNPs and von Willebrand factor levels are listed in **Table 2**. Six of the eight SNPs were associated with von Willebrand factor levels.

The age- and sex-adjusted mean von Willebrand factor levels per quartile of the first

Figure. Von Willebrand factor levels per genetic score quartile.

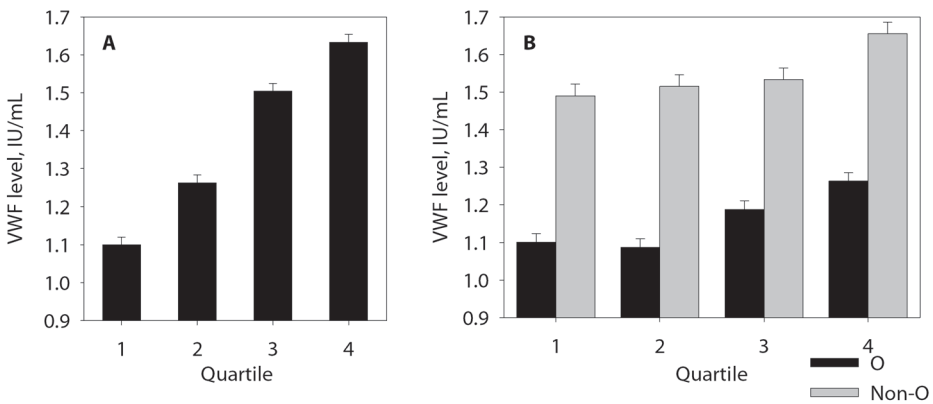

Figure A presents the mean (s.e.) von Willebrand factor level in IU/mL per quartile of the genetic score (*P* trend < 0.01). Quartile ranges: Q1, 0.50-29.68; Q2, 29.69-44.60; Q3, 44.61-56.38; Q4, 56.39-98.39.
Figure B presents the mean (s.e.) von Willebrand factor level in IU/mL per quartile of the genetic score stratified by blood group (*P* trend < 0.01 for both blood groups). Quartile ranges blood group O: Q1, 0.50-23.35; Q2, 23.36-28.43; Q3, 28.44-33.61; Q4, 33.62-54.59. Quartile ranges blood group non-O: Q1, 3.51-23.62; Q2, 23.63-28.52; Q3, 28.54-33.55; Q4, 33.56-53.27.
Von Willebrand factor levels are adjusted for age and sex.

genetic score are presented in **Figure A**. We found a strong linear association between increasing values of the genetic score and plasma von Willebrand factor level (*P* trend<0.01). Mean von Willebrand factor levels per quartile of the second genetic score (without the blood group SNP) are shown in **Figure B**. In both blood group strata (O and non-O), increasing values of the genetic score were associated with increasing von Willebrand factor levels (*P* trend<0.01).

Table 3 shows the associations between individual SNPs and risk of any stroke and cerebral infarction, adjusted for age and sex. None of the eight SNPs were associated with the

Table 3. Association between top SNPs and risk of stroke (N=5,763)

SNP	Gene	Any stroke N=632 HR (95% CI)*	Cerebral infarction N=348 HR (95% CI)*
rs9390459	STXBP5	0.97 (0.87-1.09)	0.93 (0.80-1.08)
rs2726953	SCARA5	0.99 (0.88-1.12)	1.00 (0.84-1.17)
rs687621	ABO	0.97 (0.86-1.09)	1.02 (0.88-1.20)
rs1063857	VWF	1.04 (0.92-1.16)	1.10 (0.94-1.28)
rs4981022	STAB2	1.08 (0.96-1.22)	1.08 (0.92-1.26)
rs7978987	STX2	0.97 (0.86-1.09)	0.98 (0.84-1.14)
rs10133762	TC2N	0.96 (0.86-1.07)	1.03 (0.89-1.20)
rs868875	CLEC4M	1.00 (0.85-1.17)	1.02 (0.82-1.27)

Abbreviations: SNP = single nucleotide polymorphism.
*Hazard ratios are expressed per von Willebrand factor level increasing allele and are adjusted for age and sex.

risk of any stroke or cerebral infarction. Additional adjustments for potential confounders (current cigarette smoking, hypertension, diabetes mellitus, serum total cholesterol level, body mass index and history of cardiovascular disease) did not affect the associations (results not shown).

Increasing values of the genetic scores were not associated with risk of any stroke or cerebral infarction, either in the total cohort, or in strata of blood group O and non-O (**Table 4**). Additional adjustments for potential confounders did not change any of the associations (results not shown).

Table 5 presents the mean von Willebrand factor levels per blood group genotype. Regarding the non-O genotypes, the lowest mean von Willebrand factor levels were found in the A2O genotype. The mean von Willebrand factor level per number of von Willebrand factor level increasing blood group alleles was 1.09 IU/mL for zero alleles, 1.47 for one allele and 1.63 for two alleles. The mean von Willebrand factor level per number of blood group

Table 4. Association between the genetic score and risk of stroke (N=5,763)

		Any stroke		Cerebral infarction	
Genetic score	At risk, N	Events, N	HR (95% CI)*	Events, N	HR (95% CI)*
Total cohort					
Quartile 1	1440	159	1.00 (ref)	79	1.00 (ref)
Quartile 2	1441	151	0.93 (0.75-1.16)	86	1.08 (0.79-1.46)
Quartile 3	1441	179	1.12 (0.91-1.39)	102	1.29 (0.97-1.74)
Quartile 4	1441	143	0.89 (0.71-1.11)	81	1.02 (0.75-1.39)
P trend			0.68		0.62
Blood group O					
Quartile 1	642	66	1.00 (ref)	29	1.00 (ref)
Quartile 2	643	77	1.17 (0.84-1.63)	43	1.46 (0.91-2.33)
Quartile 3	642	71	1.06 (0.76-1.49)	39	1.33 (0.82-2.15)
Quartile 4	643	70	1.05 (0.75-1.47)	39	1.32 (0.82-2.13)
P trend			0.95		0.38
Blood group Non-O					
Quartile 1	797	77	1.00 (ref)	48	1.00 (ref)
Quartile 2	797	96	1.20 (0.89-1.62)	54	1.11 (0.75-1.64)
Quartile 3	797	94	1.12 (0.83-1.51)	49	0.96 (0.64-1.43)
Quartile 4	797	80	1.03 (0.75-1.40)	46	0.96 (0.64-1.43)
P trend			0.99		0.66

*Hazard ratios are adjusted for age and sex.

non-O alleles was 1.08 IU/mL for zero alleles, 1.39 IU/mL for one allele, and 1.59 IU/mL for two alleles. However, neither the number of von Willebrand factor increasing blood group alleles, nor the number of blood group non-O alleles were associated with the risk of any stroke or the risk of cerebral infarction (results not shown).

DISCUSSION

The principal finding in this large prospective population-based cohort study is that genetic variants that strongly determine plasma von Willebrand factor levels are not associated with the risk of any stroke or cerebral infarction in the general elderly population. We constructed a score of previously identified genetic determinants of plasma von Willebrand factor levels and demonstrated that this score strongly associated with plasma von Willebrand factor levels in an elderly population. Because blood group is by far the strongest genetic determinant of plasma von Willebrand factor levels, we investigated whether the association between the

Table 5. Blood group-specific plasma von Willebrand factor levels (N=3,376)

ABO Blood group			VWF level
Genotype	Phenotype	N (%)	Mean (s.e.)*
OO	O	1,536 (45.5%)	1.08 (0.01)
A_1O	A	872 (25.8%)	1.45 (0.01)
A_2O	A	300 (8.9%)	1.14 (0.02)
BO	B	284 (8.4%)	1.50 (0.02)
A_1A_1	A	146 (4.3%)	1.67 (0.03)
A_1A_2	A	104 (3.1%)	1.52 (0.04)
A_1B	AB	83 (2.5%)	1.58 (0.04)
A_2A_2	A	17 (0.5%)	1.34 (0.09)
A_2B	AB	26 (0.8%)	1.71 (0.08)
BB	B	8 (0.2%)	1.78 (0.14)

*Mean VWF levels are adjusted for age and sex.

genetic score and plasma von Willebrand factor levels was mainly driven by the blood group variant, or influenced by other genetic variants as well. We found that in people with blood group O as well as in people with blood group non-O, the genetic score without the blood group variant still associated with plasma von Willebrand factor levels. These findings suggest that genetic constitution contributes substantially to plasma von Willebrand factor levels and strengthen the results of previous studies.[3, 4]

In the present study, SNP rs1063857, which is located within the von Willebrand factor gene, was not associated with the risk of stroke. This observation is contradictory to the previously reported association between SNP rs1063857 and stroke risk in the ATTAC study. In the ATTAC study, a hospital-based case-control study among relatively young individuals (men aged 45 or younger; women aged 55 or younger) who had a first acute myocardial infarction or cerebral infarction, an association was found between rs1063857 and cardiovascular disease, which was mainly attributable to the association with cerebral infarction.[6] The conflicting results might be explained by the large difference in age range between the ATTACK study and the Rotterdam Study. Indeed, previous studies have shown that the genetic contribution to the development of cardiovascular disease, including stroke, is stronger in younger people.[29, 30]

As far as we know, this is the first prospective cohort study reporting on the association between blood group and stroke risk. We found no association between blood group and risk of stroke or cerebral infarction, opposite to a meta-analysis of case-control studies that did report a positive association between blood group non-O and risk of cerebral infarction.[14] Most studies reporting on the association between blood group and stroke risk compared the risk in strata of blood group O and blood group non-O. However, it has been suggested

that von Willebrand factor levels in people with blood group A2 are comparable to von Willebrand factor levels in people with blood group O rather than non-O.[31, 32] We reconstructed blood group genotypes, and showed that von Willebrand factor levels were indeed lower for the A2O genotype than for all other non-O genotypes. Assuming that the association between blood group and stroke risk may be mediated by plasma von Willebrand factor levels, we hypothesized that a different definition of the potential risk increasing allele could possibly influence the observed association between blood group and stroke risk. Therefore, we investigated the association between blood group non-O alleles and risk of stroke as well as the association between the number of von Willebrand factor increasing alleles and risk of stroke. Although the different classification of risk alleles did better reflect plasma von Willebrand factor levels, there was also no association between these risk alleles and stroke risk.

Strengths of the present study are the large population based study cohort, the prospective setting and the long duration of the follow-up period. In addition, because the Rotterdam Study included only elderly people, the results of the study specifically apply to the population at the highest risk of stroke. Our study has some limitations as well. Different risk factors may be involved in different stroke subtypes. However, the number of incident stroke events was too small to study associations between the genetic determinants and risk of intracerebral hemorrhage, or to study genotype-phenotype associations underlying the subtypes of cerebral infarction. In this study, considering a power of 80% and a minor allele frequency of 30%, a minimum hazard ratio of 1.25 per risk allele could be identified. Expecting a hazard ratio of 1.25 is reasonable, because previous studies investigating the association between von Willebrand factor and stroke risk reported hazard ratios ranging from 1.33 to 1.70.[9, 11] However, this study may have been underpowered to detect hazard ratios smaller than 1.25. Another issue is that we do not know whether our results can be generalized to younger populations or to populations of other than Caucasian ethnicity.

In conclusion, we showed that von Willebrand factor levels are strongly influenced by genetic variants. However, genetic determinants of plasma von Willebrand factor levels themselves are not associated with the risk of any stroke or cerebral infarction in elderly people. Although previous studies have shown that high von Willebrand factor plasma levels are associated with an increased risk of stroke, our results suggest that von Willebrand factor is not a strong causal factor for stroke.

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CHAPTER 4

RETINA

Chapter 4.1

Retinal vascular calibers and the risk of intracerebral hemorrhage and cerebral infarction

ABSTRACT

Background – Narrower retinal arteriolar calibers and wider venular calibers are associated with cardiovascular disease, including cerebral infarction. We investigated the association between retinal vascular calibers and the long-term risk of stroke and its subtypes with particular focus on intracerebral hemorrhage.

Methods – We included 5,518 participants aged 55 years or older from the prospective population-based Rotterdam Study who were stroke-free at baseline (1990–1993) and of whom digital retinal images were available. Follow-up for incident stroke was complete up to January 1, 2007. Data were analyzed with Cox proportional hazards models adjusted for age and sex and additionally for potential confounders. Arteriolar and venular calibers were entered both separately and simultaneously in the models.

Results – During an average follow-up of 11.5 years, 623 participants developed a first-ever stroke, of which 50 were classified as intracerebral hemorrhage, 361 as cerebral infarction, and 212 as unspecified stroke. Wider venular caliber was independently associated with an increased risk of stroke (HR per SD increase, 1.20; 95% CI, 1.09 to 1.33), cerebral infarction (HR, 1.28; 95% CI, 1.13 to 1.46), and intracerebral hemorrhage (HR, 1.53; 95% CI, 1.09 to 2.15). Much weaker, only borderline significant associations were found between arteriolar caliber and risk of stroke (HR per SD decrease, 1.12; 95% CI, 0.99 to 1.23), cerebral infarction (HR, 1.12; 95% CI, 0.98 to 1.27), and intracerebral hemorrhage (HR, 1.25; 95% CI, 0.87 to 1.79). Retinal vascular calibers were strongly associated with lobar hemorrhages and oral anticoagulant-related hemorrhages.

Conclusion – Wider retinal venular caliber is associated with an increased risk of stroke in the general population and, in particular, with an increased risk of intracerebral hemorrhage.

INTRODUCTION

Intracerebral hemorrhage accounts for approximately 10% to 15% of strokes and leads to high rates of death and disability in elderly people.[1] Only a few risk factors of intracerebral hemorrhage have been identified, of which hypertension is the most frequent and the most important.[2] Because the outcome of intracerebral hemorrhage is poor and present treatment results are disappointing, prevention seems the most effective approach.[3, 4] To identify people at risk for intracerebral hemorrhage, detection of new risk factors and risk indicators is extremely important.

Pathological studies have shown that the majority of intracerebral hemorrhages result from rupture of small arteries and arterioles affected by either hypertension-related degenerative changes or cerebral amyloid angiopathy.[5, 6] These pathological vascular changes, which may develop asymptomatically until the sudden onset of intracerebral hemorrhage, are difficult to assess in vivo. The retinal vasculature, which shares many morphological and physiological properties with the cerebral vasculature, can be visualized directly and noninvasively with digitized fundus photography. Retinal vascular caliber changes are considered markers of cerebral microvascular changes and can be used as a model to study the relationship between cerebral microvascular pathology and intracerebral hemorrhage.[7, 8]

Retinal arteriolar and retinal venular caliber changes are considered to mark different pathological processes, because they are related differently to cardiovascular risk factors and disease. High blood pressure is the major systemic determinant of narrower arteriolar caliber,[9] whereas wider venular caliber is related to smoking, glucose levels, and markers of atherosclerosis and inflammation.[10–12] Both narrower arteriolar caliber and wider venular caliber have been associated with an increased risk of coronary heart disease.[13] In contrast, only wider venular caliber was reportedly associated with the risk of stroke, cerebral infarction, and progression of cerebral small vessel disease.[14–16] Data on the association between retinal vascular calibers and intracerebral hemorrhage are limited. Recently, a hospital-based cross-sectional study among acute stroke patients found that retinal vascular calibers were similar in patients with deep intracerebral hemorrhage and lacunar infarction, but patients with deep intracerebral hemorrhage were more likely to have narrower arterioles and wider venules than patients with nonlacunar stroke.[17] The longitudinal relationship between retinal vascular calibers and incident intracerebral hemorrhage has not been investigated.

The aim of the present study was to investigate the association between retinal vascular calibers and the long-term risk of stroke and its subtypes in the general elderly population. We particularly focused on the association between retinal vascular calibers and intracerebral hemorrhage.

METHODS

Source population

This study is part of the Rotterdam Study, a prospective population-based cohort study, which started in 1990 and is still ongoing.[18] All inhabitants who were 55 years of age or older and living in Ommoord, a district in the city of Rotterdam in the Netherlands, were invited to participate, and 7,983 persons agreed (response rate 78%). Invitation into the study occurred in random order. Baseline examinations consisted of an interview at home and two visits to the research center for physical examination and blood sampling. Because the ophthalmologic part of the study became operational after the main study had begun, a smaller number of participants (N=6,780) underwent the ophthalmologic examination. All participants were followed for a variety of diseases that are common in the elderly, including stroke.[18] The study was approved by the Medical Ethics Committee of the Erasmus University Medical Center in Rotterdam. Written informed consent was obtained from all participants.

Assessment of stroke

History of stroke at baseline was assessed during the baseline interview and verified by reviewing medical records. After enrollment, participants were continuously monitored for incident stroke through automated linkage of the study database with files from general practitioners. Nursing home physicians' files and files from general practitioners of participants who moved out of the district were examined as well. Additional information was obtained from hospital records. Potential strokes were reviewed by research physicians and verified by an experienced stroke neurologist (P.J.K.).

Strokes were classified as cerebral infarction or intracerebral hemorrhage on the basis of neuroimaging reports. If neuroimaging was lacking, strokes were classified as unspecified. Intracerebral hemorrhages were further categorized into one of two categories: lobar, when arising in the frontal, parietal, temporal, or occipital lobes; and deep, when occurring in the basal ganglia, thalamus, brain stem, or cerebellum. Very large hematomas with ventricular extension were also categorized as deep. Purely intraventricular hemorrhages and hemorrhages of undetermined localization were not categorized. Data on the use of oral anticoagulants at time of stroke were collected from medical records to classify hemorrhages as anticoagulation-related or non-anticoagulation-related. Cerebral infarctions were further categorized as cortical or lacunar based on clinical symptoms alone, or, when visible on CT or MRI, based on clinical and imaging features.[19] Subarachnoid hemorrhages were excluded.

Participants were followed from baseline to stroke; death; last health status update when they were known to be free of stroke; or January 1, 2007, whichever came first. Follow-up was complete up to January 1, 2007 for 96.2% of potential person years.[20]

Retinal vascular caliber measurement

Participants underwent a full eye examination at baseline, including simultaneous stereoscopic fundus color photography of the optic disc (20° field, Topcon Optical Company, Tokyo, Japan) after pharmacological mydriasis. The transparencies from both eyes were digitized with a high-resolution scanner (Nikon LS-4000, Nikon Corporation), and, for each participant, the digitized image with the best quality (left or right eye) was analyzed with the Retinal Vessel Measurement System (Retinal Analysis Optimate; Department of Ophthalmology & Visual Science, University of Wisconsin, Madison).[21] The rationale and procedures to measure and summarize retinal vascular calibers have been described.[10, 21] Summary measures for arteriolar and venular calibers were based on improved Parr-Hubbard formulas and were corrected for magnification changes attributable to refractive errors of the eye. Four trained graders performed the assessments, blinded to the clinical characteristics of the participants. In a random subsample of 100 participants, we found no differences between the right and left eyes for the arteriolar and venular calibers. Pearson's correlation coefficients for intergrader agreement were 0.67 to 0.80 for arteriolar caliber and 0.91 to 0.94 for venular caliber. Corresponding figures for intragrader agreement were 0.69 to 0.88 and 0.90 to 0.95.[10]

Cardiovascular risk factors at baseline

Blood pressure was calculated as the mean of two measurements with the random-zero sphygmomanometer at the right brachial artery while the subject was in a sitting position. Hypertension was defined as a diastolic blood pressure of ≥90 mm Hg and/or a systolic blood pressure of ≥140 mm Hg and/or the use of blood pressure-lowering medication. Diabetes mellitus was defined as a nonfasting or postload serum glucose level of ≥11.1 mmol/L and/or the use of glucose-lowering drugs. Total cholesterol, high-density lipoprotein (HDL-)cholesterol, and C-reactive protein were measured in nonfasting serum with an automated enzymatic procedure. Prevalent heart failure and left ventricular hypertrophy were determined as described previously.[22, 23] Smoking behavior, alcohol intake, and current medication use were assessed during a standardized interview.[24]

Study population

From the 6,780 participants who underwent the ophthalmologic examination, we excluded participants who had had a stroke before baseline (N=199) or had refused informed consent for the collection of follow-up data from general practitioners (N=34). Of the remaining 6,547 participants at risk for stroke, 1,029 persons could not be included in the analyses because fundus transparencies were not available or not gradable. In total, 5,518 participants were included in the analyses.

Statistical analysis

We used Cox proportional hazards regression to determine hazard ratios and 95% confidence intervals for the associations between baseline retinal vascular calibers and any incident stroke, intracerebral hemorrhage, and cerebral infarction. Only first-ever strokes were included in the analyses. Hazard ratios for stroke and its subtypes were calculated by analyzing arteriolar calibers per SD decrease and venular calibers per SD increase. To verify the linearity of associations, we also categorized vessel calibers in quartiles. We constructed four models: (1) age and sex plus either the arteriolar or the venular caliber; (2) age and sex plus both the arteriolar and the venular caliber; (3) age, sex, both vascular calibers, and cardiovascular risk factors (hypertension, diabetes mellitus, current smoking, serum total cholesterol level, serum HDL-cholesterol level, C-reactive protein level, body mass index); and (4) as model 3, plus additional putative confounders (systolic blood pressure, blood pressure-lowering medication use, alcohol intake, left ventricular hypertrophy, and heart failure). Missing values in covariates were imputed with a linear regression model based on age and sex.

We further explored the relationship between retinal vascular calibers and intracerebral hemorrhage by categorizing intracerebral hemorrhages as lobar or deep and by classifying hemorrhages as anticoagulation-related or non-anticoagulation-related. Associations were adjusted for age, sex, and the fellow-vessel caliber.

RESULTS

During 63,306 person years of follow-up (mean 11.5 years), 623 participants developed a stroke, of which 50 were classified as intracerebral hemorrhage, 361 as cerebral infarction, and 212 as unspecified. The localization of intracerebral hemorrhage was lobar in 25 and deep in 22 (3 did not fit in either of the categories). Among the 50 intracerebral hemorrhages, 13 were related to anticoagulation use and 37 were not. Baseline characteristics of the study population are shown in **Table 1**. At baseline, the mean age was 67.8 years, and 59.1% of

Table 1. Baseline characteristics (N=5,518)

Age, years	67.8 (8.1)
Female sex, %	59.1
Current smoking, %	23.4
Nonfasting serum glucose level, mmol/L	6.8 (2.6)
Diabetes mellitus, %	10.7
Hypertension, %	55.4
Systolic blood pressure, mm Hg	138.3 (22.0)
Diastolic blood pressure, mm Hg	73.7 (11.3)
Blood pressure-lowering medication use, %	30.1
Body mass index, kg/m^2	26.3 (3.7)
Serum total cholesterol level, mmol/L	6.6 (1.2)
Serum HDL-cholesterol level, mmol/L	1.4 (0.4)
C-reactive protein level, mg/L	3.1 (6.1)
Alcohol intake, gram/day	10.3 (15.0)
Heart failure, %	3.0
Left ventricular hypertrophy, %	3.8
Retinal arteriolar caliber, µm	146.9 (14.4)
Retinal venular caliber, µm	220.0 (20.8)

Values are means (SD) or percentages.

the participants were women.

Associations between retinal arteriolar caliber and stroke and its major subtypes are shown in **Table 2**. When adjusted for age and sex only, narrower arteriolar caliber was not associated with risk of any stroke or its subtypes (Model 1). When we adjusted for venular caliber (Model 2) and cardiovascular risk factors (Model 3), narrower arteriolar caliber became weakly, but nonsignificantly, associated with risk of any stroke and cerebral infarction. However, a significant trend toward an increased risk of intracerebral hemorrhage across decreasing quartiles of arteriolar caliber became apparent (*P* trend=0.03).

