Endocrine Factors, Retinal Vessels, and Risk of Dementia

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Endocriene factoren, retinavaten en de kans op dementie

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Voor Lia, Nynke en Thijs

‘Maar de wijsheid – waar wordt zij gevonden,
en waar toch is de verblijfplaats van het inzicht?’
Job 28:12

‘Zie, de vreze des HEREN – dat is wijsheid,
en van het kwade te wijken is inzicht.’
Job 28:28b

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Chapter 1

Introduction

First described 100 years ago by Alois Alzheimer, the clinico-pathological entity that we now call Alzheimer disease was initially presented as a clinically unusual type of dementia.[1] Nowadays, Alzheimer disease is recognized as the main cause of dementia and one of the most disabling and burdensome health conditions worldwide.[2,3] The number of people affected by dementia is estimated to double every 20 years to over 80 million by 2040.[3] This will not only affect patients and their caregivers, but will also put an enormous demand on health care and welfare resources.

Despite numerous studies that have attempted to find risk factors for Alzheimer disease, its etiology remains largely unknown. Different mechanisms have been proposed, including hormonal and vascular factors. As for hormones, they have been suggested to be of importance in Alzheimer pathogenesis, mainly based on the observation that hormonal dysregulation may severely affect cognitive performance and may even lead to dementia, for instance in hypothyroidism and glucocorticoid excess.[4,5] In addition, some experimental studies show thyroid hormones and glucocorticoids, but also adrenal androgens and sex hormones to induce changes in amyloid precursor processing or the deposition of amyloid-β, the major component of amyloid deposits found in the brain of Alzheimer patients.[6-9] Moreover, glucocorticoids have been reported to be neurotoxic especially to neurons in the hippocampus,[10] a structure in the medial temporal lobe of the brain, which is affected long before a clinical diagnosis of Alzheimer disease can be made.[11,12] Conversely, adrenal androgens, and sex hormones protect hippocampal neurons exposed to amyloid-β.[13-15] Epidemiological evidence for the involvement of hormones in dementia mainly comes from cross-sectional studies or studies with a short follow-up.[16,17] However, given that Alzheimer disease has a long preclinical period, it is impossible to discern whether hormonal disturbances measured at time of diagnosis or thereafter are a cause or a consequence of the dementia process. Prospective studies with a long follow-up for dementia are therefore needed to determine whether hormonal disturbances precede the onset of dementia and whether they might be involved in the development of Alzheimer disease. An alternative approach would be to study hormonal disturbances in relation to early preclinical markers of Alzheimer disease, e.g. hippocampal and amygdalar volume on brain imaging in non-demented elderly.[18]

As for the involvement of vascular risk factors in dementia, increasing evidence suggests that vascular pathology plays an important role in the clinical course and progression of Alzheimer disease.[19,20] Whether cerebrovascular changes are involved in the development of Alzheimer disease remains unclear.[21] The cerebral circulation is difficult to assess and most non-invasive indicators of vascular pathology relate to vessel beds outside the brain. Retinal vessels may provide a way to more directly study the relation between vascular pathology and Alzheimer disease, since embryological, anatomical and physiological characteristics are similar to cerebral vessels and the retina is easy to visualize non-invasively.[22,23] During the late 1990s, a semi-automated system became available to reliably

quantify retinal arteriolar and venular diameters.[24] A lower arteriolar-to-venular ratio (AVR) was suggested to reflect generalized arteriolar narrowing due to hypertension[22] and was subsequently associated with an increased risk of cardiovascular and cerebrovascular disorders, including cerebral small vessel disease and stroke.[25-28] However, the vascular mechanisms underlying a lower AVR remain unclear. Mechanisms other than hypertension may be of importance as a lower AVR was also related to atherosclerosis and markers of inflammation.[29] In particular it remains unclear how these vascular mechanisms are related to the separate arteriolar and venular diameters, as both arteriolar narrowing and venular widening may have contributed to a lower AVR.