Table 2. Association between retinal arteriolar caliber and stroke

		Model 1† HR (95% CI)	Model 2‡ HR (95% CI)	Model 3§ HR (95% CI)
Any stroke (N=623)				
Per SD*		1.00 (0.93–1.08)	1.14 (1.04–1.26)	1.12 (0.99–1.23)
Quartile 4	[155.6–235.7]	1.00 (ref)	1.00 (ref)	1.00 (ref)
Quartile 3	[146.2–155.6]	1.06 (0.85–1.32)	1.21 (0.96–1.53)	1.18 (0.94–1.49)
Quartile 2	[137.4–146.2]	0.97 (0.77–1.21)	1.20 (0.94–1.55)	1.15 (0.89–1.48)
Quartile 1	[92.2–137.4]	1.03 (0.82–1.29)	1.43 (1.09–1.87)	1.35 (1.03–1.77)
Intracerebral hemorrhage (N=50)				
Per SD*		0.95 (0.72–1.25)	1.27 (0.89–1.80)	1.25 (0.87–1.79)
Quartile 4	[155.6–235.7]	1.00 (ref)	1.00 (ref)	1.00 (ref)
Quartile 3	[146.2–155.6]	0.59 (0.25–1.43)	0.84 (0.34–2.10)	0.83 (0.33–2.06)
Quartile 2	[137.4–146.2]	1.12 (0.53–2.36)	1.99 (0.86–4.57)	1.99 (0.86–4.58)
Quartile 1	[92.2–137.4]	1.02 (0.48–2.18)	2.35 (0.93–5.95)	2.33 (0.91–5.94)
Cerebral infarction (N=361)				
Per SD*		0.97 (0.88–0.97)	1.16 (1.02–1.32)	1.12 (0.98–1.27)
Quartile 4	[155.6–235.7]	1.00 (ref)	1.00 (ref)	1.00 (ref)
Quartile 3	[146.2–155.6]	1.08 (0.81–1.43)	1.28 (0.95–1.72)	1.22 (0.91–1.64)
Quartile 2	[137.4–146.2]	0.86 (0.63–1.16)	1.12 (0.81–1.56)	1.06 (0.76–1.47)
Quartile 1	[92.2–137.4]	0.91 (0.68–1.23)	1.37 (0.96–1.95)	1.27 (0.89–1.80)

*Hazard ratios are expressed per standard deviation decrease in retinal arteriolar caliber.
†Model 1: adjusted for age and sex.
‡Model 2: adjusted for age, sex, and retinal venular caliber.
§Model 3: as Model 2, additionally adjusted for hypertension, diabetes mellitus, current smoking, serum total cholesterol level, serum HDL-cholesterol level, C-reactive protein level, and body mass index.

Table 3 shows the association between venular caliber and stroke risk. After adjustment for age and sex, wider venular caliber was associated with an increased risk of any stroke, intracerebral hemorrhage, and cerebral infarction (Model 1). Adjustment for arteriolar caliber strengthened the associations (Model 2). Additional adjustments for hypertension, diabetes mellitus, current smoking, serum total cholesterol level, serum HDL-cholesterol level, C-reactive protein level, and body mass index (Model 3) only minimally attenuated

Table 3. Association between retinal venular caliber and stroke

		Model 1†	Model 2‡	Model 3§
		HR (95% CI)	HR (95% CI)	HR (95% CI)
Any stroke (N=623)				
Per SD*		1.15 (1.07–1.25)	1.25 (1.14–1.38)	1.20 (1.09–1.33)
Quartile 1	[135.1–207.7]	1.00 (ref)	1.00 (ref)	1.00 (ref)
Quartile 2	[207.7–221.4]	1.18 (0.94–1.48)	1.24 (0.99–1.57)	1.21 (0.96–1.53)
Quartile 3	[221.4 –235.1]	1.20 (0.95–1.51)	1.33 (1.04–1.70)	1.28 (1.00–1.64)
Quartile 4	[235.1–313.6]	1.50 (1.20–1.88)	1.76 (1.35–2.29)	1.61 (1.24–2.10)
Intracerebral hemorrhage (N=50)				
Per SD*		1.40 (1.07–1.83)	1.61 (1.15–2.25)	1.53 (1.09–2.15)
Quartile 1	[135.1–207.7]	1.00 (ref)	1.00 (ref)	1.00 (ref)
Quartile 2	[207.7–221.4]	0.88 (0.36–2.13)	0.94 (0.38–2.31)	0.93 (0.38–2.31)
Quartile 3	[221.4 –235.1]	1.21 (0.53–2.75)	1.36 (0.56–3.30)	1.34 (0.55–3.26)
Quartile 4	[235.1–313.6]	1.89 (0.88–4.05)	2.28 (0.92–5.63)	1.99 (0.79–5.01)
Cerebral infarction (N=361)				
Per SD*		1.23 (1.11–1.37)	1.35 (1.19–1.53)	1.28 (1.13–1.46)
Quartile 1	[135.1–207.7]	1.00 (ref)	1.00 (ref)	1.00 (ref)
Quartile 2	[207.7–221.4]	1.40 (1.02–1.92)	1.49 (1.08–2.06)	1.42 (1.03–1.96)
Quartile 3	[221.4 –235.1]	1.44 (1.05–1.98)	1.62 (1.15–2.27)	1.52 (1.08–2.14)
Quartile 4	[235.1–313.6]	1.91 (1.41–2.60)	2.29 (1.61–3.26)	2.03 (1.42–2.90)

*Hazard ratios are expressed per standard deviation increase in retinal venular caliber.
†Model 1: adjusted for age and sex.
‡Model 2: adjusted for age, sex, and retinal arteriolar caliber.
§Model 3: as Model 2, additionally adjusted for hypertension, diabetes mellitus, current smoking, serum total cholesterol level, serum HDL-cholesterol level, C-reactive protein level, and body mass index.

the associations, and further adjustments for systolic blood pressure, blood pressure-lowering medication use, alcohol consumption, left ventricular hypertrophy, and heart failure did not influence the results (Model 4, results not shown). We found a particularly strong association between wider venular caliber and risk of intracerebral hemorrhage, which was only slightly weakened after adjustment for confounders.

We further found a strong association between venular widening and risk of lobar hemorrhage (HR per SD increase, 2.02; 95% CI, 1.28 to 3.19) but not deep hemorrhage (HR, 0.90; 95% CI, 0.53 to 1.55). Arteriolar narrowing was associated with neither type of hemorrhage. Retinal vascular calibers were also strongly associated with risk for anticoagulation-related hemorrhage. The hazard ratio per SD decrease in arteriolar caliber was 2.59 (95% CI, 1.28 to 5.23) and 2.48 per SD increase in venular caliber (95% CI, 1.30 to 4.76).

DISCUSSION

In this prospective cohort study, we found that wider retinal venular caliber was associated with an increased risk of stroke and its subtypes cerebral infarction and intracerebral hemorrhage, independent of cardiovascular risk factors. Narrower retinal arteriolar caliber was nonsignificantly associated with the risk of any stroke or cerebral infarction, although we did find a trend toward an increased risk of intracerebral hemorrhage when we took venular caliber into account. Wider venular caliber was strongly associated with lobar intracerebral hemorrhage. Both narrower arteriolar calibers and wider venular calibers increased the risk of anticoagulation-related hemorrhage.

In interpreting these findings, we have to consider some methodological issues. The strengths of this study are its prospective and population-based design, the large number of participants, the standardized procedures for retinal vascular caliber measurements, and the long duration of follow-up. Thorough stroke monitoring procedures and the nearly complete follow-up (loss of potential years only 3.8%) allowed us to identify virtually all incident stroke events, even in participants who had not been referred to a hospital, for example, people living in nursing homes or participants who had a fatal stroke. Nevertheless, we may have missed some strokes presenting with symptoms too subtle for the participant to visit a physician. Because the outcome measure of the study was symptomatic stroke, we have not collected data on the occurrence of asymptomatic stroke before baseline or during follow-up. Another implication of our stroke monitoring approach was that neuroimaging was often lacking. As a result, 33% of strokes were classified as unspecified. Intracerebral hemorrhages were further categorized as lobar or deep based on their localization on CT. Likewise, we aimed to categorize cerebral infarctions as cortical or lacunar. Unfortunately, because most infarctions were not visible on CT and subtyping of infarctions based on clinical symptoms alone is vulnerable to misclassification,[25] we decided not to perform analysis on subtypes of infarctions.

This study describes the association between retinal vascular calibers and risk of intracerebral hemorrhage. We found that wider retinal venular caliber was associated with an increased risk for intracerebral hemorrhage. The association was stronger in lobar than in deep intracerebral hemorrhages. The mechanism underlying the association is unclear. Previous studies have shown that wider retinal venules are associated with an increased risk for stroke, including cerebral infarction.[14, 15] Furthermore, studies have shown that wider retinal venular caliber is associated with cardiovascular risk factors, such as cigarette smoking, serum glucose levels, and serum cholesterol levels and with markers of atherosclerosis, inflammation, and obesity.[10–12] In the present study, adjustment for these factors only minimally influenced the results, suggesting that other mechanisms must be involved. Other possible mechanisms underlying retinal venular dilation include retinal hypoxia, venous insufficiency,

and endothelial dysfunction.[12] Whether any of these proposed mechanisms underlies the association between retinal venular caliber and intracerebral hemorrhage requires additional investigations.

A priori we had expected to find an association between narrower retinal arteriolar caliber and risk for intracerebral hemorrhage, given that hypertension is the major established risk factor of intracerebral hemorrhage and known to lead to retinal arteriolar narrowing.[2, 9, 11] Remarkably, however, the association between retinal arteriolar caliber and intracerebral hemorrhage was not significant and smaller than the association we found between retinal venular caliber and intracerebral hemorrhage. However, when we classified intracerebral hemorrhages as anticoagulation-related or non-anticoagulation-related, we found that both arteriolar narrowing and venular widening strongly increased the risk for anticoagulation-related hemorrhage.

Although this finding was based on a small number of events, it may have important clinical implications. Intracerebral hemorrhage is a major concern when prescribing oral anticoagulants to elderly people and clinical decision making would benefit from additional risk predictors to identify those at risk for bleeding complications. However, further research is needed to establish the association and to investigate possible implications of retinal vascular caliber measurements.

We have previously shown that wider retinal venular caliber, but not narrower arteriolar caliber, is associated with an increased risk of stroke and cerebral infarction.[15] In the present analysis, following more recent insights on how to adjust for confounding, we corrected for the confounding effect of the complementary retinal vessel by entering both vascular calibers simultaneously in the regression models.[26] In comparison with the previously reported results, these additional adjustments resulted in somewhat stronger associations between retinal vascular calibers and risk of cerebral infarction. However, they did not affect our previous conclusion that wider retinal venular caliber is associated with an increased risk for cerebral infarction, whereas narrower arteriolar caliber is not.[15]

The present study suggests that retinal venular caliber is a novel risk determinant of intracerebral hemorrhage. Because of the poor prognosis after intracerebral hemorrhage, identification of people at risk and treatment of risk factors is extremely important. To date, only a limited number of detectable and modifiable risk factors and risk indicators have been identified. Our finding that retinal vascular caliber is a strong risk determinant of intracerebral hemorrhage may help the early identification of people at risk for intracerebral hemorrhage, and in particular of people at risk for anticoagulation-related hemorrhage, but may also provide new directions for further research into the pathophysiology of intracerebral hemorrhage.

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Chapter 4.2

Are retinopathy signs associated with the risk of stroke in the general elderly population?

Chapter 4.3

Age-related macular degeneration and the risk of stroke

ABSTRACT

Background and purpose – Age-related macular degeneration (AMD) and stroke are both frequent diseases in the elderly. A link between AMD and stroke has been suggested, because both disorders have many risk factors in common. The aim of this study was to investigate the association between AMD and stroke and the subtypes cerebral infarction and intracerebral hemorrhage in the general elderly population.

Methods – This study was part of the population-based Rotterdam Study and included 6,207 participants aged 55 years or older who were stroke-free at baseline (1990 to 1993). Signs of AMD were assessed on fundus photographs at baseline and at regular follow-up examinations and were categorized in 5 stages (0 to 4) representing an increasing severity. Late AMD (Stage 4) was subdivided into dry and wet AMD. Follow-up for incident stroke was complete up to January 1, 2007. Data were analyzed using time-dependent Cox regression models adjusted for age, sex, and potential confounders.

Results – During a median follow-up of 13.6 years, 726 participants developed a stroke, of which 397 were classified as cerebral infarction, 59 as intracerebral hemorrhage, and 270 as unspecified. Late AMD was associated with an increased risk of any stroke (HR, 1.56; 95% CI, 1.08 to 2.26) due to a strong association with intracerebral hemorrhage (HR, 6.11; 95% CI, 2.34 to 15.98). In contrast, late AMD was not associated with cerebral infarction. Earlier AMD stages were not associated with risk of stroke or any of its subtypes.

Conclusion – We found that late AMD is strongly associated with risk of intracerebral hemorrhage, but not with risk of cerebral infarction, in the general elderly population.

INTRODUCTION

Age-related macular degeneration (AMD) is a chronic disorder and the major cause of permanent visual impairment among elderly people in developed countries.[1] Its early signs are the presence of drusen and pigmentary abnormalities in the macula. These early abnormalities are frequent in elderly people and cause only minimal visual symptoms. Early AMD may progress to late AMD, often associated with severe and irreversible loss of vision.[2]

Several risk factors for stroke, including smoking, hypertension, carotid artery disease, cholesterol levels, diabetes mellitus, and obesity, have also been associated with an increased risk of AMD.[3–6] Because of the overlap in risk factors, a common causal pathway has been hypothesized to underlie the development of both stroke and AMD.[3] Previous studies that investigated the association between AMD and stroke reported inconsistent results.[7–11] Most studies did not distinguish between early and late AMD nor did they subdivide late AMD into dry (atrophic) or wet (neovascular) AMD. Besides, except for a study based on medical reimbursement claims, no previous study presented separate associations for the subtypes cerebral infarction and intracerebral hemorrhage.[8]

The purpose of the present study was to investigate in a large population-based cohort of elderly people if AMD was associated with risk of stroke and its subtypes cerebral infarction and intracerebral hemorrhage. In addition, we analyzed if any of these associations also held for the various AMD stages.

METHODS

Study population

This study is part of the Rotterdam Study, an ongoing prospective population-based cohort study, which started in 1990.[12] All inhabitants who were age 55 years or older and living in Ommoord, a district in Rotterdam in the Netherlands, were invited to participate. Invitation to the study occurred in random order. Baseline examinations, including a standardized interview, physical examination, and blood sampling, took place between 1990 and 1993. These were followed by a first follow-up examination from 1993 to 1994, a second from 1997 to 1999, and a third from 2000 to 2004. After enrollment, participants were continuously monitored for a variety of diseases that are frequent in the elderly, including stroke.[12] The study was approved by the Medical Ethics Committee of the Erasmus Medical Center in Rotterdam. Written informed consent was obtained from all participants.

Of the initial cohort of 10,725 eligible individuals, 7,983 participated in the baseline interview. Because the ophthalmologic part of the study became operational after the main study had started, a smaller number of participants (N=6,780) underwent the ophthalmologic examination. Gradable fundus photographs were available of 6,418 participants. After

exclusion of participants who had a stroke before baseline (N=182) or had refused informed consent for the collection of follow-up data from general practitioners (N=31), a total of 6,207 participants was included in the present analyses.

Assessment of stroke

Stroke was defined as rapidly developing clinical signs of focal (or global) disturbance of cerebral function with no apparent cause other than a vascular origin.[13] History of stroke at baseline was assessed during the baseline interview and verified in medical records. After enrollment in the Rotterdam Study, participants were continuously monitored for incident stroke through automated linkage of the study database with files from general practitioners. Nursing home physicians' files and files from general practitioners of participants who moved out of the district were scrutinized as well. Additional information was obtained from hospital records. Potential strokes were reviewed by research physicians and verified by an experienced stroke neurologist (P.J.K.). Strokes were further classified as cerebral infarction or intracerebral hemorrhage based on neuroimaging reports. If neuroimaging was lacking, a stroke was classified as unspecified. Subarachnoid hemorrhages were excluded.

Participants were followed from baseline to stroke, death, last health status update when they were known to be stroke-free, or January 1, 2007, whichever came first. Follow-up was complete up to January 1, 2007, for 96.1% of potential person years.

Definition of age-related macular degeneration

Fundus photographs covering a 35° field centered on the macula were taken at each visit (Topcon TRV-50VT fundus camera; Topcon Optical Co, Tokyo, Japan) after pharmacological mydriasis. Signs of AMD were graded according to the modified international classification and grading system by the same two trained professionals who graded AMD from baseline to the present under the supervision of senior ophthalmologists (J.R.V. and P.T.V.M.d.J.), who were masked for all other determinants.[14–16] We categorized these signs into five mutually exclusive stages 0 to 4 that represent an increasing severity of AMD. No AMD was defined as Stage 0, no signs of AMD at all or only small, hard drusen (<63 μm); Stage 1 was defined as soft distinct drusen (≥63 μm) or only pigmentary abnormalities. Stage 2 was defined as soft indistinct drusen (≥125 μm), reticular drusen only, or soft distinct drusen (≥63 μm) with pigmentary abnormalities. Stage 3 included soft indistinct drusen (≥125 μm) or reticular drusen with pigmentary abnormalities. Late AMD (Stage 4) was subdivided into dry (atrophic) AMD and wet (neovascular) AMD. Persons were classified in Stage 0 to 4 based on the eye with the more severe stage.

Lesions that were considered to be the result of generalized disease such as diabetic retinopathy, chorioretinitis, high myopia, trauma, congenital diseases, or photocoagulation for reasons other than for choroidal neovascularization were excluded from AMD classifica-

tion.

Other measurements

Blood pressure was calculated as the mean of two measurements with the random-zero sphygmomanometer at the right brachial artery at the time the subject was in a sitting position. Hypertension was defined as a diastolic blood pressure of ≥90 mm Hg and/or a systolic blood pressure of ≥140 mm Hg and/or the use of blood pressure-lowering medication. Total serum cholesterol level and high-density lipoprotein (HDL-) cholesterol level were measured in nonfasting blood with an automated enzymatic procedure. Diabetes mellitus was defined as a nonfasting or postload serum glucose level ≥11.1 mmol/L and/or the use of glucose-lowering medication. Serum C-reactive protein levels were determined by the Rate Near Infrared Particle Immunoassay method (Immage high sensitive CRP; Beckman Coulter). Smoking behavior, medication use, and alcohol consumption were assessed as part of the interview.[17] Atherosclerotic plaques were visualized with ultrasonography at three sites (common carotid artery, bifurcation, and internal carotid artery, both left and right).[18] Participants were genotyped for the complement factor H (*CFH*) Y402H polymorphism in genomic DNA with an allelic discrimination assay (TaqMan; Applied Biosystems, Foster City, CA).[19] Apolipoprotein E (*APOE*) genotype was determined on DNA samples using a polymerase chain reaction followed by enzymatic digestion.[20]

Statistical analysis

We calculated the hazard ratio and 95% confidence interval of the risk of stroke in AMD cases using a Cox regression model with AMD stage as a time-dependent exposure variable. Stage 0 (no AMD) was defined as the reference category. We also determined the risk of stroke associated with dry AMD and wet AMD relative to no AMD. All analyses were adjusted for age and sex (Model 1) and for the following potential confounders: diabetes mellitus, systolic blood pressure, blood pressure-lowering medication, current smoking, serum total cholesterol level, serum HDL-cholesterol level, carotid artery plaques, body mass index, alcohol intake and C-reactive protein level (Model 2), *APOE* (Model 3), and CFH (Model 4). To examine the presence of effect modification by *APOE* or *CFH*, we performed analyses according to strata of *APOE* and strata of *CFH* and by entering interaction terms into the models. Missing values in covariates were imputed with a linear regression model based on age and sex.

RESULTS

During 69,152 person years of follow-up (median, 13.6 years), 726 participants developed a stroke, which was classified in 397 as cerebral infarction, in 59 as intracerebral hemorrhage,

Table 1. Baseline characteristics (N=6,207)

Age, years	67.6 (61.7–74.8)
Female sex, %	59.6
Current smoking, %	23.1
Systolic blood pressure, mm Hg	137 (123–153)
Hypertension, %	57.0
Blood pressure-lowering medication use, %	31.0
Diabetes mellitus, %	11.4
Serum total cholesterol level, mmol/L	6.6 (5.8–7.4)
Serum HDL-cholesterol level, mmol/L	1.3 (1.1–1.6)
Body mass index, kg/m^2	26.0 (23.8–28.4)
C-reactive protein level, mg/L	1.8 (0.9–3.5)
Alcohol intake, gram/day	3.5 (0.2–14.8)
Carotid artery plaques (No. ≥4), %	16.6
Complement factor H genotype, %	
Y402Y	41.4
Y402H	44.9
H402H	13.6
Apolipoprotein E genotype, %	
ε2ε2 or ε2ε3	13.5
ε3ε3	58.3
ε3ε4 or ε4ε4	25.6
Age-related macular degeneration stage, %	
0	63.6
1	27.6
2	6.1
3	1.2
4	1.5
Dry	0.6
Wet	0.9

Values are medians (interquartile range) or percentages.

and in 270 as unspecified. Baseline characteristics of the study population are shown in **Table 1**. At baseline, the median age was 67.6 years, and 59.6% of the participants were women.

Table 2 shows the associations between successive AMD stages and risk of any stroke, cerebral infarction, and intracerebral hemorrhage. There was no association between early AMD (Stage 1 to 3) and any stroke. Late AMD (Stage 4) was associated with an increased risk of any stroke independent of potential confounders. We found a strong association between Stage 4 AMD and intracerebral hemorrhage, be it that the numbers were small and the 95% confidence intervals large. The association between AMD and intracerebral hemorrhage was only slightly attenuated after adjustment for known stroke risk factors (Model 2) and remained significant after adjustment for *APOE* (HR, 7.05; 95% CI, 2.68 to 18.57) and *CFH* (HR, 9.46; 95% CI, 3.56 to 26.11). In contrast, AMD was not associated with risk of cerebral infarction.

In **Table 3**, we present the associations between dry AMD and wet AMD and the risk of stroke and its subtypes. Both dry and wet AMD were associated with intracerebral hemorrhage. Neither dry nor wet AMD was associated with cerebral infarction. The association between late AMD and any stroke was not modified by *APOE* or *CFH*. However, because of

Table 2. Association between age-related macular degeneration and stroke

AMD Stage	Events, N	Model 1* HR (95% CI)	Model 2† HR (95% CI)
Any stroke			
0	326	1.00 (ref)	1.00 (ref)
1	275	1.03 (0.87–1.21)	1.05 (0.89–1.24)
2	73	0.91 (0.70–1.17)	0.88 (0.68–1.14)
3	19	0.77 (0.48–1.22)	0.77 (0.48–1.23)
4	33	1.60 (1.11–2.30)	1.56 (1.08–2.26)
Cerebral infarction			
0	188	1.00 (ref)	1.00 (ref)
1	155	1.03 (0.83–1.28)	1.06 (0.86–1.32)
2	36	0.90 (0.63–1.29)	0.88 (0.61–1.27)
3	10	0.86 (0.45–1.64)	0.87 (0.46–1.65)
4	8	0.92 (0.45–1.89)	0.89 (0.43–1.82)
Intracerebral hemorrhage			
0	21	1.00 (ref)	1.00 (ref)
1	25	1.49 (0.83–2.67)	1.49 (0.81–2.61)
2	6	1.41 (0.56–3.53)	1.31 (0.52–3.29)
3	1	0.85 (0.11–6.39)	0.82 (0.11–6.19)
4	6	6.81 (2.63–17.65)	6.11 (2.34–15.98)

Abbreviations: AMD = age-related macular degeneration.
* Model 1: Adjusted for age and sex.
† Model 2: Adjusted for age, sex, diabetes mellitus, systolic blood pressure, blood pressure-lowering medication, current smoking, serum total cholesterol level, serum HDL-cholesterol level, body mass index, carotid artery plaques, C-reactive protein level, and alcohol intake.

relatively small numbers of intracerebral hemorrhage, no reliable analyses on the presence of effect modification by *APOE* or *CFH* in this subgroup were feasible.

DISCUSSION

In this prospective population-based cohort study among elderly people, we found that persons with late AMD had a higher risk of stroke than persons without AMD. Dividing stroke into its subtypes cerebral infarction and intracerebral hemorrhage revealed that late AMD was strongly associated with intracerebral hemorrhage but not with cerebral infarction. Early AMD was not associated with the risk of stroke or any of its subtypes.

There are two possible explanations for the lack of a gradual relationship between AMD stage and intracerebral hemorrhage. First of all, there seems to be a threshold, that is, AMD

Table 3. Association between late stages of age-related macular degeneration and stroke

AMD Stage	Events, N	Model 1* HR (95% CI)	Model 2† HR (95% CI)
Any stroke			
No AMD	326	1.00 (ref)	1.00 (ref)
Dry AMD	11	1.17 (0.64–2.15)	1.10 (0.60–2.02)
Wet AMD	22	1.95 (1.26–3.02)	1.98 (1.28–3.07)
Cerebral infarction			
No AMD	188	1.00 (ref)	1.00 (ref)
Dry AMD	4	1.02 (0.38–2.76)	0.95 (0.35–2.59)
Wet AMD	4	0.84 (0.31–2.28)	0.83 (0.31–2.25)
Intracerebral hemorrhage			
No AMD	21	1.00 (ref)	1.00 (ref)
Dry AMD	2	4.96 (1.13–21.82)	4.26 (0.96–18.93)
Wet AMD	4	8.35 (2.76–25.24)	7.76 (2.56–23.58)

Abbreviations: AMD = age-related macular degeneration.
*Model 1: Adjusted for age and sex.
†Model 2: Adjusted for age, sex, diabetes mellitus, systolic blood pressure, blood pressure-lowering medication, current smoking, serum total cholesterol level, serum HDL-cholesterol level, body mass index, carotid artery plaques, C-reactive protein level, and alcohol intake.

seems only associated with intracerebral hemorrhage once it has progressed to an advanced stage. This suggests that there might be a shared underlying process that leads to progression of early to late AMD as well as to intracerebral hemorrhage. Second, we performed time-varying analyses in which we took into account not only prevalent AMD stage, but also incident AMD and progression of AMD over time. If we had limited the analyses to prevalent AMD, we probably would have found a more gradual relationship between AMD stage and intracerebral hemorrhage. However, using time-varying analysis, we were able to detect that, besides persons who had late AMD at baseline, only persons with early AMD at baseline who progressed to late AMD during follow-up had an increased risk of intracerebral hemorrhage.

Recently, several population-based studies have reported on the association between AMD and stroke or stroke-related mortality. The Atherosclerosis Risk in Communities Study found early AMD to be associated with stroke in middle-aged people (aged 49 to 73 years).[11] In contrast, the Cardiovascular Health Study reported that neither early nor late AMD increased the risk of stroke in elderly people (aged 69 to 97 years).[9] The Blue Mountains Eye Study found that early and late AMD were not associated with stroke mortality,[10] whereas a study from Taiwan found that neovascular AMD increased the risk of stroke-

related death.[7] Only one study divided stroke into subtypes and found that the risks of both cerebral infarction and intracerebral hemorrhage were increased in people with either neovascular or nonneovascular AMD.[8] However, a limitation of the study was that the classification of stroke and AMD were both based on medical reimbursement claims and therefore vulnerable to misclassification. Because the studies conducted so far were heterogeneous with respect to exposure categories and outcome definitions, results are not easily comparable. In addition to these earlier studies, we aimed to study the whole spectrum of AMD abnormalities in relation to stroke and particularly to distinguish intracerebral hemorrhage from cerebral infarction.

We found that late AMD was associated with an increased risk of intracerebral hemorrhage independent of stroke risk factors, *APOE* genotype, and *CFH* genotype. The mechanism underlying this association is unknown. Although we cannot exclude a causal role of AMD in the development of intracerebral hemorrhage, a common underlying causal pathway seems more likely. Several mechanisms for the association between AMD and stroke have been proposed, one of which is atherosclerosis.[21] The Rotterdam Study previously reported that subclinical manifestations of atherosclerosis, that is, carotid artery plaques and intima-media thickness, are associated with AMD.[5, 6] However, our finding that AMD is associated with intracerebral hemorrhage rather than cerebral infarction indicates that atherosclerosis is probably not a major link between AMD and stroke. Also, adjustment for the presence of carotid artery plaques and other measures of atherosclerosis did not influence our findings.

Another common underlying mechanism that has been proposed is inflammation. Recent studies have shown that the complement system plays an important role in the development of AMD, emphasizing an inflammatory pathogenesis of AMD.[22, 23] The role of inflammation in intracerebral hemorrhage is unclear. Inflammation and complement activation contribute to the development of secondary brain injury after intracerebral hemorrhage,[24] but there is no evidence for their contribution to the onset of intracerebral hemorrhage.[25] In the present study, the association between AMD and intracerebral hemorrhage remained unchanged after adjustment for C-reactive protein levels or *CFH*, suggesting that inflammation and complement activation are less likely to explain the association. Nevertheless, we cannot rule out the possibility of other inflammatory proteins or complement components being involved in a common pathway.

Polymorphisms of the *APOE* gene affect both AMD and stroke and have also been suggested as a common mechanism. Carriers of the *APOE* ε2 allele are at a slightly increased risk of developing AMD, whereas the ε4 allele appears to be protective.[26] Regarding stroke, the *APOE* ε2 allele seems to be associated with intracerebral hemorrhage and the ε4 allele with cerebral infarction.[27] In the current study, however, late AMD was associated with intracerebral hemorrhage independently of *APOE* genotype.

Although the possibility of residual confounding cannot be excluded, our findings suggest

that the association between AMD and intracerebral hemorrhage is largely independent of atherosclerosis, inflammation, complement activation, and *APOE*. Therefore, it seems most likely that the association is due to determinants that we did not measure in our study.

The strengths of this study are the prospective and population-based design, the large number of participants, and the long duration of follow-up. Thorough stroke monitoring procedures and the nearly complete follow-up (loss of potential person years only 3.8%) allowed us to identify virtually all incident stroke events, even in participants who had not been admitted to a hospital, for example, people living in nursing homes or participants who had a fatal stroke. However, an implication of this approach was that neuroimaging was often lacking; hence, 36% of strokes could not be classified as intracerebral hemorrhage or cerebral infarction. Another advantage of this study was that we were able to use detailed data on AMD abnormalities at four time points (at baseline and follow-up visits), which increased the power of our study. However, because the numbers of late AMD cases and intracerebral hemorrhages in our study were limited, analyses were based on small numbers of events. Therefore, our findings require replication in other cohorts.

In conclusion, in this large prospective population-based cohort study among elderly people, we found a strong association between late AMD and risk of intracerebral hemorrhage. In contrast, AMD was not associated with the risk of cerebral infarction. The mechanism of the association is yet unknown and needs further investigation.

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CHAPTER 5

METABOLISM

Chapter 5.1

Serum lipid levels and the risk of intracerebral hemorrhage

ABSTRACT

Background and purpose – Low serum total cholesterol levels are associated with an increased risk of symptomatic intracerebral hemorrhage and with presence of asymptomatic cerebral microbleeds. The relative contribution of lipid fractions to these associations is unclear and requires investigation. We determined whether serum HDL-cholesterol, LDL-cholesterol, and triglycerides are associated with risk of intracerebral hemorrhage and presence of cerebral microbleeds.

Methods and results – Nine thousand sixty-eight stroke-free community-dwelling persons aged 55 or older were followed from baseline (1990–2001) up to January 1, 2009, of whom 85 suffered from intracerebral hemorrhage during follow-up. Brain MRI was carried out in 789 healthy participants, of whom 162 had cerebral microbleeds. Triglycerides were strongly and inversely associated with intracerebral hemorrhage, independently of HDL-cholesterol, LDL-cholesterol, and potential confounders (HR for highest versus lowest quartile, 0.20; 95% CI, 0.06 to 0.69). Triglycerides were also associated with deep or infratentorial microbleeds (OR for highest versus lowest quartile, 0.37; 95% CI, 0.14 to 0.96), but not with strictly lobar microbleeds. No associations were found for HDL-cholesterol or LDL-cholesterol.

Conclusion – Low serum triglyceride levels were associated with an increased risk of intracerebral hemorrhage and with the presence of deep or infratentorial cerebral microbleeds. This provides novel insights into the role of lipid fractions, particularly triglycerides, in the etiology of intracerebral hemorrhage.

INTRODUCTION

Intracerebral hemorrhage accounts for about 10% to 15% of strokes and is a devastating disease for which there are currently no curative treatment options.[1, 2] Therefore, identification of modifiable risk factors is highly important. Low levels of serum total cholesterol have long been recognized as a possible risk factor for intracerebral hemorrhage.[3] The exact role of cholesterol in the pathogenesis of intracerebral hemorrhage is unclear, although some studies suggest that low cholesterol levels make the cerebrovascular endothelium fragile and vulnerable for leakage and rupture.[4–6] Low total cholesterol levels also relate to the presence of cerebral microbleeds,[7, 8] which are thought to be asymptomatic precursors of symptomatic intracerebral hemorrhage.[9, 10] Establishing overlap in risk factors for intracerebral hemorrhage and cerebral microbleeds may thus aid in the early detection of persons at an increased risk of intracerebral hemorrhage.

Although various cohort studies show that serum total cholesterol levels are inversely related with intracerebral hemorrhage,[11–22] it is unclear how various serum lipid fractions associate with intracerebral hemorrhage. Studies investigating lipid fractions, i.e., LDL-cholesterol, HDL-cholesterol, and triglycerides, have reported inconsistent results.[13–17, 19, 23–25] However, recent evidence suggests that the association between total cholesterol levels and risk of intracerebral hemorrhage is mainly driven by low triglyceride levels.[19, 23] Still, further confirmation of these results is needed. Moreover, it is unclear whether similar patterns of lipid fractions also underlie the association of cholesterol with cerebral microbleeds.

Therefore, we investigated in a large population-based cohort of community-dwelling elderly people whether serum total cholesterol and in particular the levels of LDL-cholesterol, HDL-cholesterol, and triglycerides are associated with the risk of intracerebral hemorrhage. Because we also aimed to investigate the potential of these lipid fractions as risk factors for preclinical disease, we studied the associations between lipid fractions and the presence of cerebral microbleeds.

METHODS

Source population

The Rotterdam Study is an ongoing prospective population-based cohort study that focuses on causes and consequences of diseases that are frequent in the elderly. The rationale and design of the study have been described extensively elsewhere.[26] Briefly, the cohort started in 1990 and included 7,983 participants who were aged 55 years or older and living in Ommoord, a district in Rotterdam in the Netherlands (Rotterdam Study I). In 2000, the cohort was expanded with 3,011 participants who had reached the age of 55 or had moved into the district since the start of the study (Rotterdam Study II). All participants underwent

a comprehensive set of baseline examinations which were repeated during regular follow-up visits, approximately every 3 to 5 years. In 2005, a random subset of Rotterdam Study II underwent brain MRI. The study was approved by the Medical Ethics Committee of the Erasmus University Medical Center and all participants gave written informed consent to participate in the study.

Measurement of serum lipid levels

Venous blood samples were obtained from all participants at each visit (**Figure**). In 1990, nonfasting serum total cholesterol and HDL-cholesterol levels were measured, using enzymatic colorimetric methods (Kone Specific Analyzer, Kone Instruments). From 1997 onwards, fasting total cholesterol and HDL-cholesterol as well as fasting triglyceride levels were determined using comparable enzymatic procedures (Hitachi Analyzer, Roche Diagnostics). Lipid measurements were carried out at the Erasmus Medical Center in two laboratories (Department of Epidemiology laboratory and Clinical Chemistry laboratory), which both participated in the Dutch National Cholesterol Standardization Program, analogous to the Center for Disease Control and Prevention quality assurance and standardization programs (Atlanta, GA). All measurements fulfilled the WHO criteria for precision and accuracy of lipid measurements. Non-HDL-cholesterol was calculated by subtracting HDL-cholesterol from total cholesterol. The Friedewald equation was used to estimate LDL-cholesterol.[27] Pearson's correlation coefficients for the various lipid fractions were weak to modest: HDL-cholesterol and triglycerides, r=0.50; HDL-cholesterol and LDL-cholesterol, r=0.07; and LDL-cholesterol and triglycerides, r=0.20. Triglyceride levels were natural log-transformed because their distribution was severely skewed to the right.

Population for analysis

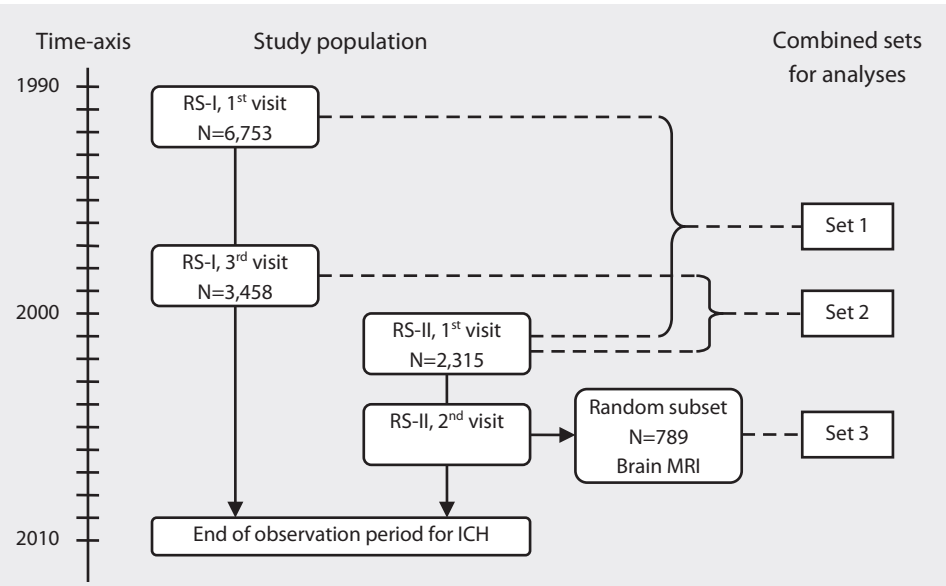
Because in different examination visits either nonfasting or fasting blood samples were drawn, we performed our analyses based on the following 3 combined sets of participants. This was done in order to obtain the largest numbers of intracerebral hemorrhage (**Figure**):

- Set 1: participants of the first examination of Rotterdam Study I (baseline 1990–1993) and the first examination of Rotterdam Study II (baseline 2000–2001);
- Set 2: persons who took part in the third examination of Rotterdam Study I (1997–1999) and the first examination of Rotterdam Study II (baseline 2000–2001); and
- Set 3: a random subset of participants of the second examination of Rotterdam Study II (2004–2005) who underwent brain MRI.

Of the total of 10,994 Rotterdam Study participants, we excluded persons who had had a stroke before their first examination (N=363), participants who had not given consent for the collection of follow-up data from general practitioners (N=195), participants who had not visited the research center for blood sampling due to death, refusal or physical inability

(N=929), and participants of whom blood draw or storage had failed (N=412). Because the Friedewald equation is not valid if triglyceride levels exceed 4.52 mmol/L (400 mg/dL),[27] we further excluded participants with triglyceride levels above this value (N=31). This resulted in a total of 9068 participants for Set 1 (Rotterdam Study I, N=6,753; Rotterdam Study II, N=2,315). Of the 4,797 persons who took part in the third examination of Rotterdam Study I, we excluded 243 participants who had a prevalent stroke at that examination, 5 participants who had not, given informed consent for the collection of follow-up data, 1,072 participants of whom lipid levels were not available, and 24 participants with triglyceride levels ≥4.52 mmol/L. Combining these 3,458 persons with 2,315 from Rotterdam Study II resulted in 5,773 participants in Set 2. Set 3 comprised a random subset of Rotterdam Study II participants who underwent brain MRI and of whom we had lipid measurements available (N=789).

Figure. Schematic overview of the Rotterdam Study population.



Abbreviations: RS-I = Rotterdam Study I; RS-II = Rotterdam Study II; ICH = intracerebral hemorrhage.
Lipid measurements in Set 1: total cholesterol, HDL-cholesterol and non-HDL-cholesterol (nonfasting).
Lipid measurements in Set 2 and 3: total cholesterol, HDL-cholesterol, non-HDL-cholesterol, LDL-cholesterol and triglycerides (fasting).

Assessment of stroke

Stroke was defined according to WHO criteria as a syndrome of rapidly developing clinical signs of focal (or global) disturbance of cerebral function, with symptoms lasting 24 hours

or longer or leading to death, with no apparent cause other than of vascular origin.[28] History of stroke at baseline was assessed during the baseline interview and verified by reviewing medical records. After enrollment, participants were continuously monitored for incident stroke through automated linkage of the study database with files from general practitioners. Nursing home physicians' files and files from general practitioners of participants who moved out of the district were checked on a regular basis as well. Additional information was obtained from hospital records. Potential strokes were reviewed by research physicians, and verified by an experienced stroke neurologist (P.J.K.). Strokes were further classified as cerebral infarction or intracerebral hemorrhage on the basis of neuroimaging reports. If neuroimaging was lacking, a stroke was classified as unspecified. Subarachnoid hemorrhages due to ruptured aneurysms were not considered stroke events.

Participants were followed from study entry to stroke, death, last health status update when they were known to be stroke-free, or January 1, 2009, whichever came first. Follow-up was complete up to January 1, 2009 for 98.4% of potential person years.

Brain MRI and rating of cerebral microbleeds

A multisequence MRI protocol was carried out on a 1.5-T scanner (GE Healthcare, Milwaukee, WI).[7] A custom-made accelerated 3-dimensional T2*-weighted gradient-recalled echo (3D T2* GRE) sequence with high spatial resolution and long echo time was used for microbleed detection.[29] All 3D T2* GRE scans were reviewed by 1 of 2 trained raters who recorded the presence, number, and location of cerebral microbleeds.[7] Cerebral microbleeds were categorized into 1 of 3 locations: lobar (cortical gray and subcortical or periventricular white matter), deep (deep gray matter: basal ganglia and thalamus, and the white matter of the corpus callosum, internal, external, and extreme capsule), and infratentorial (brain stem and cerebellum).[7]

Other measurements

Trained research physicians visited all participants at home for standardized questionnaires about their health status and medical history, including questions about current medication use, cigarette smoking behavior and average amount of alcohol intake.[30] Subsequently, all participants visited the research center twice for physical examination and blood sampling. Blood pressure was calculated as the average of 2 measurements at the right brachial artery with a random-zero sphygmomanometer after 5 minutes of rest while the subject was in a sitting position. Hypertension was defined as a diastolic blood pressure of ≥90 mm Hg and/or a systolic blood pressure of ≥140 mm Hg, and/or the use of blood pressure-lowering medication.[31] Diabetes mellitus was defined as a fasting serum glucose level ≥7.0 mmol/L, a nonfasting or postload serum glucose level ≥11.1 mmol/L and/or the use of blood glucose-lowering drugs. Fasting serum insulin level was determined by metric assay (Biosource Diag-

nostics, Camarillo, CA).[32] Body mass index was calculated as weight (in kilograms) divided by the square of height (in meters).

Statistical analysis

Cox proportional hazards regression was used to calculate hazard ratios and 95% confidence intervals for the associations between lipid levels and risk of intracerebral hemorrhage, expressed per standard deviation increase in serum lipid level. Associations between total cholesterol, HDL-cholesterol and non-HDL-cholesterol, and intracerebral hemorrhage were calculated in Set 1 (**Figure**); associations between LDL-cholesterol and triglycerides, and intracerebral hemorrhage were calculated in Set 2 (**Figure**). For both sets, we constructed 2 models. In model 1 we adjusted for age, sex, lipid-lowering medication use, and Rotterdam Study subcohort (RS-I/RS-II). In model 2, we adjusted for age, sex, and a propensity score that included the following potential confounders: lipid-lowering medication use, systolic blood pressure, blood pressure-lowering medication use, diabetes mellitus, serum glucose level, current cigarette smoking, body mass index, antithrombotic medication use, alcohol intake, and subcohort. We adjusted for a propensity score instead of individual confounders because the number of intracerebral hemorrhages was small compared to the large number of potential confounders.[33, 34]

Statin treatment might modify the association between lipid levels and intracerebral hemorrhage.[35] Therefore, we investigated whether the associations between lipid levels and intracerebral hemorrhage differed across strata of lipid-lowering medication use and we tested the presence of interaction. These analyses were performed in Set 2. Hazard ratios were expressed per standard deviation increase in lipid level and were adjusted for age, sex, and a propensity score of potential confounders (systolic blood pressure, blood pressure lowering-medication use, diabetes mellitus, serum glucose level, serum insulin level, current cigarette smoking, body mass index, antithrombotic medication use, alcohol intake, and subcohort). Subsequently, we investigated whether the associations between HDL-cholesterol, LDL-cholesterol, triglycerides, and risk of intracerebral hemorrhage were independent of each other, by entering these lipid fractions simultaneously in the Cox regression models. The analyses were performed in Set 2. We adjusted for age, sex, a propensity score of potential confounders (lipid-lowering medication use, systolic blood pressure, blood pressure-lowering medication use, diabetes mellitus, serum glucose level, serum insulin level, current cigarette smoking, body mass index, antithrombotic medication use, alcohol intake, and subcohort) and for the other lipid fractions. To verify the log-linearity of associations, we also categorized lipid levels in quartiles using the lowest quartile as the reference category.

Finally, we determined the associations between HDL-cholesterol, LDL-cholesterol, and triglycerides, and the presence of deep or infratentorial microbleeds (with or without lobar microbleeds) versus strictly lobar cerebral microbleeds, using logistic regression models.

These analyses were carried out in Set 3. Associations were adjusted for age, sex, a propensity score (lipid-lowering medication use, systolic blood pressure, blood pressure-lowering medication use, diabetes mellitus, serum glucose level, serum insulin level, current cigarette smoking, body mass index, antithrombotic medication use, and alcohol intake), and the complementary lipid fractions. Odds ratios were expressed per standard deviation increase and in quartile categories.

In each set, covariate adjustments were based on data that were collected during the same visit as the blood sample in which the lipid levels were determined. We did not have complete data on all covariates. Alcohol intake was missing in 15.4%; other covariates were missing in less than 4% of participants. Missing values in covariates were imputed with a linear regression model based on age and sex. All analyses were performed using SPSS for Windows, version 16.0 (SPSS Inc., Chicago, IL).

RESULTS

During 97,956 person years of follow-up (median, 9.7 years), 1,005 participants developed a first-ever stroke, which was classified in 85 as intracerebral hemorrhage, in 561 as cerebral infarction, and in 359 as unspecified. Of the 85 intracerebral hemorrhages, 73 had occurred in Rotterdam Study I (33 after the start of the third visit), and 12 in Rotterdam Study II. Cerebral microbleeds were present in 162 (20.5%) of the 789 participants who underwent brain MRI; microbleeds were localized in deep or infratentorial brain regions in 65 participants and were strictly lobar in location in 97. Baseline characteristics of the Rotterdam Study population are shown in the **Figure** and in **Table 1**. Alcohol intake and antithrombotic medication use were higher in 1997 and 2000 than in 1990, whereas median total cholesterol and non-HDL-cholesterol levels were much higher in 1990 than in 1997 and 2000. Lipid-lowering medication use was more common 1997 and 2000 than in 1990 (13.1% and 12.0% versus 2.4%).

Table 2 shows the associations between serum lipid levels and risk of intracerebral hemorrhage. As expected, decreasing levels of total serum cholesterol were associated with an increasing risk of intracerebral hemorrhage. This was particularly due to an inverse association between the non-HDL-cholesterol fraction and intracerebral hemorrhage. LDL-cholesterol was not associated with risk of intracerebral hemorrhage. Triglyceride levels showed a strong inverse association with risk of intracerebral hemorrhage, independently of age, sex, lipid-lowering medication use, and multiple potential confounders.