The objective of this thesis was twofold: to gain more insight in the role of hormones in the etiology of dementia (part I), and to quantify the role of retinal vessel diameters as markers of vascular pathology in cerebrovascular disease and dementia (part II). The research was conducted within the setting of the Rotterdam Study, a large prospective population-based cohort study among 7,983 elderly aged 55 years and older, designed to study determinants of diseases in the elderly.[30] In Part I, I first describe the role of thyroid function in dementia (chapter 2). The focus is on serum thyroid hormone levels in chapter 2.1 and on genetic variations that potentially alter thyroid hormone bioactivity in chapter 2.2. I also examine serum thyroid hormone levels and the risk of dementia within the Honolulu-Asia Aging Study, a prospective population-based cohort study among elderly Japanese American men (chapter 2.3). In chapter 3, the role of steroid hormones in dementia is investigated. Chapter 3.1 concentrates on testosterone and chapter 3.2 on the influence of genetically determined variation in glucocorticoid sensitivity. Part II starts with four studies on determinants of retinal vessel diameters (chapter 4). I studied blood pressure and atherosclerosis in chapter 4.1, inflammation markers in chapter 4.2, genetic variation in complement factor H, an important inhibitor of the complement pathway, in chapter 4.3, and arterial oxygen saturation and total cerebral blood flow in chapter 4.4. In the following chapter, I examine retinal arteriolar and venular diameters in relation to cerebrovascular disease and dementia. The focus is on cerebral small vessel disease in chapter 5.1 and on brain atrophy in chapter 5.2. Retinal vessel diameters are studied in relation to the risk of dementia in chapter 5.3. In chapter 6, I discuss the main findings in the context of current knowledge and give suggestions for future research.

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Part I

Endocrine factors and risk of dementia

Chapter 2

Thyroid hormones and risk of dementia

Chapter 2.1

Thyroid hormones, dementia, and atrophy of the medial temporal lobe

ABSTRACT

Context: *Thyroid function has been related to Alzheimer disease (AD), but it remains unclear whether thyroid dysfunction results from or contributes to developing AD.*

Objective: *The objective of the study was to determine the association between thyroid function and both medial temporal lobe atrophy on brain magnetic resonance imaging (MRI) as putative early sign of AD and risk of dementia.*

Design and participants: *This was a population-based cohort study among 1,077 elderly subjects aged 60-90 years and dementia free at baseline (1995-1996).*

Main outcome measures: *Non-fasting serum levels of TSH, free T_4 (fT_4), T_3 and rT_3 were available in 1,025 subjects followed up for incident dementia until 2005. In a subset of 489 non-demented elderly, we assessed volumes of the hippocampus and amygdala on brain MRI. Subjects using thyroid medication were excluded.*

Results: *During 5,657 person-years of follow-up (mean 5.5 years), 63 subjects were diagnosed with dementia (46 with AD). TSH and thyroid hormones were not associated with risk of dementia or AD. TSH and T_3 were also not related to brain atrophy, whereas non-demented subjects with higher fT_4 levels had more hippocampal and amygdalar atrophy on MRI. Similar associations were found for rT_3. Excluding subjects with thyroid disorders or incipient AD did not change the results.*

Conclusion: *In our study, TSH was related neither to risk of AD nor with early MRI markers thereof, arguing against an important role of thyroid function in the development of AD. Whether the association of higher fT_4 and rT_3 levels with brain atrophy on MRI has functional significance remains to be elucidated.*

INTRODUCTION

Clinical thyroid disorders may negatively influence cognitive performance,[1] which is the reason why thyroid function is evaluated when a diagnosis of dementia is considered.[2] It is, however, uncertain whether thyroid function also influences the risk of developing the most frequent subtype of dementia, Alzheimer disease (AD). Studies showed inconsistent results, reporting either no significant association[3,4] or an association between hypothyroidism and AD.[5,6] Yet, these studies had several methodological shortcomings, including selection bias due to the case-control design, retrospectively obtained information on thyroid function and small numbers of subjects. An earlier study from our group suggested subclinical hyperthyroidism to be associated with a higher risk of AD, especially in the presence of serum antibodies to thyroid peroxidase (TPO-Abs).[7] Conversely, others have shown low T_4 to increase risk of cognitive impairment among physically impaired elderly women.[8]