Stratification by lipid-lowering medication use did not reveal differences across strata, although numbers were small. Consequently, *P* values for interaction were all nonsignificant (**Table 3**).

In **Table 4**, we present the independent effects of each of the lipid fractions on risk of

Tabel 1. Baseline characteristics of the Rotterdam Study population

	Rotterdam Study I		Rotterdam Study II
	1990–1993	1997–1999	2000–2001
	N=6,753	N=3,458	N=2,315
Age, years	68.1 (62.0-75.5)	71.0 (66.5-76.6)	61.8 (58.8-68.5)
Female sex, %	60.0	58.5	55.0
Systolic blood pressure, mm Hg	138 (123-153)	141 (128-156)	141 (128-156)
Diastolic blood pressure, mm Hg	73 (66-81)	75 (68-82)	78 (71-86)
Hypertension, %	55.5	70.6	62.4
Blood pressure-lowering medication, %	31.5	39.5	27.0
Serum glucose level, mmol/L	6.2 (5.5-7.4)*	5.6 (5.2-6.1)†	5.6 (5.3-6.1)†
Diabetes mellitus, %	10.6	13.2	10.5
Insulin level, pmol/L	ND	66 (46-94)†	70 (50-97)†
Current cigarette smoking, %	20.8	15.8	19.6
Alcohol intake, gram/day	3.4 (0.2-14.8)	4.3 (0.7-15.7)	7.4 (0.7-20.0)
Body mass index, kg/m2	25.9 (23.8-28.4)	26.4 (24.2-29.0)	26.7 (24.4-29.4)
Antithrombotic medication, %	4.6	22.6	13.5
Total cholesterol, mmol/L	6.6 (5.8-7.4)*	5.8 (5.2-6.5)†	5.8 (5.1-6.4)†
HDL-cholesterol, mmol/L	1.3 (1.1-1.6)*	1.3 (1.1-1.6)†	1.3 (1.1-1.6)†
Non-HDL-cholesterol, mmol/L	5.2 (4.4-6.0)	4.4 (3.8-5.1)	4.4 (3.7-5.0)
LDL-cholesterol, mmol/L	ND	3.7 (3.2-4.3)	3.7 (3.1-4.3)
Triglycerides, mmol/L	ND	1.3 (1.0-1.8)†	1.4 (1.0-1.8)†
Lipid-lowering medication, %	2.4	13.1	12.0

Abbreviations: ND = not determined.
Values are medians (interquartile range) or percentages.
* Nonfasting blood samples (1990 to1993).
†Fasting blood samples (1997 to1999; 2000 to 2001).

intracerebral hemorrhage. The borderline association between HDL-cholesterol and intracerebral hemorrhage was strongly attenuated after adjustment for triglyceride levels. In contrast, the strong association between increasing triglyceride levels and decreasing risk of intracerebral hemorrhage was not affected by adjustments for HDL-cholesterol levels or LDL-cholesterol levels.

Associations between HDL-cholesterol, LDL-cholesterol, triglycerides, and presence of cerebral microbleeds are shown in **Table 5**. Serum triglyceride levels were strongly and inversely associated with the presence of deep or infratentorial microbleeds, but not with strictly lobar microbleeds. There was also a trend toward an inverse relationship between HDL-cholesterol and LDL-cholesterol and presence of deep or infratentorial microbleeds,

Table 2. Association between serum lipid levels and risk of intracerebral hemorrhage (Set 1 or Set 2)

	Population for analysis*	At risk N	Events N	Model 1§ HR (95% CI)†	Model 2¶ HR (95% CI)†
Total cholesterol	Set 1	9,068	85	0.74 (0.58-0.94)	0.75 (0.59-0.95)
HDL-cholesterol	Set 1	9,068	85	1.15 (0.96-1.38)	1.17 (0.98-1.39)
Non-HDL-cholesterol	Set 1	9,068	85	0.71 (0.56-0.90)	0.71 (0.56-0.90)
LDL-cholesterol	Set 2	5,773	45	0.96 (0.71-1.29)	0.96 (0.71-1.30)
Triglycerides‡	Set 2	5,773	45	0.69 (0.50-0.94)	0.62 (0.44-0.86)

* Analyses are based on Set 1 or Set 2 (Figure).
†Hazard ratios are expressed per SD increase in lipid level.
‡Triglyceride levels are natural log-transformed.
§Model 1: Adjusted for age, sex, lipid-lowering medication use and subcohort.
¶Model 2: Adjusted for age, sex, and a propensity score of potential confounders (lipid-lowering medication use, systolic blood pressure, blood pressure-lowering medication use, diabetes mellitus, serum glucose level, current cigarette smoking, body mass index, antithrombotic medication use, alcohol intake and subcohort).
Note: hazards ratios obtained from Set 1 and Set 2 are not directly comparable because of different datasets and log-transformation of triglyceride levels.

but these associations were far from significant.

DISCUSSION

In this prospective population-based cohort study among people aged 55 years or older who were free from stroke at baseline, we confirmed that serum total cholesterol levels were inversely associated with the risk of intracerebral hemorrhage. When investigating the various lipid fractions, we found that the association was due to a strong inverse relationship between triglyceride levels and risk of intracerebral hemorrhage, and not due to HDL-cholesterol or LDL-cholesterol levels. Similarly, we found an inverse association between triglyceride levels and the presence of cerebral microbleeds in the deep or infratentorial brain regions.

Strengths of this study include the prospective and population-based design, the large number of participants, and the long duration and completeness of follow-up. Furthermore, we were able to study lipid levels in association with both asymptomatic microbleeds and symptomatic intracerebral hemorrhage. However, the study also has limitations. We did not include 1,368 participants in the analysis because of incomplete data on lipid levels. These participants were older (median age 73 versus 66 years), more often female (66% versus 59%), and more likely to have cardiovascular risk factors. It is possible that exclusion of these participants introduced a selection bias that could have affected the estimates. Loss to follow-up, another potential source of selection bias, was only 1.6%.

Another issue is that 36% of strokes were classified as unspecified because neuroimaging had not been performed, which is similar to unspecified stroke rates reported in other

Table 3. Associations between serum lipid levels and risk of intracerebral hemorrhage stratified by lipid-lowering medication use at baseline (Set 2)*

	No lipid-lowering medication use	**Lipid-lowering medication use**	
	N=40†	N=5†	*P*
	HR (95% CI)‡	HR (95% CI)‡	interaction
Total cholesterol	0.92 (0.67-1.28)	1.29 (0.51-3.29)	0.84
HDL-cholesterol	1.21 (0.94-1.55)	1.86 (0.76-4.55)	0.75
Non-HDL-cholesterol	0.85 (0.62-1.17)	1.06 (0.41-2.76)	0.75
LDL-cholesterol	0.94 (0.68-1.28)	1.23 (0.49-3.07)	0.94
Triglycerides §	0.65 (0.46-0.92)	0.64 (0.24-1.70)	0.59

* Analyses are based on Set 2 (Figure).
†N indicates the number of intracerebral hemorrhages per stratum.
‡Hazard ratios are expressed per SD increase in lipid level and are adjusted for age, sex, and a propensity score of potential confounders (systolic blood pressure, blood pressure-lowering medication use, diabetes mellitus, serum glucose level, serum insulin level, current cigarette smoking, body mass index, antithrombotic medication use, alcohol intake and subcohort).
§Triglyceride levels are natural log-transformed.

population-based or even hospital-based studies.[36, 37] Therefore it is likely that an unknown number of intracerebral hemorrhages were misclassified as unspecified. Apart from conventional stroke risk factors, major determinants of unspecified stroke risk are older age, living in a nursing home and dementia prior to stroke. Although this misclassification was independent of exposure measurement and therefore nondifferential, it may have resulted in an underestimation of the true associations between lipid levels and intracerebral hemorrhage. However, because we observed very similar patterns between lipid levels and cerebral microbleeds, we think that misclassification, if any, has not importantly influenced our results. In addition, differences were observed in cholesterol levels, use of lipid-lowering medication, and some other covariates between the sets of 1990, 1997, and 2000. These differences are likely to be explained by time-effects between successive follow-up visits. Given that we adjusted for these variables in the analyses, these differences probably did not have a strong impact on the results. MRI scans were carried out approximately 5 years after blood samples were drawn. However, previous studies have shown that, once present, cerebral microbleeds rarely disappear.[38]

We found that low triglyceride levels are associated with an increased risk of intracerebral hemorrhage, independently of lipid-lowering medication use, levels of LDL-cholesterol and HDL-cholesterol, and other potential confounders. This finding is in agreement with results from the Three-City Study, which reported a similar inverse association between low triglyceride levels and intracerebral hemorrhage,[23] and with results from a pooled cohort study among Atherosclerosis Risk in Communities Study participants and Cardiovascular Health

Table 4. Associations between lipid levels and intracerebral hemorrhage, adjusted for the complementary lipid fractions (Set 2)*

	mmol/L	At risk, N	Events, N	HR (95% CI)	HR (95% CI)	HR (95% CI)	HR (95% CI)
HDL-cholesterol				**Basic model‡**	**+ LDL-C**	**+ TG**	**+ LDL-C & TG**
Per SD		5,773	45	1.25 (0.99-1.57)	1.26 (0.99-1.61)	1.11 (0.81-1.51)	1.12 (0.81-1.54)
Quartile 1	[0.4-1.1]	1,473	9	1.00 (ref)	1.00 (ref)	1.00 (ref)	1.00 (ref)
Quartile 2	[1.1-1.3]	1,417	8	0.91 (0.35-2.39)	0.91 (0.35-2.38)	0.76 (0.29-2.03)	0.76 (0.28-2.01)
Quartile 3	[1.3-1.6]	1,454	9	1.05 (0.40-2.73)	1.05 (0.40-2.73)	0.76 (0.28-2.07)	0.74 (0.27-2.03)
Quartile 4	[1.6-5.5]	1,429	19	2.13 (0.89-5.11)	2.14 (0.89-5.15)	1.30 (0.49-3.46)	1.29 (0.48-3.45)
LDL-cholesterol				**Basic model‡**	**+ HDL-C**	**+ TG**	**+ HDL-C & TG**
Per SD		5,773	45	0.96 (0.71-1.30)	1.01 (0.74-1.37)	1.07 (0.78-1.46)	1.07 (0.78-1.46)
Quartile 1	[0.1-3.2]	1,443	11	1.00 (ref)	1.00 (ref)	1.00 (ref)	1.00 (ref)
Quartile 2	[3.2-3.7]	1,444	11	0.93 (0.40-2.16)	0.96 (0.41-2.24)	1.03 (0.44-2.44)	1.03 (0.44-2.41)
Quartile 3	[3.7-4.3]	1,443	14	1.21 (0.54-2.71)	1.30 (0.58-2.93)	1.47 (0.64-3.33)	1.47 (0.65-3.33)
Quartile 4	[4.3-7.9]	1,443	9	0.76 (0.31-1.89)	0.82 (0.33-2.04)	0.99 (0.39-2.52)	0.98 (0.39-2.50)
Triglycerides†				**Basic model‡**	**+ LDL-C**	**+ HDL-C**	**+ LDL-C & HDL-C**
Per SD		5,773	45	0.63 (0.46-0.88)	0.62 (0.44-0.87)	0.66 (0.46-0.95)	0.64 (0.44-0.94)
Quartile 1	[0.4-1.0]	1,457	18	1.00 (ref)	1.00 (ref)	1.00 (ref)	1.00 (ref)
Quartile 2	[1.0-1.3]	1,426	10	0.52 (0.24-1.14)	0.51 (0.23-1.12)	0.54 (0.25-1.20)	0.53 (0.24-1.17)
Quartile 3	[1.3-1.8]	1,442	13	0.65 (0.31-1.33)	0.62 (0.29-1.31)	0.69 (0.32-1.50)	0.66 (0.30-1.47)
Quartile 4	[1.8-4.3]	1,448	4	0.18 (0.06-0.57)	0.18 (0.06-0.56)	0.21 (0.06-0.71)	0.20 (0.06-0.69)

Abbreviations: HDL-C = HDL-cholesterol; LDL-C = LDL-cholesterol; TG = triglycerides.
* Analyses are based on Set 2 (Figure).
†Triglyceride levels are natural log-transformed.
‡Basic model: adjusted for age, sex, and a propensity score of potential confounders (lipid-lowering medication use, systolic blood pressure, blood pressure-lowering medication use, diabetes mellitus, serum glucose level, serum insulin level, current cigarette smoking, body mass index, antithrombotic medication use, alcohol intake and subcohort).

Table 5. Lipid levels and cerebral microbleeds (Set 3)*

		Strictly Lobar Microbleeds†		Deep or Infratentorial Microbleeds‡	
	mmol/L	N	OR (95% CI)§	N	OR (95% CI)§
HDL-cholesterol					
Per SD		97	0.83 (0.62-1.12)	65	0.92 (0.66-1.27)
Quartile 1	[0.4-1.1]	28	1.00 (ref)	14	1.00 (ref)
Quartile 2	[1.1-1.3]	26	0.86 (0.47-1.57)	20	1.25 (0.58-2.68)
Quartile 3	[1.3-1.6]	21	0.63 (0.32-1.25)	16	0.78 (0.33-1.82)
Quartile 4	[1.6-5.5]	22	0.61 (0.29-1.28)	15	0.49 (0.19-1.27)
LDL-cholesterol					
Per SD		97	0.89 (0.70-1.12)	65	0.78 (0.58-1.05)
Quartile 1	[0.1-3.2]	21	1.00 (ref)	25	1.00 (ref)
Quartile 2	[3.2-3.7]	30	1.41 (0.76-2.62)	16	0.79 (0.39-1.61)
Quartile 3	[3.7-4.3]	27	1.08 (0.57-2.06)	12	0.56 (0.25-1.21)
Quartile 4	[4.3-7.9]	19	0.74 (0.37-1.48)	12	0.53 (0.24-1.19)
Triglycerides¶					
Per SD		97	0.94 (0.72-1.22)	65	0.80 (0.57-1.11)
Quartile 1	[0.4-1.0]	22	1.00 (ref)	23	1.00 (ref)
Quartile 2	[1.0-1.3]	23	0.91 (0.48-1.75)	19	0.76 (0.38-1.54)
Quartile 3	[1.3-1.8]	27	1.01 (0.52-1.96)	13	0.52 (0.23-1.17)
Quartile 4	[1.8-4.3]	25	0.81 (0.38-1.70)	10	0.37 (0.14-0.96)

* Analyses based on Set 3 (Figure).
†Presence of strictly lobar microbleeds versus absence of microbleeds.
‡Presence of deep or infratentorial microbleeds versus absence of microbleeds.
§Odds ratios are adjusted for age, sex, a propensity score (lipid-lowering medication use, systolic blood pressure, blood pressure-lowering medication use, diabetes mellitus, serum glucose level, serum insulin level, current cigarette smoking, body mass index, antithrombotic medication use and alcohol intake), and HDL-cholesterol, LDL-cholesterol and triglyceride levels when applicable.
¶Triglyceride levels are natural log transformed.

Study participants.[19] However, three other studies did not detect an association between triglyceride levels and intracerebral hemorrhage.[14–16] Analyses of the Copenhagen Heart Study and Oslo Study were based on nonfasting triglyceride levels and included only few events.[14, 15] Furthermore, results of the Oslo Study were based on 21 years of follow-up, which may have diluted the effect.[15] The lack of an association observed by the Japan Lipid Intervention Trial could be due to the fact that they only included hypercholesterolemic patients with relatively high triglyceride levels.[16]

Although the mechanism of the association between triglyceride levels and intracerebral hemorrhage is unknown, there are some possible explanations. Several studies have suggested that high triglyceride levels favor a prothrombotic state because they are positively correlated with the vitamin K-dependent coagulation factors VII and IX, and with plasminogen activator inhibitor and blood viscosity.[39] Likewise, one could hypothesize that low triglyceride

levels may result in a prohemorrhagic state. Another possible explanation is that low triglyceride levels may contribute to weakness of the vascular endothelium. Cholesterol and fatty acids are essential elements of all cell membranes. In vitro studies have shown that low cholesterol levels result in increased permeability of erythrocyte membranes,[4] and animal studies reported that low cholesterol levels cause smooth muscle degeneration and endothelial weakness in small intracerebral arteries.[18] Therefore it has been hypothesized that very low cholesterol levels may contribute to the development of a fragile endothelium, prone to leakage and rupture.[5] However, whether any of these perspectives explains the observed association between low triglyceride levels and the risk of intracerebral hemorrhage remains uncertain and requires further investigation. We also cannot exclude the possibility of residual confounding by unmeasured determinants, for example diet or physical activity, or due to the fact that lipid levels and confounders were measured only once. Therefore, studies using time-varying analyses are needed to explore whether intraindividual fluctuations in lipid levels and confounders influence the results.

We further found a comparable inverse association between triglyceride levels and presence of cerebral microbleeds, which provides accumulating support for a parallel between asymptomatic microbleeds and symptomatic intracerebral hemorrhage.[40, 41] However, although not significant, associations of HDL-cholesterol and LDL-cholesterol with cerebral microbleeds seemed somewhat different from the associations with intracerebral hemorrhage. This may indicate that intracerebral hemorrhage and cerebral microbleeds are reflections of a different stage of arteriolosclerosis. Moreover, we cannot fully rule out the possibility that intracerebral hemorrhage and microbleeds do not completely share the same underlying pathology.

Our finding that triglycerides are related to deep or infratentorial microbleeds rather than lobar microbleeds may provide etiologic clues for the association between triglycerides and intracerebral hemorrhage. In a previous study, we showed that lobar microbleeds are indicative of underlying amyloid angiopathy, whereas deep or infratentorial microbleeds are associated with known risk factors for arteriolosclerosis.[42] The association between triglycerides and deep or infratentorial microbleeds but not lobar microbleeds underscores these differences in underlying pathology and is suggestive for a role of triglyceride levels through development of arteriolosclerotic microangiopathy.

To conclude, in this large population-based cohort study among elderly people we found that low serum triglyceride levels were associated with an increased risk of intracerebral hemorrhage as well as with the presence of deep or infratentorial cerebral microbleeds. This finding provides novel insights into the role of lipid metabolism in the etiology of intracerebral hemorrhage. Though the exact mechanism of the association remains unclear, triglyceride levels may aid in the identification of people at risk for intracerebral hemorrhage.

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Chapter 5.2

Liver enzymes and the risk of intracerebral hemorrhage

ABSTRACT

Background and purpose – Severe liver disease is a risk factor for intracerebral hemorrhage through impairment of hemostasis and coagulation, but it is uncertain if variations in liver enzyme levels, as markers of mild liver dysfunction, are also associated with risk of intracerebral hemorrhage. We aimed to study the association between liver enzyme levels and risk of intracerebral hemorrhage in the general elderly population. We also explored the role of alcohol intake in this association.

Methods – The study included 3,916 stroke-free Rotterdam Study participants aged 55 years or older of whom we had serum levels of aspartate transaminase (AST), alanine transaminase (ALT), gamma-glutamyltranspeptidase (GGT), alkaline phosphatase (ALP) and lactate dehydrogenase (LDH). Participants were followed for intracerebral hemorrhage from baseline (1990–1993) up to January 1, 2009. Cox proportional hazards regression was used to determine associations between enzyme levels and intracerebral hemorrhage, adjusted for age, sex and potential confounders.

Results – During 50,375 person years of follow-up 55 intracerebral hemorrhages occurred. ALP levels were associated with risk of intracerebral hemorrhage (HR per SD, 1.30; 95% CI, 1.02 to 1.68), as were LDH levels (HR, 1.42; 95% CI, 1.11 to 1.81). These associations seemed stronger in excessive alcohol drinkers than in non-excessive alcohol drinkers. AST, ALT and GGT levels were also associated with intracerebral hemorrhage, but only in excessive alcohol drinkers.

Conclusion – In this cohort of community-dwelling elderly people, ALP and LDH were associated with risk of intracerebral hemorrhage independently of alcohol use, whereas AST, ALT and GGT were associated with risk of intracerebral hemorrhage only in combination with excessive alcohol use.

INTRODUCTION

Liver diseases are associated with an increased bleeding tendency due to hematological disturbances such as platelet dysfunction and coagulation disorders.[1] Therefore, liver dysfunction is considered a putative risk factor for intracerebral hemorrhage.[2] Various studies reported on the concurrence of liver disease and intracerebral hemorrhage.[2–8] Other studies suggested a relationship between liver dysfunction and hematoma growth or hematoma volume.[9–11] These data were mainly based on autopsy studies, case series or hospital-based case-control studies without an appropriate control group. Data from longitudinal studies are scarce. A recent follow-up study reported a nonsignificant association between intracerebral hemorrhage and liver cirrhosis.[12] Two other population-based cohort studies reported associations between mild liver enzyme elevations and intracerebral hemorrhage, but these studies were restricted to only men or to a single enzyme.[13, 14]

Liver disease and intracerebral hemorrhage share a common risk factor, which is alcohol use.[15] Whether alcohol consumption influences the presumed association between liver dysfunction and intracerebral hemorrhage is unclear.

The aim of this study was to investigate in a large prospective cohort study among community-dwelling elderly people whether variations in liver enzyme levels, as markers of mild liver dysfunction, were associated with the long-term risk of intracerebral hemorrhage. We studied five enzymes that are commonly assayed for the diagnosis of liver disease, namely aspartate transaminase (AST), alanine transaminase (ALT), gamma-glutamyltranspeptidase (GGT), alkaline phosphatase (ALP), and lactate dehydrogenase (LDH).[16] Given the interplay of alcohol with both intracerebral hemorrhage and liver disease, we also investigated whether the associations between enzyme levels and intracerebral hemorrhage were modified by alcohol intake.

METHODS

Source population

This study was based on the Rotterdam Study, an ongoing prospective population-based cohort study that focuses on causes and consequences of diseases that are frequent in the elderly. The rationale and design of the study have been described extensively elsewhere.[17] Briefly, the cohort started in 1990 and included 7,983 participants (78% of those invited) who were aged 55 years or older and living in Ommoord, a district in Rotterdam in the Netherlands. Invitation into the study occurred in random order. All participants underwent a comprehensive set of baseline examinations. Trained research physicians visited all participants at home for standardized questionnaires about their health status and medical history. Subsequently, all participants visited the research center twice for physical examination and

blood sampling. The study was approved by the Medical Ethics Committee of the Erasmus University Medical Center and all participants gave written informed consent to participate in the study.

Serum liver enzyme measurements

Nonfasting venous blood samples were drawn at the research center and stored at -80°C in 5 mL aliquots. AST, ALT, GGT, ALP, and LDH levels were determined by an enzymatic colorimetric assay (Autoanalyzer Elan, Merck Diagnostica). All measurements were performed at the Clinical Chemistry Laboratory of the Erasmus Medical Center in Rotterdam.

Assessment of stroke

Stroke was defined according to WHO criteria as a syndrome of rapidly developing clinical signs of focal (or global) disturbance of cerebral function, with symptoms lasting 24 hours or longer or leading to death, with no apparent cause other than of vascular origin.[18] History of stroke at baseline was assessed during the baseline interview and verified by reviewing medical records. After enrollment, participants were continuously monitored for incident stroke through automated linkage of the study database with files from general practitioners. Nursing home physicians' files and files from general practitioners of participants who moved out of the district were scrutinized as well. Additional information was obtained from hospital records. Potential strokes were reviewed by research physicians, and verified by an experienced stroke neurologist (P.J.K.). Strokes were classified as cerebral infarction or intracerebral hemorrhage on the basis of neuroimaging reports. If neuroimaging was lacking, strokes were classified as unspecified.

Participants were followed from baseline to stroke, death, last health status update when they were known to be free of stroke, or January 1, 2009, whichever came first. Follow-up was complete up to January 1, 2009 for 99.1% of potential person years.

Other measurements

Medication use and cigarette smoking behavior were assessed during the home interview. Alcohol consumption was assessed as part of a standardized dietary questionnaire. Methods for the computation of the average amount of ethanol intake in gram per day have been described earlier.[19] We defined excessive alcohol use as an average intake of >20 gram ethanol per day (20 gram approximates 2 standard drinks). Blood pressure was calculated as the average of two measurements at the right brachial artery with a random-zero sphygmomanometer after 5 minutes of rest while the subject was in a sitting position. Hypertension was defined as a diastolic blood pressure ≥90 mm Hg, and/or a systolic blood pressure ≥140 mm Hg, and/or the use of blood pressure-lowering medication.[20] Diabetes mellitus was defined as a nonfasting or post-load serum glucose level ≥11.1 mmol/L, and/or the use of blood

glucose-lowering drugs. Body mass index was calculated as weight (in kilograms) divided by the square of height (in meters). Nonfasting serum total cholesterol and high-density lipoprotein (HDL-) cholesterol levels were measured using enzymatic colorimetric methods (Kone Specific Analyzer, Kone Instruments).

Population for analysis

From the 7,983 Rotterdam Study participants, we excluded participants who had had a stroke before baseline (N=261) and participants who had refused consent for the collection of follow-up data from general practitioners (N=175). Of the remaining 7,547 participants at risk for first-ever stroke, 3,631 were not included in the study population because they had not visited the research center (N=668), or because they had visited the research center after December 31, 1992, when clinical chemistry assays had been stopped due to financial constraints (N=1,713), or because blood draw or storage had failed (N=292), or because of missing data on alcohol use (N=958). The remaining 3,916 participants were included in the population for analysis.

Statistical analysis

Cox proportional hazards regression was used to determine hazard ratios and 95% confidence intervals for the associations between baseline liver enzyme levels and risk of intracerebral hemorrhage. We included only first-ever intracerebral hemorrhages in the analyses. Levels of AST, ALT, GGT and ALP were natural log-transformed because of right skewness of their distributions. Hazard ratios were assessed by analyzing liver enzyme levels per standard deviation increase. Enzyme levels were also categorized in tertiles to verify the log-linearity of associations. We constructed three models. In model 1, we adjusted for age and sex. In model 2, we additionally adjusted for average alcohol intake. In model 3, we included age, sex, and a propensity score. The propensity score was constructed to be able to perform meaningful adjustments for many potential confounders (average alcohol intake, systolic blood pressure, blood pressure-lowering medication use, serum total cholesterol level, serum HDL-cholesterol level, current cigarette smoking, diabetes mellitus, body mass index and antithrombotic medication use) despite the relatively small number of events.[21, 22] Missing values in covariates (≤5%) were imputed with a linear regression model based on age and sex.

We further investigated whether the associations between liver enzyme levels and risk of intracerebral hemorrhage differed across strata of excessive alcohol use and we tested the presence of interaction on a multiplicative scale and on an additive scale.[23]

RESULTS

Table 1 shows the baseline characteristics of the study population. Baseline serum liver enzyme levels in the total study cohort and in strata of excessive alcohol use are presented in **Table 2**.

During 50,375 person years of follow-up (median, 15.5 years), 554 first-ever strokes occurred, of which 55 were classified as intracerebral hemorrhage, 317 as cerebral infarction, and 182 as unspecified.

Table 3 shows the associations between separate enzyme levels and the risk of intracerebral hemorrhage.

Table 1. Baseline characteristics (N=3,916)

Age, years	67.5 (62.0-73.7)
Female sex, %	60.8
Current cigarette smoking, %	22.3
Systolic blood pressure, mm Hg	137 (123-153)
Diastolic blood pressure, mm Hg	73 (66-81)
Hypertension, %	55.8
Blood pressure-lowering medication use, %	30.9
Nonfasting serum glucose level, mmol/L	6.2 (5.5-7.4)
Diabetes mellitus, %	9.5
Body mass index, kg/m^2	26.0 (23.9-28.5)
Serum total cholesterol level, mmol/L	6.6 (5.9-7.4)
Serum HDL-cholesterol level, mmol/L	1.3 (1.1-1.6)
Alcohol intake, gram/day	3.2 (0.1-14.8)
Excessive alcohol use (>20 gram/day), %	19.6
Antithrombotic medication use, %	4.1

Values are medians (interquartile range) or percentages.

AST and ALT were not associated with intracerebral hemorrhage. GGT levels were also not associated with intracerebral hemorrhage, although there seemed to be a pattern across increasing tertiles of GGT and risk of intracerebral hemorrhage. In contrast, increasing levels

Table 2. Baseline serum liver enzyme levels of the study population

	Total population	Alcohol intake		
		Non-excessive	Excessive	
	N=3,916	N=3,147	N=769	*P* value*
Aspartate transaminase (U/L)	19 (17-22)	19 (16-22)	21 (18-24)	<0.001
Alanine transaminase (U/L)	16 (12-21)	16 (12-21)	18 (14-24)	<0.001
Gamma-glutamyltranspeptidase (U/L)	23 (18-32)	22 (17-30)	29 (22-43)	<0.001
Alkaline phosphatase (U/L)	76 (63-90)	76 (64-91)	72 (61-87)	<0.001
Lactate dehydrogenase (U/L)	321 (287-360)	323 (288-360)	315 (286-357)	0.78

Values are medians (interquartile range).
* *P* values for the difference in enzyme levels between excessive alcohol drinkers and non-excessive alcohol drinkers are adjusted for age and sex.

Table 3. Associations between liver enzyme levels and risk of intracerebral hemorrhage

		N	Model 1† HR (95% CI)	Model 2‡ HR (95% CI)	Model 3§ HR (95% CI)
Aspartate transaminase (U/L)*					
Per SD		55	1.25 (0.98-1.60)	1.23 (0.95-1.57)	1.20 (0.93-1.54)
Tertile 1	[2.0-17.9]	15	1.00 (ref)	1.00 (ref)	1.00 (ref)
Tertile 2	[19.0-21.0]	18	0.98 (0.49-1.94)	0.96 (0.48-1.90)	0.95 (0.48-1.89)
Tertile 3	[21.1-261.0]	22	1.39 (0.72-2.68)	1.32 (0.68-2.57)	1.26 (0.64-2.45)
P trend			0.31	0.39	0.48
Alanine transaminase (U/L)*					
Per SD		55	1.21 (0.93-1.58)	1.19 (0.91-1.55)	1.23 (0.93-1.61)
Tertile 1	[2-14]	15	1.00 (ref)	1.00 (ref)	1.00 (ref)
Tertile 2	[14-19]	23	1.42 (0.74-2.72)	1.39 (0.72-2.67)	1.42 (0.74-2.74)
Tertile 3	[19-259]	17	1.16 (0.57-2.37)	1.12 (0.55-2.30)	1.17 (0.56-2.43)
P trend			0.68	0.76	0.68
Gamma-glutamyltranspeptidase (U/L)*					
Per SD		55	1.27 (0.99-1.62)	1.24 (0.96-1.59)	1.24 (0.95-1.60)
Tertile 1	[6-19]	14	1.00 (ref)	1.00 (ref)	1.00 (ref)
Tertile 2	[19-28]	20	1.52 (0.76-3.03)	1.49 (0.75-2.99)	1.46 (0.73-2.94)
Tertile 3	[28-586]	21	1.77 (0.87-3.52)	1.65 (0.81-3.36)	1.62 (0.78-3.37)
P trend			0.12	0.17	0.20
Alkaline phosphatase (U/L)*					
Per SD		55	1.35 (1.06-1.72)	1.37 (1.07-1.75)	1.30 (1.02-1.68)
Tertile 1	[4-67]	10	1.00 (ref)	1.00 (ref)	1.00 (ref)
Tertile 2	[67-84]	24	2.54 (1.21-5.31)	2.61 (1.24-5.46)	2.43 (1.16-5.09)
Tertile 3	[85-404]	21	2.32 (1.09-4.93)	2.40 (1.12-5.11)	2.15 (1.00-4.60)
P trend			0.04	0.03	0.07
Lactate dehydrogenase (U/L)					
Per SD		55	1.49 (1.13-1.96)	1.41 (1.11-1.79)	1.42 (1.11-1.81)
Tertile 1	[15-298]	11	1.00 (ref)	1.00 (ref)	1.00 (ref)
Tertile 2	[299-344]	20	1.87 (0.89-3.92)	1.85 (0.88-3.89)	1.86 (0.89-3.92)
Tertile 3	[345-797]	24	2.35 (1.14-4.85)	2.30 (1.12-4.76)	2.34 (1.12-4.91)
P trend			0.02	0.03	0.03

*Serum enzyme levels are natural log-transformed.
†Model 1: adjusted for age and sex.
‡Model 2: adjusted for age, sex and alcohol intake.
§Model 3: as model 1, additionally adjusted for a propensity score of potential confounders.

of ALP and LDH were associated with an increased risk of intracerebral hemorrhage. Adjustments for alcohol use (Model 2) and other potential confounders (Model 3) had little effect on the strength of the associations.

Associations stratified by excessive alcohol use are shown in **Table 4**. AST, ALT and GGT were not associated with intracerebral hemorrhage in non-excessive alcohol users, whereas they were associated in excessive alcohol users. The association between ALP and intracerebral hemorrhage, and particularly the association between LDH and intracerebral hemorrhage also seemed stronger in excessive alcohol users than in non-excessive alcohol users. Formal statistical testing of the presence of effect modification was hampered by the small numbers of events in the strata. Nonetheless, multiplicative interaction terms for AST and ALT with excessive alcohol use were borderline significant (AST, P=0.06; ALT, P=0.07), providing statistical support for the observed differences across strata. We found no support for the presence of interaction on an additive scale (results not shown).

DISCUSSION

In this large prospective cohort study among community-dwelling elderly people, we found that ALP and LDH levels were positively associated with the risk of intracerebral hemorrhage, independently of alcohol consumption and other potential confounders. We further found that increasing levels of AST, ALT and GGT were associated with an increased risk of intracerebral hemorrhage in excessive alcohol drinkers. These three enzymes were not associated with intracerebral hemorrhage in non-excessive drinkers or abstainers.

This study has several strengths, including the prospective and population-based design, the large number of participants, the long duration, and the completeness of the follow-up. However, this study also has some limitations. We did not include 3,631 participants in the analysis because of incomplete data on enzyme levels or alcohol use. However, although these participants were older (median age 77 versus 68 years), they only minimally differed from the study population with respect to other baseline characteristics. Therefore, we consider it unlikely that exclusion of these participants resulted in an important selection bias. Furthermore, because neuroimaging had not been performed in all stroke cases, 33% of the strokes were classified as unspecified. This proportion is comparable to other population-based studies.[24, 25] However, it is likely that an unknown number of intracerebral hemorrhages were misclassified as unspecified, which may have somewhat influenced our effect estimates. Finally, the analyses were based on quite a small number of intracerebral hemorrhages (N=55). Therefore our findings require replication in other cohorts.

Only a few previous studies have investigated liver enzyme levels in relation to incident intracerebral hemorrhage. GGT levels were associated with intracerebral hemorrhage in a hospital-based case-control study from Finland,[26] whereas a population-based case-control

Table 4. Associations in strata of excessive alcohol intake (>20 gram/day)

	No excessive alcohol intake	Excessive alcohol intake	
	N=39‡	N=16‡	*P*
	HR (95% CI)†	HR (95% CI)†	interaction
Aspartate transaminase (U/L)*	0.96 (0.68-1.36)	1.69 (1.20-2.38)	0.06
Alanine transaminase (U/L)*	1.02 (0.72-1.43)	1.75 (1.16-2.64)	0.07
Gamma-glutamyltranspeptidase* (U/L)*	1.08 (0.76-1.54)	1.51 (1.04-2.20)	0.33
Alkaline phosphatase (U/L)*	1.27 (0.94-1.70)	1.55 (0.94-2.57)	0.39
Lactate dehydrogenase (U/L)	1.27 (0.93-1.72)	2.01 (1.30-3.13)	0.14

* Serum enzyme levels are natural log-transformed.
† Hazard ratios are expressed per standard deviation increase and are adjusted for age, sex and a propensity score of potential confounders.
‡ N indicates the number of intracerebral hemorrhages per stratum.

study in France showed that AST, ALT, GGT and ALP levels were not.[27] However, in both studies enzyme levels were assessed after the event had occurred, hence results may be biased by post-stroke changes in enzyme levels. A prospective case-control study nested within a European consortium found a graded relationship between increasing GGT levels and intracerebral hemorrhage.[13] These results are in line with the results of our study, although it should be mentioned that the consortium also included Rotterdam Study data. However, the present study was based on a much longer follow-up, a considerably larger number of incident events, and four additional enzymes. Furthermore, in addition to this previous paper that assumes GGT levels to be an unbiased marker of alcohol consumption,[13] our study shows that increasing GGT levels seem particularly associated with risk of intracerebral hemorrhage in combination with excessive alcohol use. AST and ALT were also associated with risk of intracerebral hemorrhage in a 10–year follow-up study from Korea.[14] Similar to our findings, they reported stronger associations in heavy drinkers as compared with abstainers, albeit they also found an effect in the latter.[14] Because this study was limited to middle-aged men, our study confirms that the associations between AST and ALT and intracerebral hemorrhage can be extrapolated to women and elderly people.

We were particularly interested in the role of alcohol use in the association between liver enzymes and intracerebral hemorrhage. First, we presumed that alcohol use could be a confounder because alcohol intake influences liver enzyme levels and because heavy alcohol intake is a risk factor for both liver disease and intracerebral hemorrhage. However, associations remained virtually unchanged after adjustment for alcohol intake, indicating that the associations between enzyme levels and intracerebral hemorrhage are largely independent of alcohol consumption. Nonetheless, we cannot completely rule out the possibility of residual confounding, for example, because we relied on self-report for the amount of

alcohol intake. Second, alcohol could be an effect modifier. A previous study reported that alcohol and mild liver dysfunction may exert a synergistic effect on intracerebral hemorrhage.[8] When we stratified for excessive alcohol use, we found quite strong associations between AST, ALT, GGT and intracerebral hemorrhage in excessive alcohol drinkers, but no associations in non-excessive drinkers or abstainers. The association between LDH and intracerebral hemorrhage seemed also stronger in excessive alcohol drinkers than in non-excessive drinkers or abstainers, whereas the association between ALP and intracerebral hemorrhage was not clearly modified by excessive alcohol intake. Although our study found only borderline statistical evidence for interaction, the unambiguous differences across the strata suggest that the associations between liver enzymes and intracerebral hemorrhage are indeed modified by alcohol intake.

ALP and LDH are unspecific enzymes that are present in tissues throughout the entire body. Therefore, we do not know whether the associations between ALP, LDH and intracerebral hemorrhage we observed actually reflect liver dysfunction. Apart from liver disease, high ALP levels may indicate bone disorders, and highly elevated LDH levels may indicated for example hemolysis or hematologic malignancies. Assessment of tissue-specific isoenzymes is needed to further unravel the nature of the associations between ALP, LHD and intracerebral hemorrhage.

Another issue that should be discussed is that the enzyme levels which we found to be associated with risk of intracerebral hemorrhage were within the reference range. Therefore, it seems unlikely that hematological disturbances, which often accompany severe liver disease, can explain the observed associations between enzyme levels and intracerebral hemorrhage. Alternative pathways linking high-normal enzyme levels to intracerebral hemorrhage could be through hypertension or diabetes mellitus. Hypertension and diabetes mellitus are associated with intracerebral hemorrhage.[15] Moreover, high GGT levels have been associated with risk of hypertension,[28] and high AST, ALT and GGT levels have been associated with incident diabetes mellitus.[28, 29] However, adjustments for hypertension and diabetes mellitus did not change any of the associations, suggesting that these factors are no important confounders and probably not in the causal pathway.

In this cohort study among community-dwelling elderly people, we found that increasing levels of the enzymes ALP and LDH were associated with an increased risk of intracerebral hemorrhage, regardless of alcohol use. Increasing levels of the enzymes ALT, AST and GGT were only associated with an increased risk of intracerebral hemorrhage in people who drink excessive amounts of alcohol. The mechanisms of these associations are yet unknown and need to be further clarified.

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Chapter 5.3

Insulin resistance and risk of stroke and stroke subtypes in nondiabetic elderly

ABSTRACT

Background and purpose – Insulin resistance, which plays a key role in the development of diabetes mellitus, is a putative modifiable risk factor for stroke. The aim of this study was to investigate if markers of insulin resistance were associated with risk of stroke in the general elderly population.

Methods – The study included 5,234 participants of the longitudinal population-based Rotterdam Study, who were aged 55 years or older and stroke-free and diabetes-free at baseline (1997–2001). Fasting insulin levels and homeostasis model assessment for insulin resistance (HOMA-IR) were used as markers for insulin resistance. Cox regression was used to determine associations between insulin resistance markers and stroke risk, adjusted for age, sex and potential confounders.

Results – During 42,806 person years of follow-up (median 8.6 years), 366 first-ever strokes occurred of which 225 were cerebral infarctions, 42 were intracerebral hemorrhages and 99 were unspecified strokes. Fasting insulin levels were not associated with risk of any stroke or cerebral infarction or intracerebral hemorrhage. HOMA-IR, which almost perfectly correlated with fasting insulin levels, was also not associated with risk of stroke or stroke subtypes.

Conclusion – In this population-based cohort study among nondiabetic elderly, insulin resistance markers were not associated with risk of stroke or any of its subtypes.

INTRODUCTION

Type 2 diabetes mellitus is a major risk factor for vascular disease, including stroke.[1] Moreover, several studies suggest that precursor stadia of diabetes mellitus, such as impaired fasting glucose and insulin resistance, already increase the risk of vascular complications.[2, 3] However, results from studies investigating coronary heart disease or vascular disease either separately or as a composite endpoint may not apply to stroke. For example, in a large meta-analysis, fasting glucose levels below the threshold for diabetes mellitus (7.0 mmol/L) were associated with risk of coronary heart disease, but not with risk of stroke.[4] The relationship between insulin resistance and stroke risk has been studied less extensively.

The gold standard for quantifying insulin resistance is the euglycemic hyperinsulinemic glucose clamp technique.[5] Because this method is complicated and impractical for epidemiologic research, epidemiologic studies use either fasting insulin levels or the homeostasis model assessment for insulin resistance (HOMA-IR) index as surrogate markers for the degree of insulin resistance. Both markers correlate well with the clamp method.[6, 7]

Previous studies have investigated the association between insulin resistance and stroke, but results remain inconclusive. An overview of population-based cohort studies reporting on the association of fasting insulin levels and HOMA-IR index with risk of stroke in people without diabetes mellitus is given in **Tables 1** and **2**.[8–14]

Taken together, these studies do not enable us to draw firm conclusions regarding the association between insulin resistance and risk of stroke. Apart from conflicting results, most studies were based on relatively small numbers of events and included middle-aged but not elderly people, whereas elderly people are at the highest risk of stroke. Furthermore, all studies focused on any stroke or the subtype cerebral infarction. The association between insulin resistance markers and risk of intracerebral hemorrhage has yet not been investigated.

The aim of the present study was to investigate in a large population-based cohort study among elderly people without diabetes mellitus if fasting insulin levels and HOMA-IR index were associated with risk of stroke. We also investigated if fasting insulin levels and HOMA-IR index were associated with risk of cerebral infarction and intracerebral hemorrhage.

METHODS

Source population

The Rotterdam Study is an ongoing prospective population-based cohort study that focuses on causes and consequences of diseases that are frequent in the elderly. The rationale and design of the study have been described extensively elsewhere.[15] Briefly, the cohort started in 1990 and included 7,983 participants who were aged 55 years or older and living in Ommoord, a district in Rotterdam in the Netherlands (Rotterdam Study I). In the year

Table 1. Overview of prospective cohort studies reporting on the association between fasting insulin levels and risk of stroke in people without diabetes mellitus

	Age range	Women %	At risk N	FUP years	Outcome	N	Continuous	HR (95% CI)	Categorical	HR (95% CI)	Covariates
Pyorala[12] 1998 Finland	34-64	0	970	22.3 (median)	Any stroke	70	Per SD	1.07 (0.81-1.41)	Q5 vs Q1-4	1.54 (0.90-2.62)	Age, SBP, smoking, scapular skinfold
Lakka[8] 2000 Finland	42-60	0	1,521	9.4 (mean)	Any stroke	48	Per pmol/L	1.01 (1.00-1.01)	Quartile 1 Quartile 2 Quartile 3 Quartile 4	1.0 (ref) 1.5 (0.5-4.0) 1.1 (0.4-3.0) 1.4 (0.5-4.0)	Age, year, SBP, DBP, smoking, lipids, BMI, WHR, alcohol, WBC, fibrinogen, VO2 max
Lindahl[10] 2000 Sweden	25-64	37	272	12 (max)	Any stroke	94			Tertile 1 Tertile 2 Tertile 3	1.0 (ref) 1.1 (0.6-2.3) 2.0 (1.0-4.0)	None
Lawlor[9] 2007 UK	60-79	100	3,246	4.6 (median)	Any stroke	52	[e]Log per SD	1.16 (0.83-1.59)			Age, SBP, smoking, lipids, BMI, WHR, exercise, SES
Nakamura[11] 2010 Japan	35-59	0	2,548	10 (mean)	Cerebral infarction	13	[e]Log per SD	1.62 (1.03-2.57)	Q4 vs Q1	4.01 (1.10-14.67)	Age, SBP, blood pressure-lowering, drugs, smoking, lipids waist, alcohol, exercise, lipid- lowering drugs
Rasmussen[13] 2010 USA	45-64	57	12,323	16-18 (max)	Cerebral infarction	445			Quintile 1 Quintile 2 Quintile 3 Quintile 4 Quintile 5	1.00 (ref) 0.89 (0.63-1.25) 1.11 (0.82-1.51) 1.10 (0.80-1.53) 1.28 (0.92-1.78)	Age, sex, race, center, SBP, blood pressure-lowering drugs, smoking, BMI, LVH

Abbreviations: BMI = body mass index; DBP = diastolic blood pressure; FUP = follow-up; LVH = left ventricular hypertrophy; SBP = systolic blood pressure; SES = socio-economic status; VO2 max = peak oxygen uptake; WHR = waist-to-hip ratio.

Table 2. Overview of prospective cohort studies reporting on the association between HOMA-IR index and risk of stroke in people without diabetes mellitus

	Age range	Women %	At risk N	FUP years	Outcome	N	Continuous	HR (95% CI)	Categorical	HR (95% CI)	Covariates
Tanne[21] 2009 Israel	45-74	9	2,938	6.2 (mean)	Any stroke	172	[e]Log per unit	1.07 (0.90-1.26)	Tertile 1 Tertile 2 Tertile 3	1.00 (ref) 1.07 (0.72-1.58) 1.11 (0.74-1.67)	Age, sex, study arm, hypertension, smoking, lipids, BMI, CHD
Nakamura[11] 2010 Japan	35-59	0	2,548	10 (mean)	Cerebral infarction	13	[e]Log per SD	1.59 (1.00-2.54)	Quartile 1 Quartile 2 Quartile 3 Quartile 4	1.00 (ref) 1.40 (n.s.)* 1.15 (n.s.)* 3.23 (0.82-12.89)	Age, SBP, blood pressure-lowering drugs, lipids, waist, alcohol, smoking, exercise, lipid-lowering drugs
Rundek[14] 2010 USA	68	64	1,509	8.5 (mean)	Cerebral infarction	46	Per SD†	1.04 (0.90-1.19)	Quartile 1 Quartile 2 Quartile 3 Quartile 4	1.00 (ref) 1.88 (0.72-4.87)† 0.93 (0.29-2.95)† 3.11 (1.25-7.76)†	Age, sex, race, SBP, DBP, smoking, lipids, waist, alcohol, exercise, education

Abbreviations: BMI = body mass index; CHD = coronary heart disease; DBP = diastolic blood pressure; FUP = follow-up; HOMA-IR = homeostasis model assessment for insulin resistance; SBP = systolic blood pressure.
* Hazard ratios and 95% confidence intervals are plotted on a log scale, values not reported.
† Adjusted for age only.