Although thyroid dysfunction could be related to clinical symptoms of AD, the underlying mechanism is unclear. In particular it is unclear whether thyroid dysfunction results from or contributes to AD pathology. The hippocampus and amygdala are structures in the medial temporal lobe that can be easily visualized on magnetic resonance imaging (MRI) of the brain and are reduced in volume early in the process of AD.[9,10] Both structures also have a high density of thyroid hormone receptors and are an important target of thyroid hormones entering the brain.[11,12] In addition, mild Alzheimer patients have more hippocampal and amygdalar atrophy on MRI, compared with healthy elderly.[13]

The aim of this study was to assess whether thyroid function is associated with risk of dementia, including AD, and hippocampal or amygdalar atrophy on MRI of non-demented elderly.

METHODS

Study sample

The study was based on subjects in the Rotterdam Scan Study. This study was designed to investigate determinants and consequences of brain abnormalities on MRI in the elderly.[14] Between 1995 and 1996 we randomly selected subjects aged 60-90 years in strata of age (5 years) and sex from the ongoing population-based Zoetermeer and Rotterdam studies.[15,16] As part of the eligibility criteria we excluded persons who were demented or blind or had MRI contraindications at time of selection.[17] Complete information including a cerebral MRI scan was obtained in 1,077 persons who gave written informed consent (participation rate 63%). The study was conducted in accordance with the tenets of the Declaration

of Helsinki. The Medical Ethics Committee of Erasmus Medical Center, Rotterdam, The Netherlands, approved the study.

MRI Procedures

In 1995-1996 standard T1, T2 and proton-density weighted MR sequences of the brain in a 1.5 Tesla MR unit were made in all study subjects (VISION MR; Siemens, Erlangen, Germany; and Gyroscan; Philips, Best, The Netherlands).[17] For the 563 subjects of the Rotterdam Study, a custom-made, double contrast, three-dimensional (3D), half-Fourier acquisition single-shot turbo spin echo (HASTE) sequence was added for volumetric assessments of the hippocampus and amygdala. MRI acquisition parameters have been described.[18] Fifty-two persons developed claustrophobia during the scanning period, leaving 511 subjects with a 3D HASTE sequence for volumetric assessments.

Ascertainment of incident dementia

All subjects were free of dementia at baseline, and the cohort was followed up for incident dementia. The diagnosis of dementia was made following a three-step protocol.[19,20] Briefly, all subjects were screened at follow-up visits (1997-1999, 1999-2000, and 2000-2003) with two brief tests of cognition (Mini-Mental State Examination (MMSE)[21] and Geriatric Mental State Schedule (GMS)[22]). Screen-positives (MMSE score < 26 or GMS organic level > 0) underwent the Cambridge examination for mental disorders of the elderly (Camdex). Subjects who were suspected of having dementia were examined by a neuropsychologist if additional neuropsychological testing was required for diagnosis. In addition, the total cohort was continuously monitored for incident dementia through computerized linkage between the study database and digitalized medical records from general practitioners and the Regional Institute for Outpatient Mental Health Care until January 1, 2005. The diagnosis of dementia and subtype of dementia was made in accordance with internationally accepted criteria for dementia (DSM-III-R)[23] and Alzheimer disease (NINDS-ADRDA)[24] by a panel consisting of a neurologist, neuropsychologist and research physician.