2000, the cohort was expanded with 3,011 participants who had reached the age of 55 or had moved into the district since the start of the study (Rotterdam Study II). All participants underwent a comprehensive set of baseline examinations which were repeated during regular follow-up visits. The study was approved by the Medical Ethics Committee of the Erasmus University Medical Center and all participants gave written informed consent to participate in the study.

Measurement of serum glucose and insulin levels and HOMA-IR

Venous blood samples were taken at the research center after an overnight fast and stored at -80 ºC in a number of 5 mL aliquots. Serum glucose levels were determined using the glucose hexokinase method within one week after sampling.[16] Serum insulin levels were determined in samples that had been kept frozen from baseline (1997–2001) until usage in 2008 by metric assay (Biosource Diagnostics, Camarillo, CA). This assay does not cross-react with proinsulin or C-peptide. All measurements were carried out at the Clinical Chemistry Laboratory in the Erasmus University Medical Center. The following formula was used to calculate HOMA-IR: [fasting insulin (mU/L) × fasting glucose (mmol/L)] / 22.5.[7]

Definition of diabetes mellitus

Diabetes mellitus was defined as a fasting serum glucose level ≥7.0 mmol/L and/or the use of blood glucose-lowering drugs.

Assessment of stroke

Stroke was defined according to World Health Organization criteria as a syndrome of rapidly developing clinical signs of focal (or global) disturbance of cerebral function, with symptoms lasting 24 hours or longer or leading to death, with no apparent cause other than of vascular origin.[17] History of stroke at baseline was assessed during the baseline interview and verified by reviewing medical records. After enrollment, participants were continuously monitored for incident stroke through automated linkage of the study database with files from general practitioners. Nursing home physicians' files and files from general practitioners of participants who moved out of the district were checked on a regular basis as well. Additional information was obtained from hospital records. Potential strokes were reviewed by research physicians, and verified by an experienced stroke neurologist (P.J.K.). Strokes were further classified as cerebral infarction or intracerebral hemorrhage on the basis of neuroimaging reports. If neuroimaging was lacking, a stroke was classified as unspecified. Subarachnoid hemorrhages were not considered stroke events.

Participants were followed from study entry to stroke, death, last health status update when they were known to be stroke-free, or January 1, 2009, whichever came first. Follow-up was complete up to January 1, 2009 for 97.6% of potential person years.

Other measurements

Trained research physicians visited all participants at home for standardized questionnaires about their health status and medical history, including questions about current medication use and cigarette smoking behavior. Subsequently, all participants visited the research center twice for physical examination and blood sampling. Blood pressure was calculated as the average of two measurements at the right brachial artery with a random-zero sphygmomanometer after 5 minutes of rest while the subject was in a sitting position. Hypertension was defined as a diastolic blood pressure of ≥90 mm Hg and/or a systolic blood pressure of ≥140 mm Hg, and/or the use of blood pressure-lowering medication.[18] Fasting serum total cholesterol, high-density lipoprotein cholesterol and triglyceride levels were measured using enzymatic colorimetric methods (Hitachi Analyzer, Roche Diagnostics). Von Willebrand factor antigen was determined with an in-house enzyme-linked immunosorbent assay, using polyclonal rabbit antihuman von Willebrand factor antibodies (DakoCytomation, Glostrop, Denmark) for catching and tagging.[19] Body mass index was calculated as weight (in kilograms) divided by the square of height (in meters). The waist-to-hip ratio was calculated by dividing the waist circumference (in centimeters) by the hip circumference (in centimeters).

Population for analysis

The present analyses were based on participants of the third survey of Rotterdam Study I (N=5,990) and participants of the first survey of Rotterdam Study II (N=3,011). Of this pooled cohort of 9,001 participants, 8,530 persons were stroke-free at baseline and had given written informed consent for the collection of follow-up data from general practitioners. Participants who had not visited the research center for blood sampling due to death, refusal or physical inability (N=1,883), as well as participants of whom blood draw or storage had failed (N=479) and participants who had not been fasting before blood draw (N=299), were excluded. Of the remaining 5,689 participants, we excluded 635 persons with prevalent diabetes mellitus. This resulted in a diabetes-free and stroke-free study population of N=5,234 participants.

Statistical analysis

We estimated Pairwise Pearson correlation coefficients to determine the strength of the linear relationships between fasting glucose levels, fasting insulin levels, and HOMA-IR index. Insulin levels and HOMA-IR index were natural log-transformed because of non-normality of their distributions. Cox proportional hazards regression was used to calculate hazard ratios and 95% confidence intervals for the associations between fasting insulin levels, HOMA-IR index, fasting glucose levels, and the risk of stroke and the stroke subtypes cerebral infarction and intracerebral hemorrhage. Only first-ever strokes were included in the analyses. Hazard ratios were calculated by analyzing ^{e}log insulin, ^{e}log HOMA-IR and glucose per

standard deviation increase. To verify the (log-)linearity of associations, we also categorized the exposure levels in quartiles using the lowest quartile as the reference category. The linear trend across quartiles was tested by including quartile categories as a continuous variable in the model. All analyses were adjusted for age and sex (Model 1), and additionally for age, sex, and a propensity score that included the following potential confounders: systolic blood pressure, blood pressure-lowering medication use, serum total cholesterol level, high-density lipoprotein cholesterol level, triglyceride level, lipid-lowering medication use, current cigarette smoking, body mass index, waist-to-hip ratio and plasma von Willebrand factor level (Model 2).[20] The main reason to adjust for a propensity score instead of individual covariates was that the number of intracerebral hemorrhages in our study was small in comparison to the number of potential confounders. Missing values in covariates, which varied from 0.0% to 5.1% per variable, were imputed with a linear regression model based on age and sex. All analyses were performed using PASW Statistics for Windows, version 17.0 (SPSS Inc., Chicago, Illinois).

RESULTS

Baseline characteristics of the study population are shown in **Table 3**. The median age was 67.9 years and 57.6% were women. The Pairwise Pearson correlation coefficient for elog insulin and ^{e}log HOMA-IR was 0.99, indicating almost perfect linearity. Glucose and ^{e}log HOMA-IR were only weakly correlated, r=0.51, as were glucose and ^{e}log insulin, r=0.37.

During 42,806 person years of follow-up (median 8.6 years), 366 participants developed a first-ever stroke, which was classified in 225 as cerebral infarction, in 42 as intracerebral hemorrhage and in 99 as unspecified.

Table 4 shows the associations between fasting insulin levels, HOMA-IR index, fasting glucose levels,

Table 3. Baseline characteristics (N=5,234)

Age, years	67.9 (62.4-74.6)
Female sex, %	57.6
Current cigarette smoking, %	17.5
Systolic blood pressure, mm Hg	140 (127-155)
Diastolic blood pressure, mm Hg	76 (69-84)
Blood pressure-lowering medication use, %	32.2
Hypertension, %	65.3
Serum total cholesterol level, mmol/L	5.8 (5.2-6.5)
Serum HDL-cholesterol level, mmol/L	1.4 (1.1-1.6)
Serum triglycerides level, mmol/L	1.3 (1.0-1.8)
Lipid-lowering medication use, %	11.9
Body mass index, kg/m^2	26.3 (24.1-28.9)
Waist-to-hip ratio, cm/cm	0.92 (0.84-0.98)
Plasma von Willebrand factor level, IU/mL	1.2 (0.9-1.6)
Fasting serum glucose level, mmol/L	5.5 (5.2-5.9)
Fasting serum insulin level, pmol/L	65 (46-91)
HOMA-IR index	2.3 (1.6-3.3)

Abbreviations: HOMA-IR = homeostasis model assessment for insulin resistance.
Values are medians (interquartile range) or percentages.

Table 4. Associations of insulin, HOMA-IR and glucose with risk of any stroke

		At risk	Events	Model 1†	Model 2‡
		N	N	HR (95% CI)	HR (95% CI)
Insulin level, pmol/L					
Per SD*		5,234	366	0.94 (0.85-1.05)	0.86 (0.76-0.98)
Quartile 1	[10-46]	1,318	94	1.00 (ref)	1.00 (ref)
Quartile 2	[47-64]	1,269	91	0.96 (0.72-1.28)	0.91(0.68-1.22)
Quartile 3	[65-90]	1,338	94	0.98 (0.74-1.30)	0.88 (0.65-1.19)
Quartile 4	[91-430]	1,309	87	0.95 (0.71-1.27)	0.80 (0.57-1.12)
P trend				0.76	0.20
HOMA-IR					
Per SD*		5,234	366	0.95 (0.86-1.06)	0.86 (0.76-0.98)
Quartile 1	[0.3-1.6]	1,310	100	1.00 (ref)	1.00 (ref)
Quartile 2	[1.6-2.3]	1,303	84	0.78 (0.58-1.04)	0.73 (0.54-0.97)
Quartile 3	[2.3-3.3]	1,313	96	0.96 (0.73-1.28)	0.84 (0.62-1.13)
Quartile 4	[3.3-5.2]	1,308	86	0.86 (0.64-1.14)	0.68 (0.49-0.96)
P trend				0.57	0.07
Glucose level, mmol/L					
Per SD		5,234	366	1.05 (0.94-1.16)	1.00 (0.90-1.11)
Quartile 1	[3.7-5.1]	1,221	91	1.00 (ref)	1.00 (ref)
Quartile 2	[5.2-5.5]	1,555	102	0.89 (0.67-1.18)	0.86 (0.65-1.14)
Quartile 3	[5.5-5.9]	1,031	61	0.78 (0.57-1.08)	0.73 (0.52-1.01)
Quartile 4	[5.9-6.9]	1,427	112	1.06 (0.80-1.40)	0.94 (0.70-1.26)
P trend				0.74	0.61

Abbreviations: HOMA-IR = homeostasis model assessment for insulin resistance.
*Insulin levels and HOMA-IR index are natural log-transformed.
†Model 1: adjusted for age and sex.
‡Model 2: adjusted for age, sex, and a propensity score (current smoking, systolic blood pressure, blood pressure-lowering medication use, serum total cholesterol level, serum HDL-cholesterol level, serum triglyceride level, lipid-lowering medication use, plasma von Willebrand factor level, body mass index, and waist-to-hip ratio).

and risk of any stroke. Fasting insulin levels and HOMA-IR index were not associated with risk of stroke after adjustment for age and sex (Model 1). However, insulin levels and HOMA-IR index were borderline inversely associated with risk of stroke after adjustment for multiple putative confounders (Model 2). Furthermore, effect estimates for insulin levels and HOMA-IR were practically identical, as could be expected given their high correlation. Fasting glucose levels in the nondiabetic range were also not associated with risk of stroke. However, because the pattern across quartiles seemed suggestive of a curvilinear relationship, we additionally performed a quadratic trend test, which did not reach statistical significance at the conventional α=0.05 level (P=0.06).

Associations between insulin levels, HOMA-IR, glucose levels, and risk of the stroke subtypes cerebral infarction and intracerebral hemorrhage are shown in **Table 5**. Fasting insulin levels and HOMA-IR were not associated with cerebral infarction after adjusting for age and sex, but they were inversely associated with cerebral infarction after adjusting for

Table 5. Associations of insulin, HOMA-IR and glucose with risk of cerebral infarction and intracerebral hemorrhage

	Model 1†	Model 2‡
	HR (95% CI)	HR (95% CI)
Cerebral infarction (N=225)		
Insulin level per SD*	0.92 (0.81-1.06)	0.83 (0.71-0.98)
HOMA-IR index per SD*	0.93 (0.82-1.06)	0.83 (0.71-0.97)
Glucose level per SD	1.02 (0.90-1.17)	0.97 (0.85-1.12)
Intracerebral hemorrhage (N=42)		
Insulin level per SD*	1.00 (0.73-1.35)	1.08 (0.74-1.55)
HOMA-IR index per SD*	1.03 (0.76-1.39)	1.11 (0.76-1.60)
Glucose level per SD	1.19 (0.89-1.60)	1.16 (0.85-1.59)

Abbreviations: HOMA-IR = homeostasis model assessment for insulin resistance.
* Insulin levels and HOMA-IR index are natural log-transformed.
†Model 1: adjusted for age and sex.
‡Model 2: adjusted for age, sex, and a propensity score (current smoking, systolic blood pressure, blood pressure-lowering medication use, serum total cholesterol level, serum HDL-cholesterol level, serum triglyceride level, lipid-lowering medication use, plasma von Willebrand factor level, body mass index, and waist-to-hip ratio).

multiple confounders, similarly to what we found for any stroke. Fasting glucose levels were not associated with risk of cerebral infarction in either model. **Table 5** further shows that neither fasting insulin levels, nor HOMA-IR, nor fasting glucose levels were associated with risk of intracerebral hemorrhage.

Because we did not observe any association between increasing glucose or insulin levels and stroke, we checked in a post-hoc analysis whether diabetes mellitus was associated with risk of stroke in our study cohort. For this purpose, we added the participants with diabetes mellitus at baseline (N=635), who we had initially excluded from the study population, to the population for analysis. We used Cox regression to calculate the association between diabetes mellitus and risk of stroke, adjusted for age, sex, and the propensity score of potential confounders. Diabetes mellitus was indeed associated with an increased risk of stroke, independently of confounders (HR, 1.59; 95% CI, 1.06 to 2.40 [Model 2]).

DISCUSSION

In this prospective population-based cohort study among elderly people without diabetes, we found no evidence for a longitudinal relationship between increasing degrees of insulin resistance and risk of stroke. Previous studies investigating the association between fasting insulin levels and stroke risk observed either borderline significant associations or positive associations that were strongly attenuated after adjustment for confounders, particularly after

adjustment for components of the metabolic syndrome,[8–10, 12, 13] though one study reported a positive association that remained after adjustment for confounders (**Table 1**).[11] However, all studies but one were conducted among middle-aged men and women.[8, 10–13] The only study carried out in an older population was restricted to women and found no association.[9] Our finding that fasting insulin levels are not associated with risk of stroke in elderly men and women adds to these previous findings and fits with prior knowledge of diabetes mellitus being a stronger risk factor for stroke in younger than in older people.[4]

Associations between HOMA-IR index and stroke risk have also been investigated before.[11, 14] Because HOMA-IR was reported to correlate better with the euglycemic hyperinsulinemic glucose clamp technique than fasting insulin levels, recent epidemiologic studies tend to use HOMA-IR as a marker for insulin resistance.[6, 7] However, in normoglycemic conditions, fasting insulin levels also accurately reflect insulin resistance.[7] Therefore, it was not surprising that we observed an almost perfect correlation between fasting insulin level and HOMA-IR index (r=0.99). Nonetheless, for the sake of comparability with other studies, we presented the results for both fasting insulin level and HOMA-IR index. Three follow-up studies addressed the association between HOMA-IR and stroke with conflicting results (**Table 2**). One study found no association but was conducted in a highly selected study population of people with pre-existing coronary heart disease.[21] Two other studies, one of which included only 13 events,[11] the other only 46 events,[14] reported (borderline) positive associations. However, because the effect estimates for successive HOMA-IR quartiles in these studies were fluctuating, the interpretation of these findings remains questionable. We showed in the largest study so far that HOMA-IR, like fasting insulin level, is not associated with stroke or cerebral infarction. Furthermore, our study shows that in normoglycemic elderly, HOMA-IR does not provide any additional information beyond fasting insulin level.

It should be noted that, though we found null-associations between insulin and HOMA-IR index and stroke in minimally adjusted analyses, we found inverse associations when we adjusted for multiple putative confounders. Based on prior knowledge, we consider a protective effect of increasing insulin resistance on stroke risk highly unlikely. Instead, we consider it more likely that the inverse association is a spurious relationship introduced after the adjustment for strong confounders.

Because diabetes mellitus is not only associated with risk of thromboembolic vascular disease but also with risk of intracerebral hemorrhage, we also investigated the relationship between insulin resistance markers and intracerebral hemorrhage.[4] We found that neither fasting insulin levels nor HOMA-IR were associated with an increased risk. We are not aware of any studies reporting on the association between fasting insulin level or HOMA-IR and risk of intracerebral hemorrhage. Therefore, our study provides new information that insulin resistance does not play a major role in the occurrence intracerebral hemorrhage. However,

because our results were based on only 42 intracerebral hemorrhages, additional studies are needed to confirm these findings.

Diabetes mellitus is diagnosed if the fasting glucose level exceeds 7.0 mmol/L.[22] The rationale for this threshold is that the presence of retinopathy lesions increases at higher levels.[22] The observation that diabetes mellitus, as well as glucose levels above 7.0 mmol/L associate with stroke risk, whereas precursor stadia of diabetes mellitus do not, indicates that a similar threshold may also hold for stroke risk.[4] Besides, because the retinal microvasculature and the cerebral microvasculature have many characteristics in common,[23] it is not surprising that the deleterious effects of hyperglycemia, hyperinsulinemia and insulin resistance start at similar thresholds in both retinopathy and stroke.

Strengths of the study include the prospective and population-based design, the large number of participants, and the long duration and completeness of the follow-up. However, this study also has some limitations. We did not include 2,477 participants in the analysis because of incomplete data on fasting glucose levels or fasting insulin levels. These participants were older (median age 74.5 versus 67.9 years) and more often female (68% versus 58%) in comparison to the study population. Therefore, it is possible that exclusion of these participants resulted in some selection bias. However, in longitudinal studies a more important source of selection bias is loss to follow-up, which was only 2.4% in this study. Another consideration is that, inherent to the population-based design and rigorous stroke monitoring procedure, neuroimaging had not been performed in all stroke cases. As a result, 27% of the strokes could not be classified as either cerebral infarction or intracerebral hemorrhage. However, because the association between fasting insulin levels and unspecified stroke risk (HR per SD, 0.86; 95% CI, 0.68 to 1.08 [Model 2]) was not materially different from the association between insulin levels and cerebral infarction or intracerebral hemorrhage (**Table 4**), we consider this misclassification of limited importance. In addition, fasting insulin levels were measured only once so we could not investigate whether results were influenced by intra-individual fluctuations in insulin resistance.

To conclude, in this population-based cohort study among nondiabetic elderly people we found no evidence for an association between markers of insulin resistance and risk of any stroke, cerebral infarction, or intracerebral hemorrhage. Taken together with previous findings that fasting glucose levels below the threshold for diabetes mellitus are not associated with stroke risk,[4] these results indicate that, in contrast with overt diabetes mellitus, precursor stadia of diabetes mellitus, as measured by fasting glucose levels, fasting insulin levels and HOMA-IR index, do not seem to be important risk factors for stroke, cerebral infarction, or intracerebral hemorrhage.

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CHAPTER 6

GENERAL DISCUSSION

The main goals of my thesis were: (1) to study temporal trends in stroke incidence rates and conventional stroke risk factors in the Netherlands during the past twenty years; (2) to identify new risk indicators for stroke and more specifically for cerebral infarction and intracerebral hemorrhage. The studies described in this thesis were based on data from the Rotterdam Study, an ongoing population-based cohort study among elderly people living in Ommoord, a district in the city of Rotterdam in the Netherlands.

In the following, I will first describe and comment on the main findings of this thesis. Then, I will consider methodological issues regarding these findings. Finally, I will discuss potential clinical implications of my findings and give recommendations for future research.

MAIN FINDINGS

Stroke epidemiology

Stroke incidence rates increase exponentially with age.[1] Because the number of elderly people is expected to rise steeply in the years ahead, the number of people that will experience the burden of stroke is predicted to increase enormously, putting a large appeal on health care facilities and budgets.[2] On the other hand, stroke is increasingly considered a disease that can be prevented and numerous efforts have been undertaken to prevent stroke both at the population level and at the individual level. In several high-income countries, age-specific stroke incidence rates have decreased in recent decades, probably as a result of improvements in primary prevention.[3] In chapter 2.1, I investigated whether these observations also hold for the Netherlands. I studied changes in stroke incidence rates and in the prevalence and treatment of stroke risk factors from 1990 to 2008 by comparing two separate Rotterdam Study subcohorts. The first subcohort was followed for the occurrence of stroke from 1990 to 1998; the second subcohort was followed from 2000 to 2008. Inclusion criteria for these subcohorts were identical.[4] The major finding was a 34% decrease in stroke incidence rates in men, but no change at all in women. Remarkably though, trends in vascular risk factors were not indisputably favorable, except for cigarette smoking which had decreased substantially in men. Other risk factors, such as atrial fibrillation, low high-density lipoprotein cholesterol level and diabetes mellitus were unchanged, whereas high blood pressure and obesity had even increased. However, the proportion of men and women taking preventive medication, particularly lipid-lowering drugs, antithrombotic agents and medication for the treatment of diabetes mellitus had increased enormously.

These results suggest that strategies to reduce stroke risk have been implemented quite successfully in men, but not in women. Previous studies have demonstrated that physicians tend to underestimate the importance of vascular risk factors in women, as their perceived risk is often lower than their calculated risk.[5] This may result in relative undertreatment of women. In addition, given the projected rise in the number of older people in our popu-

lation, current achievements in primary prevention may be insufficient to counterbalance the expected increase in stroke incidence due to demographic changes, particularly in women.[2]

NOVEL RISK FACTORS AND RISK MARKERS FOR CEREBRAL INFARCTION AND INTRACEREBRAL HEMORRHAGE

Von Willebrand factor

Among the first steps in establishing targets for primary prevention are the identification of modifiable risk factors and the identification of risk markers that aid in the stratification of people at risk for disease. I studied von Willebrand factor as a potential target (chapter 3). Von Willebrand factor is a multimeric glycoprotein that is synthesized and stored in endothelial cells.[6] Endothelial dysfunction, the initial step of atherosclerosis, results in elevated secretion of stored von Willebrand factor into the circulation.[7] High circulating von Willebrand factor levels are therefore thought to indicate endothelial dysfunction.[8] In addition, von Willebrand factor plays a key role in primary hemostasis in situations of high shear stress, for example in stenotic arteries.[9] In chapter 3.1, I describe the association between von Willebrand factor plasma levels and risk of any stroke and cerebral infarction. High von Willebrand factor levels were associated with an increased risk of stroke and cerebral infarction independently of age and sex, ABO blood group, and other potential confounders.

Plasma von Willebrand factor levels are influenced by non-genetic factors, such as age, hormones and vascular risk factors, but they are also strongly heritable.[10, 11] Therefore, I subsequently tried to find out to what extent the association between plasma von Willebrand factor levels and risk of stroke and cerebral infarction was attributable to its genetic components (chapter 3.2). We created a genetic score of SNPs that have been shown to associate with plasma von Willebrand factor levels,[12] and assessed whether this genetic score was also associated with stroke risk. Although increasing values of the genetic score strongly associated with increasing plasma von Willebrand factor levels, the genetic score showed no association with risk of stroke or cerebral infarction.

In addition, because ABO blood group is the strongest genetic determinant of von Willebrand factor levels,[13] and because several cross-sectional studies have suggested a relationship between ABO blood group and cardiovascular disease,[14] we also investigated the associations between ABO blood group genotypes and phenotypes and stroke risk. However, we found no support for a longitudinal relationship between ABO blood group and stroke risk.

Together, these studies suggest that von Willebrand factor levels are associated with an increased risk of stroke and cerebral infarction. This association is probably due to environmental influences on von Willebrand factor levels and not due to genetically determined elevations. Moreover, this implies that von Willebrand factor is a marker of stroke risk, but

probably not an important causal factor.