Hippocampal and amygdalar volumes

For the 511 subjects with a 3D HASTE sequence, we reconstructed a series of coronal brain slices (contiguous 1.5 mm slices) aligned to be perpendicular to the long axis of the hippocampus. The procedure of segmenting the hippocampus and amygdala has been described and was performed by two raters without knowledge of clinical information.[18] Briefly, we manually traced the boundaries of the hippocampi and amygdalae on each slice by means of a mouse-driven pointer. We proceeded from posterior to anterior, starting on the slice where the crux of the fornices was in full profile. Entering the outlined areas (mm^2) into a spreadsheet program, we multiplied the summed areas on each side

with slice thickness to yield estimates of the left and right hippocampal and amygdalar volume (ml). Total hippocampal or amygdalar volume was calculated by summing the left and right hippocampal or amygdalar volume. Fourteen randomly selected scans were used to evaluate intra- and interrater agreement, which showed good overall agreement (all intra- and interclass correlation coefficients exceeded $r = 0.77$).

We measured midsagittal area (cm^2) by tracing the inner skull to obtain a proxy for intracranial volume. Head size differences across individuals were corrected for by dividing the uncorrected volumes by the participant's calculated head size area and subsequently multiplying this ratio by the average head size area (men and women separately).[13]

Assessment of thyroid function

At time of MRI, non-fasting blood samples were collected and put on ice immediately. Within 30 minutes serum was separated by centrifugation and stored at –80°C. Multiple biochemical markers were used to investigate thyroid function. TSH, free T_4 (fT_4) and T_3 were all measured by chemoluminescence assays (Vitros ECI Immunodiagnostic System, Ortho-Clinical Diagnostics, Rochester, USA). rT_3 was measured with an in-house radioimmunoassay.[25] TPO-Abs were assessed with an immunometric assay (DPC, Los Angeles, CA, USA).

Hypothyroidism was defined as a concentration of serum TSH above the upper limit of the reference range (0.4-4.3 μU/dL) and fT_4 or T_3 concentrations below the reference range (0.85-1.94 ng/dL and 92.8-162.9 ng/dL respectively). Hyperthyroidism was defined as a concentration of serum TSH below the reference range and fT_4 or T_3 concentrations above the reference range. Serum TSH levels were high in 20 and low in 87 subjects (10 and 44 in the subset with hippocampal and amygdalar atrophy). However, in the majority of those with high or low TSH levels, fT_4 and T_3 concentrations were within the reference range. Of the 20 subjects with high TSH levels, 3 subjects had hypothyroidism whereas 17 subjects had an isolated high TSH indicating subclinical hypothyroidism. Of the 87 subjects with low TSH levels, 18 subjects had hyperthyroidism whereas 69 had isolated low TSH levels, which may be consistent with either subclinical hyperthyroidism or may be due to non-thyroidal illness or drug effects. Although useful to indicate thyroid status, reference values might be less appropriate in an elderly population since thyroid hormone concentrations change with aging.[26] Therefore, we also assessed thyroid function according to the continuous distribution of the thyroid hormones in our population.

Evaluation of thyroid function in the elderly is complicated by an increased prevalence of non-thyroidal illness.[27] Several conditions including malnutrition, starvation, and inflammatory processes accompanying disease are known to alter thyroid hormone and TSH concentrations, without overt thyroid dysfunction being present. In these situations, T_4 is converted preferentially to rT_3 instead of T_3.[28] We assessed rT_3 (reference range 9.1-22.1 ng/dL), an inactive metabolite of T_4, to obtain an indicator of peripheral

thyroid hormone metabolism. The ratio of T_3 over rT_3 (T_3/rT_3) was considered to be a marker of non-thyroidal illness.[29] Serum antibodies to thyroid peroxidase, which may be the cause of either hypo- or hyperthyroidism, were determined to indicate autoimmune activity against the thyroid gland. We considered subjects TPO-Abs positive if serum levels exceeded 35 IU/ml.

Complete thyroid hormone assessments were available in 1,047 subjects. Those using thyromimetic or thyrostatic medications (n = 22) were excluded from the analyses, leaving 1,025 subjects for the analyses on dementia and 489 subjects for the analyses on hippocampal and amygdalar atrophy on MRI.

Covariates

Several variables may confound an association between thyroid hormones and measures of brain atrophy, such as age at time of thyroid assessment, sex, educational level, depressive symptoms, smoking habits, medication use, atrial fibrillation, diabetes mellitus, body mass index (BMI) (calculated as weight in kilograms divided by height in meters squared), cholesterol, creatinine, homocysteine levels and apolipoprotein E (*APOE*) genotype. Educational status was defined as the highest education according to UNESCO and dichotomized into primary education only, and more than primary education.[30] Depressive symptoms were assessed with a validated Dutch version of the Center for Epidemiologic Studies Depression (CES-D) scale).[31] Smoking status was categorized into current, former and never smoking. We included reported use of cardiac medication (including amiodarone), β-blocking agents and systemic use of corticosteroids (Anatomical Therapeutical Chemical codes c01, c07 and h02). Presence of atrial fibrillation was based on analysis of digitally stored standard 12-lead electrocardiograms using the Modular Electrocardiogram Analysis System (MEANS).[32] Diabetes mellitus was defined as reported use of oral anti-diabetic treatment or insulin, or a random serum glucose concentration greater than or equal to 202 ng/dL. Serum total and HDL cholesterol and creatinine levels were determined using an automated enzymatic procedure. Homocysteine levels were determined by a fluorescence polarization immunoassay.[33] Apolipoprotein E (*APOE*) genotype testing was performed on coded DNA samples.[34]

Statistical analyses

Cox proportional hazards models were used to estimate the association between thyroid function and dementia. Duration of follow-up for each participant was calculated from baseline examination until death, diagnosis of dementia, or the end of follow-up, whichever came first. Multivariate linear regression models were used to quantify the relation between thyroid function and measures of brain atrophy.

The association between thyroid function and the outcome measures was analyzed in two ways. First, thyroid status was analyzed in categories based on the cut-off values

of TSH (> 4.3 μU/dl (high), 0.4-4.3 μU/dl (normal) and < 0.4 μU/dl (low)). In these analyses, the reference group consisted of subjects with TSH levels within the reference range. Second, thyroid hormone levels were analyzed according to their distribution, in quintiles as well as continuously (per SD increase) if the observed association was not obviously non-linear.

All analyses were adjusted for age and sex. To account for changes in thyroid hormone levels due to concomitant disease, the models were additionally adjusted for T_3/rT_3. To investigate whether associations were modified by autoimmune thyroid disease, analyses were repeated according to presence or absence of TPO-Abs (In the analysis on abnormal serum TSH levels in combination with positive TPO-Abs, subjects without raised TPO-Abs who were euthyroid were reference). Further adjustments included educational level and depressive symptoms, cigarette smoking, cardiac medication, β-blocking agents, systemic corticosteroid use, atrial fibrillation, diabetes mellitus, BMI, total and HDL cholesterol, creatinine and homocysteine levels. In addition, all analyses were repeated after exclu-

Table 1. Baseline characteristics of the total study sample and of the subset with data on hippocampal and amygdalar volume*

Characteristic	Total study sample	Subset†
Number (n)	1,025	489
Age (years)	72.3 (7.4)	73.4 (8.0)
Sex (% female)	51.2	48.3
Education (% primary only)	34.9	31.1
Depressive symptoms (%)	7.5	7.4
Smoking (% current)	17.0	17.5
Use of β-blocking agents (%)	14.1	17.8
Use of cardiac medication (%)	10.2	13.3
Use of systemic corticosteroids (%)	1.1	1.0
Atrial fibrillation (%)	2.6	2.7
Diabetes mellitus (%)	7.1	6.5
Body mass index (kg /m^2)	26.6 (3.6)	26.3 (3.6)
Total cholesterol (mg/dL)	227.8 (38.6)	223.9 (42.5)
HDL cholesterol (mg/dL)	50.2 (11.6)	50.2 (19.4)
Serum creatinine (mg/dL)	0.98 (0.2)	0.99 (0.2)
Total homocysteine (mg/dL)	1.6 (0.7)	1.6 (0.6)
TSH (μU/dL)‡	1.2 (0.7; 1.7)	1.2 (0.7; 1.7)
fT_4 (ng/dL)	1.4 (0.2)	1.4 (0.3)
T_3 (ng/dL)	129.8 (19.5)	129.8 (19.5)
rT_3 (ng/dL)	19.5 (6.5)	25.6 (6.5)
TPO-Abs (% > 35 IU/mL)	11.7	12.5