Retinal determinants

The eye offers a window to the cerebral vasculature. Because the retinal and cerebral vasculatures share several embryologic, morphologic and physiologic properties, pathologic retinal vascular changes are thought to reflect concurrent pathology in the cerebral vasculature.[15, 16] Furthermore, the retina and its vasculature are directly accessible and can be visualized easily and non-invasively. In chapter 4, I described the relationship between three different retina disorders and risk of stroke, cerebral infarction and intracerebral hemorrhage.

First, I studied retinal vascular calibers in association with stroke subtypes (chapter 4.1). Previous studies have shown that retinal vascular caliber changes are associated with conventional vascular risk factors, and also with incident stroke and cerebral infarction, and with progression of cerebral small vessel disease.[17–24] I investigated the association between retinal arteriolar and venular calibers and stroke, with particular focus on intracerebral hemorrhage. Wider retinal venular calibers were associated with an increased risk of intracerebral hemorrhage, and this association was much stronger with lobar than with deep intracerebral hemorrhages. In addition, both narrower arteriolar caliber and wider venular caliber were associated with an increased risk of anticoagulation-related intracerebral hemorrhage.

Second, I assessed the association between presence and severity of retinopathy and risk of stroke and stroke subtypes (chapter 4.2). Retinopathy is a common microvascular complication of diabetes mellitus and hypertension, but also occurs without these conditions.[25, 26] Furthermore, like retinal vascular caliber changes, retinopathy signs may be indicators of concurrent cerebrovascular pathology.[15, 16] Although I could confirm the previously reported association between retinopathy and stroke, the increased risk could not be attributed to either cerebral infarction or intracerebral hemorrhage. Instead, the strongest association was found between retinopathy and the unspecified stroke subtype. Because about 85% to 90% of all strokes are cerebral infarctions,[27] it is likely that a similar percentage of unspecified strokes are in fact cerebral infarctions. However, given that retinopathy showed no association with cerebral infarction, it is uncertain if cerebral infarction underlies the observed association. Instead, this association may be confounded by characteristics that increase the likelihood of stroke remaining unspecified. Although the association between retinopathy and unspecified stroke was only partly attenuated and still significant after adjustment for many putative confounders, such as age, vascular risk factors, living situation and dementia prior to stroke, the association may still be explained by residual confounding. Retinal dot hemorrhages and microaneurysms are unspecific microvascular lesions that also occur in the absence of hypertension or diabetes mellitus. Therefore, these lesions might be markers of general frailty, or biologic aging (rather than chronologic aging), or fragile vessels. The frailty concept is not clearly defined, but mostly includes impairments of the domains strength, function, nutri-

tion, mobility, and cognition.[28–31] Frailty is associated with an increased risk of cardiovascular disease, and cardiovascular disease in turn is associated with an increased risk of developing frailty.[32] Furthermore, frailty often goes hand in hand with comorbidities and disabilities.[33] Therefore, frailty might be associated with stroke risk, and might also be a reason for not being referred to a hospital in the event of a stroke. Unfortunately, because we were not able to adjust for frailty in our analyses, the question whether frailty explains the association between retinopathy and unspecified stroke remains unanswered. Besides, instead of being a confounder, frailty could also be a common antecedent of both retinopathy and unspecified stroke. Anyhow, further research is needed to clarify if frailty is involved in the unexpected and yet unexplained association between retinopathy and unspecified stroke.

Third, I investigated the association between age-related macular degeneration (AMD) and risk of stroke, cerebral infarction and intracerebral hemorrhage (chapter 4.3). Although several vascular risk factors are associated with AMD, it is not generally considered a vascular disorder.[34–37] Nevertheless, because AMD and stroke share many risk factors, there might be a common pathophysiologic mechanism underlying both disorders.[35] I found that late AMD, the most advanced stage, was strongly associated with risk of intracerebral hemorrhage, but not with risk of cerebral infarction. Early AMD was not associated with either subtype. This finding suggests that progression from early to late AMD and the occurrence of intracerebral hemorrhage may share a common underlying pathway. Further research is required to unravel the precise mechanisms.

Altogether, these studies show that retinal determinants provide valuable information on vascular pathology ongoing in the brain and they particularly suggest that retinal determinants may aid in the identification of people at risk for intracerebral hemorrhage.

Metabolic disturbances

In chapter 5, I studied the associations between several metabolic disturbances and risk of cerebral infarction or intracerebral hemorrhage. In chapter 5.1, I focused on lipid metabolism. Whereas high total cholesterol levels are associated with an increased risk of cerebral infarction, low total cholesterol levels are reportedly associated with an increased risk of intracerebral hemorrhage.[38–53] I aimed to further elucidate this association and more specifically to determine which lipid fractions explain the inverse relationship. Likewise, I also determined the associations between lipid fractions and cerebral microbleeds. Cerebral microbleeds are presumed to be the remnants of small amounts of blood that have leaked into the brain.[54] Deep and infratentorial located microbleeds are considered markers of arteriolosclerosis, whereas lobar microbleeds may mark amyloid angiopathy.[54–56] Moreover, cerebral microbleeds have been associated with an increased risk of intracerebral hemorrhage.[57] Therefore, cerebral microbleeds and intracerebral hemorrhage might be manifestations of similar microvascular pathology. I found that low triglyceride levels, but not LDL-cholesterol or

HDL-cholesterol levels, were associated with an increased risk of intracerebral hemorrhage. In addition, I found that low triglyceride levels were associated with the presence of microbleeds in the deep or infratentorial brain regions, but not with presence of microbleeds in the lobar brain regions. This suggests that triglyceride levels may play a role in the development of arteriolosclerotic microangiopathy.

Liver dysfunction is another potential risk factor for intracerebral hemorrhage because of the important role of the liver in maintaining normal hemostasis and coagulation.[58] I studied whether variations in liver enzyme levels are associated with risk of intracerebral hemorrhage (chapter 5.2). Because alcohol is a risk factor for liver dysfunction and for intracerebral hemorrhage, I also investigated the role of alcohol intake in this association.[38] Alkaline phosphatase and lactate dehydrogenase were associated with an increased risk of intracerebral hemorrhage, with somewhat stronger associations of particularly lactate dehydrogenase in excessive alcohol drinkers. Moreover, higher levels of aspartate transaminase, alanine transaminase and gamma-glutamyltranspeptidase were associated with intracerebral hemorrhage only in people who drink excessive amounts of alcohol. This finding implies that higher liver enzyme levels are particularly indicative of an increased risk of intracerebral hemorrhage in people who drink excessive amounts of alcohol.

Lastly, I studied the association between precursor stadia of diabetes mellitus and risk of any stroke, cerebral infarction and intracerebral hemorrhage (chapter 5.3). 'Prediabetes', which is a modifiable condition, is characterized by insulin resistance and impaired fasting glucose.[59, 60] Furthermore, prediabetes is associated with an increased risk of developing diabetes mellitus and with risk of vascular disease.[59, 60] Fasting glucose levels within the nondiabetic range were previously shown to associate with risk of coronary heart disease, but not with risk of stroke.[61] In addition to these findings, I found that fasting insulin levels and the homeostasis model assessment for insulin resistance index, which are both considered markers for insulin resistance, were not associated with risk of stroke, cerebral infarction and intracerebral hemorrhage in elderly people without diabetes mellitus. Whereas diabetes mellitus is a strong risk factor for stroke and its subtypes,[61] these findings suggest that below the threshold for diabetes mellitus, neither fasting glucose levels nor insulin resistance markers are associated with risk of stroke, cerebral infarction or intracerebral hemorrhage.

METHODOLOGICAL CONSIDERATIONS

The studies described in this thesis were conducted within the framework of the Rotterdam Study, an ongoing population-based cohort study. Major advantages of the Rotterdam Study are the prospective and longitudinal design, the long duration of the follow-up period, the large number of participants, the large number of stroke events and the meticulous case ascertainment methods. However, the stringent case finding procedures also lead to an ever

returning point of concern, which I will discuss in the following paragraphs.

Stroke diagnosis and classification

The Rotterdam Study uses rigorous monitoring procedures for incident stroke. From the day of inclusion up to the present day, all participants are continuously monitored for the occurrence of stroke, through linkage of our study database with files from general practitioners, who are the gatekeepers of the Dutch healthcare system. Furthermore, nursing home physician's files and files from general practitioners of participants who moved out of the district are regularly checked. If a stroke is reported, additional information is obtained from hospital records. Due to these stringent procedures, we are able to identify all stroke events that occur in the study population, even in participants who are not referred to a hospital; for example in participants who live in a nursing home or in participants who suffered a fatal stroke. All information is reviewed by research physicians and verified by an experienced vascular neurologist. A stroke is recorded if it fulfills WHO criteria, that is, 'rapidly developing clinical signs of focal disturbance of cerebral function, with symptoms lasting at least 24 hours or leading to death, which are most likely of a vascular origin'.[62] Strokes are then graded as possible, probable or definite.[1] If the diagnosis is based on typical clinical symptoms and on neuroimaging results, the stroke is considered 'definite'. If clinical symptoms are typical for stroke, but neuroimaging has not been performed, the stroke is considered 'probable'. If the treating physician considers a possible stroke, or if clinical symptoms are less typical, the stroke is qualified 'possible'. We include only definite and probable strokes in our analyses.

Definite and probable strokes are further classified as intracerebral hemorrhage, cerebral infarction, or unspecified. Originally, criteria were as follows: intracerebral hemorrhage was diagnosed if a relevant hemorrhage was shown on CT or MRI scan, or if the person lost consciousness permanently or died within hours after the onset of focal signs; cerebral infarction was diagnosed if a CT or MRI scan carried out within four weeks after the event ruled out other diagnoses, or if indirect evidence (neurological deficit limited to one limb or completely recovered within 72 hours, or atrial fibrillation in the absence of anticoagulant therapy) indicated that the stroke was of an ischemic nature.[1] Strokes that did not match any of these criteria were classified as unspecified. Currently, however, classification of stroke subtypes without imaging data is increasingly considered unacceptable. Although certain clinical symptoms increase the likelihood of a stroke being either of ischemic or hemorrhagic origin, reliable distinction between the subtypes requires neuroimaging.[27] Furthermore, since the start of the Rotterdam Study, CT and MRI have become widely available. Therefore, we have decided to update our classification methods and, except for the study described in chapter 3.1, we currently classify strokes as cerebral infarction or intracerebral hemorrhage based on neuroimaging data only. If neuroimaging is lacking the stroke is called unspecified.

Of the 1,029 first-ever stroke events that occurred in the Rotterdam Study I subcohort between 1990 and 2009, 40% could not be classified as cerebral infarction or intracerebral hemorrhage, mainly because of missing imaging data. Although this percentage may seem large in comparison to other studies, some population-based studies also reported similar percentages.[63]

There are several reasons for the large number of unspecified strokes in our study population. First of all, the main reason for missing neuroimaging data is that the general practitioner or nursing home physician who first diagnosed the stroke did not refer the patient to a hospital for further neurological evaluation and treatment. In the Rotterdam Study I subcohort, 65% of stroke patients were referred to a neurologist, 61% underwent neuroimaging and 53% were hospitalized. Because several advances in acute stroke care were achieved since the 1990s, such as increased availability of CT scans, implementation of thrombolytic therapy for cerebral infarction and the introduction of well-equipped stroke units, we had expected that the percentage of stroke patients that underwent neuroimaging would have increased substantially during the past twenty years, resulting in a decrease in the percentage of unspecified strokes. **Figure 1**, however, shows only a modest decline in unspecified strokes over time, from 50% in 1993 to 32% in 2008.

Secondly, the proportion of stroke patients that undergoes neuroimaging is highly dependent on age. **Figure 2** shows that the proportion of unspecified strokes increases steadily from 13% in the 65–69 year age category to 74% in the 90–plus age category. Furthermore, **Figure 3** confirms that age is a major risk factor for unspecified stroke: the association between age and the unspecified stroke subtype increases exponentially with age, whereas the association of age with cerebral infarction and intracerebral increases up to a certain age, but decreases in the older age groups, probably because the likelihood of being referred to a hospital decreases with age.

Thirdly, a major reason for general practitioners and nursing home physicians not to refer the patient to a hospital is dementia prior to stroke. In the Rotterdam Study, dementia prior to stroke is strongly associated with risk of unspecified stroke (HR, 2.61; 95% CI, 2.06 to 3.31), whereas dementia prior to stroke is associated with a lower risk of being diagnosed with cerebral infarction (HR, 0.69; 95% CI, 0.47 to 1.00) and an also somewhat lower risk of being diagnosed with intracerebral hemorrhage (HR, 0.88; 95% CI, 0.37 to 2.09).

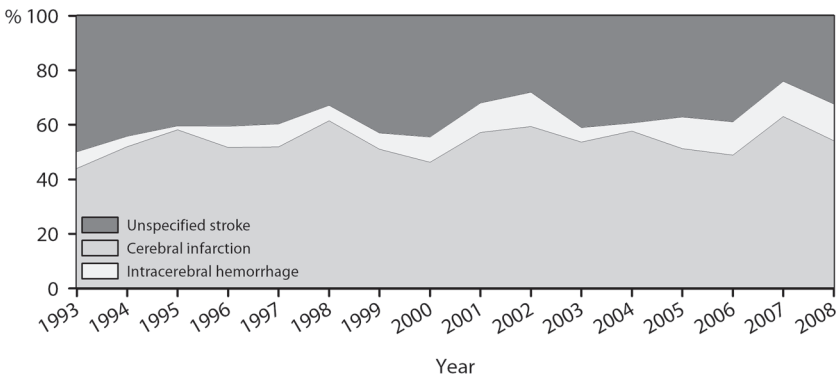
Altogether, these observations suggest that the older the patient, the greater the likelihood that the general practitioner or nursing home physician decides not to refer him or her to a hospital. Similarly, dementia increases the likelihood of not being referred to a hospital in the event of a stroke.

Consequences of misclassification

Non-differential misclassification of outcome events results in a bias towards the null,

whereas differential misclassification results either in bias towards the null, or in bias away from null.[64] In the following two examples, I will demonstrate what bias may have occurred due to misclassification of stroke subtypes, assuming (1) that no false positive diagnoses of stroke occurred and (2) that no false positive diagnoses of cerebral hemorrhage or intracerebral hemorrhage occurred. **Table 1** shows the associations between hypertension and stroke subtypes in the Rotterdam Study I subcohort. The median follow-up time is 15 years. The observed associations of hypertension with cerebral infarction, intracerebral hemorrhage

Figure 1. Stroke subtypes per calendar year



and unspecified stroke are shown in **Table 1.1**. The association between hypertension and unspecified stroke was weaker than the association between hypertension and cerebral infarction or intracerebral hemorrhage, suggesting that misclassification of the outcome event was not independent from exposure and thus differential. Therefore, underascertainment of cere-

Figure 2. Stroke subtypes per age category

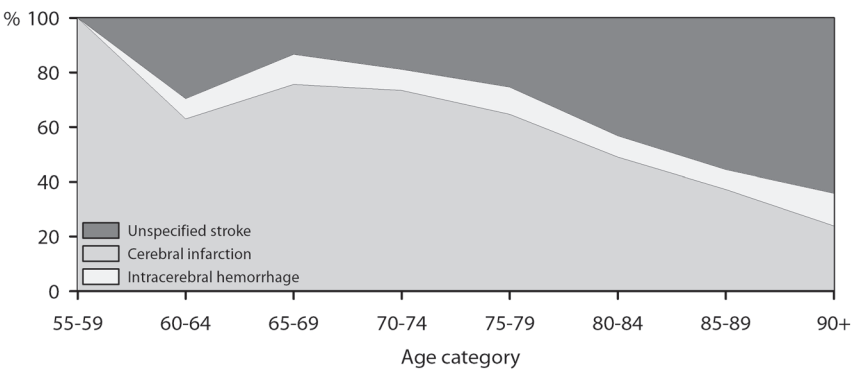
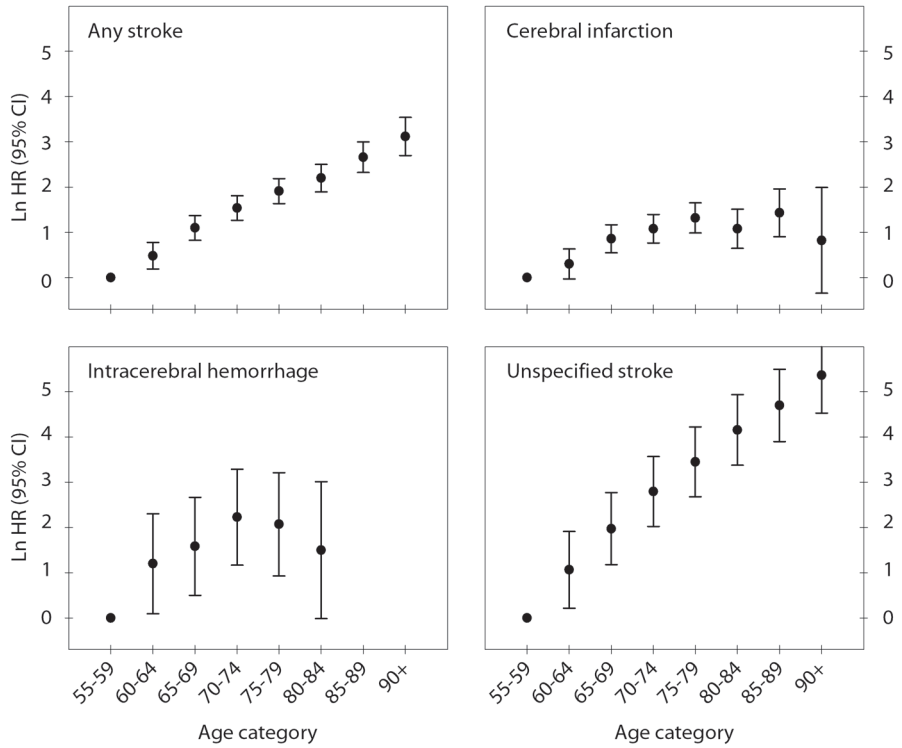


Figure 3. Associations between age and risk of stroke subtypes



bral infarction and intracerebral hemorrhage may have resulted in either underestimation or overestimation of the effect estimates. We simulated what the effect estimates would be if all unspecified strokes had been classified as cerebral infarction or intracerebral hemorrhage by randomly reclassifying unspecified strokes as cerebral infarction or intracerebral hemorrhage according to the expected 85:15 ratio,[65] using a thousand permutations. The resulting mean hazard ratios and empirical 95% confidence intervals are presented in **Table 1.2**. This example shows that, because the association between hypertension and unspecified stroke was weaker than the association between hypertension and cerebral infarction or intracerebral hemorrhage, the associations with cerebral infarction and intracerebral hemorrhage were overestimated.

Another example is shown in **Table 2**. The association between diabetes mellitus and unspecified stroke is somewhat stronger than the association between diabetes mellitus and cerebral infarction or intracerebral hemorrhage (**Table 2.1**). **Table 2.2** presents the hazard ratios after random reclassification of unspecified strokes as cerebral infarctions or intracere-

bral hemorrhages and shows that the originally obtained effect estimates were slightly underestimated.

In these examples, we assumed that 85% of unspecified strokes were cerebral infarctions and 15% were intracerebral hemorrhages. However, we do not know the true distribution of cerebral infarctions and intracerebral hemorrhage among unspecified strokes. **Figure 4** shows how the effect estimates change depending on the ratio between cerebral infarctions and intracerebral hemorrhages, ranging from 100:0 to 0:100. Effect estimates were determined after a thousand permutations.

I had expected that the effect estimate for the association between a certain exposure variable and the outcome unspecified stroke would be somewhere in between the association for cerebral infarction or intracerebral hemorrhage. However, these findings show that this is not necessarily true, probably because unspecified strokes do not occur randomly, but as the result of specific characteristics such as higher age or dementia. I further showed that

Table 1. Associations between hypertension and stroke subtypes

1. Risk estimates based on the actual subtype classification

	Hypertension	At risk, N	Events, N	HR (95% CI)*
Cerebral infarction	Exposed	3,493	312	1.98 (1.58-2.47)
	Non-exposed	2,213	109	
Intracerebral hemorrhage	Exposed	3,493	47	1.67 (0.97-2.85)
	Non-exposed	2,213	20	
Unspecified stroke	Exposed	3,493	215	1.38 (1.05-1.82)
	Non-exposed	2,213	72	

2. Risk estimates based on random reclassification of unspecified into cerebral infarction and intracerebral hemorrhage, ratio 85:15

	Hypertension	At risk, N	Events, N	HR (95% CI)*
Cerebral infarction	Exposed	3,493	495	1.72 (1.65-1.80)
	Non-exposed	2,213	170	
Intracerebral hemorrhage	Exposed	3,493	79	1.55 (1.20-1.95)
	Non-exposed	2,213	31	

*Hazard ratios are adjusted for age and sex.

it is possible to estimate the bias that occurs due to misclassification of stroke subtypes and to estimate hypothetical unbiased effect estimates if no misclassification of subtypes would have occurred, assuming a certain underlying distribution of cerebral infarctions and intracerebral hemorrhages.

In the preceding paragraphs, I assumed that no misdiagnosis of stroke events had occurred. However, the accuracy of the clinical diagnosis of stroke largely depends on the skills of the examiner, neurologists being better trained for diagnosing stroke than general practitioners or nursing home physicians.[66–69] In the Rotterdam Study, medical records of all events suspicious for stroke were meticulously reviewed by trained physicians and an experienced stroke neurologist. However, given that unspecified strokes were mainly reported by general practitioners and nursing home physicians, whereas nearly all cerebral infarctions and intracerebral hemorrhage were reported by a neurologist, misdiagnosis of non-strokes (so called stroke mimics) as stroke events will probably have occurred more often in the unspecified stroke subgroup. Furthermore, apart from distinguishing between cerebral infarction and intracerebral hemorrhage, neuroimaging aids in the identification of stroke mimics, such as tumors

Table 2. Associations between diabetes mellitus and stroke subtypes

1. Risk estimates based on the actual subtype classification

	Diabetes mellitus	At risk, N	Events, N	HR (95% CI)*
Cerebral infarction	Exposed	708	67	1.68 (1.29-2.19)
	Non-exposed	4,998	354	
Intracerebral hemorrhage	Exposed	708	11	1.81 (0.94-3.49)
	Non-exposed	4,998	56	
Unspecified stroke	Exposed	708	62	1.85 (1.39-2.46)
	Non-exposed	4,998	225	

2. Risk estimates based on random reclassification of unspecified into cerebral infarction and intracerebral hemorrhage, ratio 85:15

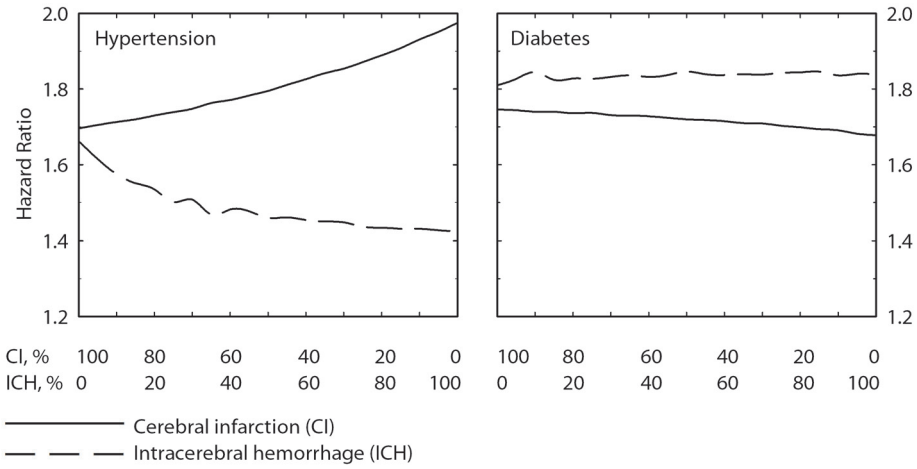
	Diabetes mellitus	At risk, N	Events, N	HR (95% CI)*
Cerebral infarction	Exposed	708	120	1.74 (1.67-1.82)
	Non-exposed	4,998	545	
Intracerebral hemorrhage	Exposed	708	20	1.82 (1.34-2.41)
	Non-exposed	4,998	90	

*Hazard ratios are adjusted for age and sex.

or subdural hematoma.[70] Because unspecified strokes did not undergo neuroimaging, this subgroup will probably include more stroke mimics than the subgroups cerebral infarction and intracerebral hemorrhage. By qualifying strokes as definite, probable or possible, and not including possible strokes in the analyses, we aimed to minimize the number of false positive stroke events in the analyses. Nevertheless, it is likely that misclassification of non-strokes as stroke events preferably involved unspecified strokes.

The examples shown in **Table 1** and **2** show that hypertension and diabetes mellitus associate differently with unspecified stroke than with cerebral infarction or intracerebral hemorrhage and that the effect estimates for unspecified stroke are not, as would be expected, some-

Figure 4. Associations after reclassification of unspecified stroke events



where in between the effect estimates for cerebral infarction and intracerebral hemorrhage. Although this may indicate differential misclassification of outcome events, it may also indicate that a certain proportion of unspecified strokes were in fact misdiagnosed as stroke events.

CLINICAL IMPLICATIONS AND FUTURE PERSPECTIVES

The observation that stroke incidence rates decreased in men but not in women has several important implications. First of all, it suggests that primary prevention strategies have been implemented quite successfully in men. On the other hand, the stable results in women are disappointing and alarming in light of predicted demographic changes. Several drugs for the treatment of modifiable risk factors, such as blood-pressure lowering medication and HMG-CoA reductase inhibitor mediation, as well as lifestyle interventions such smoking cessation, proper diet, increased physical activity, and appropriate weight loss, have shown effective in the primary prevention of stroke.[71–75] Furthermore, platelet aggregation inhibitors are recommended in high risk patients, that is, if the benefits of treatment outweigh the risks.[71] However, although aspirin was shown particularly effective for the primary prevention of stroke in women, women are less likely to receive these drugs than men.[76] Therefore, increasing awareness and treatment of vascular risk factors in women is mandatory to compete with the upcoming stroke epidemic.

The finding that von Willebrand factor is associated with risk of stroke and cerebral infarction has potential implications for clinical practice, although I would not recommend

von Willebrand factor itself as a target for intervention. Von Willebrand factor is a marker for endothelial dysfunction, a condition that predisposes for atherosclerosis and cerebral infarction.[77] Because treatment of vascular risk factors such as hypertension, diabetes mellitus, smoking, dyslipidemia and obesity results in a decrease of plasma von Willebrand factor level,[77] von Willebrand factor might be used as a marker for monitoring the degree of endothelial dysfunction and for monitoring the combined effects of preventive interventions on endothelial dysfunction and vascular disease risk. Future studies are needed to investigate whether a reduction in von Willebrand factor levels, achieved by treatment of stroke risk factors, results in a reduction of stroke events.

Recently, there is increasing interest for prediabetes as a potential target for the prevention of diabetes mellitus and cardiovascular disease. However, whereas previous studies have shown that prediabetes, characterized by impaired fasting glucose levels or insulin resistance, increases the risk of incident diabetes mellitus, coronary heart disease and recurrent stroke, prediabetes seems not to be associated with an increased risk of first-ever stroke, cerebral infarction or intracerebral hemorrhage.[61] Nevertheless, although treatment of prediabetes will not directly result in a reduction of stroke risk, treatment of prediabetes in order to prevent diabetes mellitus and coronary heart disease is worth consideration.

In this thesis, I also described several determinants of intracerebral hemorrhage. Intracerebral hemorrhage accounts for only 10% to 15% of strokes, but is responsible for more than 30% of stroke-related mortality.[78] Currently, there are no effective treatment options for intracerebral hemorrhage.[79, 80] Furthermore, preventive strategies have lumped all strokes together.[71] Although there is evidence for overlap in risk factors, there are also important pathogenetic differences between cerebral infarction and intracerebral hemorrhage, indicating that primary prevention may benefit from subtype-specific targets. We identified new determinants of intracerebral hemorrhage, namely low triglyceride levels, high liver enzyme levels, wider retinal venular calibers and late stage age-related macular degeneration. Unfortunately, there are several reasons why our findings cannot be directly translated to clinical practice. First of all, our results were based on quite a small number of intracerebral hemorrhages. Therefore, our findings need to be confirmed in other population-based cohorts or consortia. Secondly, possible implications depend on whether a determinant is causally or non-causally related to the disease, and, if causal, whether the determinant represents a treatable or untreatable condition. Mild liver dysfunction and low triglyceride levels may well contribute to causal pathways leading to intracerebral hemorrhage, though further research is needed to unravel the possible mechanisms and to investigate whether treatment of these conditions results in a reduction of intracerebral hemorrhage risk. In contrast, a causal role for retinal vascular calibers and age-related macular degeneration in intracerebral hemorrhage seems unlikely. Instead, we consider it more likely that retinal vascular changes mark the simultaneous presence of cerebral small vessel disease predisposing for intracerebral hemorrhage.

Therefore these retinal characteristics may improve our understanding of the pathophysiologic concept of intracerebral hemorrhage. Moreover, retinal characteristics may be useful for the stratification of people at risk for intracerebral hemorrhage. However, whether retinal vascular caliber measurements and assessment of age-related macular degeneration are useful determinants for the prediction of intracerebral hemorrhage requires investigation. Besides, I am not aware of any prediction rules specifically targeting intracerebral hemorrhage. This is particularly important because some drugs have opposite effects on the risk of cerebral infarction and intracerebral hemorrhage. Well-known examples are oral anticoagulation therapy, aspirin, and combinations of antiplatelet therapies, but there are also some controversies regarding lipid-lowering drugs.[81–85] Therefore, subtype-specific risk stratification may help in the identification of people who should or who should not receive certain therapies, thus increasing the likelihood of successful prevention.

To conclude, this thesis contributes new insights into the overlap and differences in etiology between cerebral infarction and intracerebral hemorrhage, and provides new potential targets for the primary prevention of these diseases. Though stroke is considered a disease that can be prevented, a tremendous amount of work is yet to be done to counterbalance the upcoming stroke epidemic.

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CHAPTER 7

SUMMARY / SAMENVATTING

Summary

Stroke is a frequent disorder in elderly people. Despite improvements in primary prevention, the burden of stroke remains high and is predicted to even increase in the near future due to aging of the population. Therefore, additional targets for primary prevention are urgently needed. Most previous epidemiological studies have not specifically studied the two main subtypes of stroke, i.e., cerebral infarction and intracerebral hemorrhage, whereas differences in etiology between these subtypes suggest that they may have different risk profiles. Therefore, primary prevention may benefit from the identification of subtype-specific determinants. The aims of this thesis were to study trends in stroke incidence rates and stroke risk factors in the past twenty years, and to identify new determinants of any stroke, cerebral infarction, and intracerebral hemorrhage. The studies described in this thesis were part of the Rotterdam Study, a large population-based cohort study among elderly people who were followed from 1990 up to the present day for the occurrence of stroke and other diseases that are frequent in the elderly.

Changes in stroke incidence rates and stroke risk factors in Rotterdam in the Netherlands between 1990 and 2008 were described in chapter 2. The major finding was a 34% decrease in stroke incidence rates in men, but no change in women. The prevalence of some risk factors, particularly hypertension and obesity, had increased, whereas cigarette smoking in men had decreased. Other risk factors, such as atrial fibrillation, low high-density lipoprotein cholesterol and diabetes mellitus were unchanged. The proportion of men and women taking preventive medication had increased enormously. However, the divergent trends in stroke incidence rates in men and women stress the need for adequate recognition and treatment of stroke risk factors in women.

In chapter 3, I reported my findings regarding the association of the plasma protein von Willebrand factor, which plays an important role in hemostasis but also marks the presence of endothelial dysfunction, with stroke and its subtype cerebral infarction. Higher von Willebrand factor levels were associated with an increased risk of stroke and cerebral infarction (chapter 3.1). However, genetic determinants of von Willebrand factor levels were not associated with stroke risk, either individually or joint in a weighted sum score (chapter 3.2). These findings suggest that von Willebrand factor is a marker for stroke risk, but probably not an important causal factor.

Chapter 4 includes three studies concerning the association between retinal characteristics and risk of any stroke, cerebral infarction and intracerebral hemorrhage. In the first study I showed that wider retinal venular calibers were not only associated with an increased risk of cerebral infarction, but also with an increased risk of intracerebral hemorrhage (chapter 4.1). Furthermore, these associations were stronger for lobar than deep intracerebral hemorrhages. Both narrower arteriolar calibers and wider venular calibers were strongly associated with risk

of anticoagulation-related intracerebral hemorrhage. In the second study I showed that the presence of retinopathy signs was associated with an increased risk of any stroke, but that this association was due to an increased risk of unspecified stroke and not due to an increased risk of either cerebral infarction or intracerebral hemorrhage (chapter 4.2). In the third study I addressed the association between age-related macular degeneration and risk of stroke and I showed that late stage age-related macular degeneration was associated with an increased risk of intracerebral hemorrhage, but not with risk of cerebral infarction (chapter 4.3). Altogether, the results of these three studies suggest that several retinal characteristics may be used as markers for future or present cerebrovascular pathology.

Chapter 5 contains three studies on metabolic determinants of stroke. In chapter 5.1, I described the association between serum lipid fractions and risk of intracerebral hemorrhage and presence of cerebral microbleeds, and showed that low triglyceride levels are associated with an increased risk of intracerebral hemorrhage, and with the presence of deep or infratentorial microbleeds, but not with lobar microbleeds. Because deep and infratentorial microbleeds are considered markers of arteriolosclerosis, whereas lobar microbleeds more likely reflect amyloid angiopathy, these findings suggest that low triglyceride levels may play a role in the development of arteriolosclerotic microangiopathy. In chapter 5.2, I addressed the association between liver enzyme levels and risk of intracerebral hemorrhage, and the role of alcohol in this association. Alkaline phosphatase and lactate dehydrogenase were associated with an increased risk of intracerebral hemorrhage independently of alcohol intake. However, aspartate transaminase, alanine transaminase and gamma-glutamyltranspeptidase were associated with intracerebral hemorrhage only in people who drink excessive amounts of alcohol. In chapter 5.3, I described the associations between insulin resistance markers and risk of any stroke, cerebral infarction and intracerebral hemorrhage in nondiabetic elderly. Insulin resistance markers were not associated with risk of any stroke or its subtypes. Although diabetes mellitus is a strong risk factor for stroke, these findings suggest that below the threshold for diabetes mellitus, the degree of insulin resistance is not associated with risk of any stroke, cerebral infarction or intracerebral hemorrhage.

Chapter 6 includes a general discussion of the main findings, methodological issues concerning the classification of stroke subtypes, potential clinical implications of the main findings and future perspectives.

To conclude, in this thesis I reported on several new determinants of cerebral infarction and intracerebral hemorrhage. Future research should further unravel the pathophysiologic mechanisms and investigate the potential of these determinants as risk predictors or as targets for preventive intervention.

Samenvatting

Een beroerte is een veelvoorkomende aandoening bij ouderen. Ondanks toenemende en succesvolle primaire preventie, zoals behandeling van hoge bloeddruk, blijft de prevalentie van beroerte hoog. Vanwege de vergrijzing verwacht men dat de totale incidentie en prevalentie in the nabije toekomst verder zullen stijgen. Daarom is er dringend behoefte aan nieuwe aangrijpingspunten voor primaire preventie. Eerdere epidemiologische studies naar determinanten van beroerte hebben vaak geen onderscheid gemaakt tussen herseninfarcten en hersenbloedingen, terwijl verschillen in etiologie suggereren dat deze subtypes een ander risicoprofiel zouden kunnen hebben. Primaire preventie kan daarom baat hebben bij de ontdekking van subtype-specifieke determinanten. Het eerste doel van dit proefschrift was om in kaart te brengen of de incidentie van beroerte en de prevalentie van conventionele risicofactoren in de afgelopen twintig jaar in Nederland zijn veranderd, en of het gebruik van medicatie voor de behandeling van die risicofactoren in dezelfde periode is veranderd. Het tweede doel van dit proefschrift was om nieuwe determinanten te ontdekken voor beroerte in brede zin, maar ook specifiek voor de subtypes herseninfarct en hersenbloeding. De studies die ik beschrijf in dit proefschrift zijn onderdeel van de Rotterdam Studie, een grootschalig bevolkingsonderzoek onder ouderen in de wijk Ommoord in Rotterdam. De deelnemers aan het onderzoek worden sinds 1990 gevolgd voor het optreden van beroerte en andere aandoeningen die vaak voorkomen bij ouderen.

In hoofdstuk 2 beschrijf ik de veranderingen in de incidentie van beroerte en in de prevalentie van risicofactoren die plaatsvonden tussen 1990 en 2008. De belangrijkste bevinding was een 34% afname van de incidentie van beroerte bij mannen, terwijl de incidentie bij vrouwen constant was gebleven. De prevalentie van sommige risicofactoren, waaronder hypertensie en overgewicht, was overwegend toegenomen, terwijl de prevalentie van atriumfibrilleren, laag HDL-cholesterol en diabetes mellitus niet duidelijk was veranderd. Opvallend was dat het percentage mannelijke rokers was aanzienlijk gedaald, terwijl het percentage vrouwelijke rokers hetzelfde was gebleven. Het gebruik van preventieve medicatie was zowel bij mannen als bij vrouwen enorm gestegen. De bevinding dat incidentiecijfers wel zijn afgenomen bij mannen maar niet bij vrouwen onderstreept het belang van adequate herkenning en behandeling van risicofactoren bij vrouwen.

In hoofdstuk 3 beschrijf ik de associatie tussen het plasma eiwit von Willebrand factor, een belangrijk stollingseiwit maar ook een marker voor endotheelschade, en het risico op beroerte. Hogere von Willebrand factor concentraties in het plasma waren geassocieerd met een hoger risico op een beroerte en in het bijzonder met het risico op een herseninfarct (hoofdstuk 3.1). Echter, genen die in belangrijke mate de hoogte van de von Willebrand factor concentratie in het plasma bepalen waren niet geassocieerd met het risico op beroerte, noch individueel, noch samengevoegd in een gewogen somscore (hoofdstuk 3.2). Dit sugge-

reert dat von Willebrand factor wel een marker is van het risico op beroerte, maar dat het waarschijnlijk geen belangrijke causale rol speelt.

Hoofdstuk 4 bevat drie studies over het verband tussen (vaat)afwijkingen van de retina, het netvlies van het oog, en het risico op beroerte. In de eerste studie toon ik aan dat wijdere retinavenen geassocieerd zijn met een grotere kans op een herseninfarct, maar ook met een grotere kans op een hersenbloeding (hoofdstuk 4.1). Dit verband was sterker voor bloedingen in de hersenkwabben dan voor bloedingen in de diepe hersengebieden of in de kleine hersenen. Verder waren zowel nauwere arteriolen als wijdere venulen sterk geassocieerd met antistolling-gerelateerde bloedingen. In de tweede studie beschrijf ik dat ook tekenen van retinopathie geassocieerd zijn met een hoger risico op het krijgen van een beroerte (hoofdstuk 4.2). Deze associatie lijkt echter niet te verklaren door een hoger risico op een herseninfarct of hersenbloeding, maar door een hogere kans op een 'niet nader gespecificeerde' beroerte. In de derde studie toon ik aan dat er een verband bestaat tussen leeftijdgebonden maculadegeneratie en het risico op beroerte (hoofdstuk 4.3). Eindstadia van leeftijdgebonden maculadegeneratie waren geassocieerd met een hoger risico op het krijgen van een hersenbloeding, maar niet met het risico op een herseninfarct. Alles bij elkaar suggereren deze drie studies dat verschillende (vaat)aandoeningen van de retina gebruikt zouden kunnen worden als markers voor bestaande of toekomstige vaatschade in de hersenen.

Hoofdstuk 5 bestaat uit drie studies over metabole verstoringen en het risico op beroerte. In hoofdstuk 5.1 beschrijf ik de associatie tussen lipidenconcentraties en symptomatische hersenbloedingen en de associatie tussen lipidenconcentraties en asymptomatische microbloedingen in de hersenen. Lagere triglyceridenconcentraties waren geassocieerd met een hoger risico op een hersenbloeding. Lagere triglyceridenconcentraties waren ook geassocieerd met het vaker voorkomen van microbloedingen in de diepe hersengebieden en in de kleine hersenen, maar niet met microbloedingen in de hersenkwabben. Omdat microbloedingen in de diepe breingebieden geassocieerd zijn met arteriolosclerose, terwijl microbloedingen in de hersenkwabben geassocieerd zijn met amyloid angiopathie, suggereren deze bevindingen dat lage triglyceridenconcerentraties een rol zouden kunnen spelen in het ontstaan van arteriolosclerose. In hoofdstuk 5.2 bespreek ik de associatie tussen leverenzymconcentraties en het risico op een hersenbloeding, en ook de rol van alcohol in dit verband. Alkalische fosfatase en lactaat dehydrogenase waren geassocieerd met een hoger risico op een hersenbloeding ongeacht de hoeveelheid alcoholconsumptie. Aspartaat transaminase, alkaline transaminase en gamma-glutamyltranspeptidase waren echter alleen geassocieerd met hersenbloedingen bij mensen die teveel alcohol drinken (meer dan 2 glazen per dag). In hoofdstuk 5.3 beschrijf ik de associatie tussen de mate van insulineresistentie en het risico op een beroerte, herseninfarct of hersenbloeding, bij ouderen zonder diabetes. Insulineresistentie was niet geassocieerd met het risico op beroerte of met een van beide subtypes, wat impliceert dat onder de drempelwaarde voor diabetes, de mate van insulineresistentie geen bijdrage levert aan het risico op

een herseninfarct of hersenbloeding.

Hoofdstuk 6 bestaat uit een algemene discussie van de belangrijkste bevindingen beschreven in dit proefschrift, de methodologische beperkingen van het onderzoek, de mogelijke implicaties voor de praktijk, en suggesties voor toekomstig onderzoek.

Tot slot, in dit proefschrift beschrijf ik welke nieuwe determinanten van herseninfarcten en hersenbloedingen ik heb ontdekt. Verder onderzoek moet erop gericht zijn de onderliggende mechanismen te ontrafelen, uit te zoeken of deze determinanten bruikbaar zijn als voorspellers voor wie de aandoening gaat krijgen en wie niet, en uit te zoeken of deze determinanten bruikbaar zijn als aangrijpingspunt voor preventieve maatregelen.

CHAPTER 8

DANKWOORD
PHD PORTFOLIO
LIST OF PUBLICATIONS
ABOUT THE AUTHOR

Dankwoord

Op het moment dat ik dit schrijf is het precies drieënhalf jaar geleden dat ik begon aan het onderzoek dat de basis vormde voor dit proefschrift. Hoewel de jaren voorbij zijn gevlogen is het een enerverende tijd geweest waarin ik veel nieuwe ervaringen heb opgedaan en waaraan ik waardevolle herinneringen bewaar. Dit proefschrift zou er niet zijn geweest zonder de bijdragen van anderen. Op deze plaats wil ik graag iedereen bedanken met wie ik de afgelopen jaren heb samengewerkt. Een aantal van hen wil ik graag in het bijzonder bedanken.

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PhD Portfolio – Summary of PhD training and teaching activities

Name	R.G. Wieberdink
Erasmus MC Departments	Epidemiology and Neurology
Research Schools	NIHES, COEUR
Supervisors	Prof.dr. M.M.B. Breteler, Prof.dr. P.J. Koudstaal, Dr. M.A. Ikram

Research skills

2008-2010	MSc in Clinical Epidemiology, Netherlands Institute for Health Sciences, Erasmus University Rotterdam, the Netherlands (including courses on methodology, study design, and statistical analysis)

General academic skills

2009	Biomedical English Writing and Communication, Erasmus MC, Rotterdam, the Netherlands

In-depth courses

2009	Thrombosis and Hemostasis, Netherlands Platform for Cardiovascular Research, Netherlands Heart Foundation, Papendal, the Netherlands
2009	Peripheral and Intracranial Obstructive vascular disease, COEUR, Rotterdam, the Netherlands
2008	Course on SNPs and Human Diseases, Molecular Medicine, Erasmus MC, Rotterdam, the Netherlands

National and international conferences

2011	20th European Stroke Conference, Hamburg, Germany Oral presentation 'Serum liver enzyme levels and the risk of stroke: the Rotterdam Study'
2011	4th Symposium on Heart failure, Rotterdam, the Netherlands Oral presentation 'Neurological complications in heart failure patients'
2011	1st Erasmus MC meeting of the interdisciplinary working group on healthy aging, Rotterdam, the Netherlands
2011	International Stroke Conference, Los Angeles, USA Oral presentation 'Age-related macular degeneration and the risk of stroke: the Rotterdam Study'

2010	7th Forum of European Neuroscience, Amsterdam, the Netherlands
2010	19th European Stroke Conference, Barcelona, Spain Oral presentation 'Retinal vascular calibers and the risk of intracerebral hemorrhage: the Rotterdam Study' Poster presentation 'High von Willebrand factor levels increase the risk of stroke: the Rotterdam Study'
2009	Thrombosis and Hemostasis, Netherlands Platform for Cardiovascular Research, Netherlands Heart Foundation, Papendal, the Netherlands Poster presentation 'High von Willebrand factor levels increase the risk of stroke: the Rotterdam Study'
2009	18th European Stroke Conference, Stockholm, Sweden Oral presentation 'Trends in stroke incidence and stroke risk factors in the Netherlands from 1990-2006'
2008	Research Institute for Diseases in the Elderly Symposium, Amsterdam, the Netherlands

Teaching activities

2011	Supervising practicals at NIHES, Erasmus MC, Rotterdam, the Netherlands
2008-2010	Teaching practicals in epidemiology to 4th year medical students, Erasmus MC, Rotterdam, the Netherlands

List of publications and manuscripts

Wieberdink RG*, van Schie MC*, Koudstaal PJ, Witteman JCM, Hofman A, de Maat MPM, Leebeek FWG, Breteler MMB. *Shared first authorship. High plasma von Willebrand factor levels increase the risk of stroke: the Rotterdam Study. *Stroke*. 2010;41:2151-2156.

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Wieberdink RG, Koudstaal PJ, Hofman A, Breteler MMB, Ikram MA. Insulin resistance and the risk of stroke and stroke subtypes in nondiabetic elderly: the Rotterdam Study. *Submitted*.

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About the author

Renske Wieberdink was born on April 3, 1980 in The Hague, the Netherlands. She attended Gymnasium Sorghvliet in The Hague and graduated in 1998. Subsequently, she started her medical education at the Catholic University of Louvain in Belgium. In 2004, after she had finished the theoretical part of the study, she decided to move back to the Netherlands and continued her medical training with two years of internships in various hospitals aligned to the Erasmus University in Rotterdam. Shortly after graduation in December 2006, she started working as a resident at the department of Neurology of the St Franciscus General Hospital in Rotterdam (dr. S.L.M. Bakker). In June 2008, she started working on the project described in this thesis under the supervision of prof.dr. M.M.B. Breteler at the department of Epidemiology, in close collaboration with the department of Neurology (prof.dr. P.J. Koudstaal). As part of her research training, she obtained a Master of Science degree in Clinical Epidemiology at the Netherlands Institute of Health Sciences (NIHES). Her research primarily focused on the identification of novel determinants for cerebral infarction and intracerebral hemorrhage in the general elderly population. She presented her work at the European Stroke Conferences in Stockholm (2009), Barcelona (2010), and Hamburg (2011), and at the International Stroke Conference in Los Angeles (2011). As of December 2011, she is continuing her training in neurology as a resident at the department of Neurology of the Erasmus University Medical Center Rotterdam (prof.dr. P.A.E. Sillevis Smitt).