* Values are unadjusted means (SD) or percentages

† Subset includes subjects with data on hippocampal and amygdalar volume

‡ Median (interquartile range)

sion of subjects with hypo- (n = 3) or hyperthyroidism (n = 18). Finally, we repeated the analyses in strata of *APOE* genotype. We classified subjects into those with and without an ε4 allele. Those with *APOE* genotype ε2/ε4 were excluded. All statistical analyses were performed using SPSS statistical software version 11 (SPSS Inc., Chicago, Illinois).

RESULTS

Baseline characteristics are shown in Table 1. During a total of 5,657 person-years of follow-up (mean per person 5.5 years) 63 subjects developed dementia, of whom 46 were diagnosed with AD. When we made cut-offs in serum TSH values, the risk of dementia did not differ significantly between subjects with TSH concentrations within the reference range and those with a high or low TSH concentration (Table 2). Subjects with low TSH in the presence of TPO-Abs had a nearly 4-fold increased risk of dementia, although

Table 2. Thyroid status and risk of dementia in the total sample (n=1,025)

	Dementia (n=63)		Alzheimer disease (n=46)	
	N*	Hazard Ratio (95% CI)†	N*	Hazard Ratio (95% CI)†
TSH 0.4-4.3 µU/dL	53	1.00 (ref.)	40	1.00 (ref.)
TSH > 4.3 µU/dL	3	1.28 (0.39; 4.23)	2	0.94 (0.22; 4.04)
TSH > 4.3 µU/dL and TPO-Abs positive‡	1	0.96 (0.13; 7.10)	1	1.02 (0.14; 7.63)
TSH < 0.4 µU/dL	7	1.13 (0.51; 2.52)	4	0.77 (0.27; 2.19)
TSH < 0.4 µU/dL and TPO-Abs positive	2	3.78 (0.90; 15.9)	1	2.16 (0.29; 16.0)

* Number of dementia cases

† Values are age and sex adjusted hazard ratios (95% confidence intervals (CI)) for subjects with reduced or elevated TSH compared to euthyroid subjects (reference). In the analyses on TPO-Abs, euthyroid subjects without TPO-Abs were reference.

‡ TPO-Ab level > 35 IU/mL

Table 3. Thyroid hormone levels and risk of dementia and AD in the total sample (n=1,025)

	Dementia (n=63)	AD (n=46)
	Hazard Ratio (95% CI)*	Hazard ratio (95% CI)*
TSH (per SD: 1.5 µU/dL)	1.00 (0.84; 1.18)	0.97 (0.73; 1.28)
fT_4 (per SD: 0.3 ng/dL)	0.99 (0.69; 1.43)	0.97 (0.63; 1.50)
T_3 (per SD: 19.5 ng/dL)	1.00 (0.97; 1.03)	1.00 (0.96; 1.03)
rT_3 (per SD: 6.5 ng/dL)	1.00 (0.99; 1.01)	1.00 (0.98; 1.01)
T_3/rT_3 (per SD: 2.1 U)	1.00 (0.79; 1.25)	0.98 (0.74; 1.29)

* Values are age and sex adjusted hazard ratios per SD increase in thyroid hormone level (95% confidence intervals (CI))

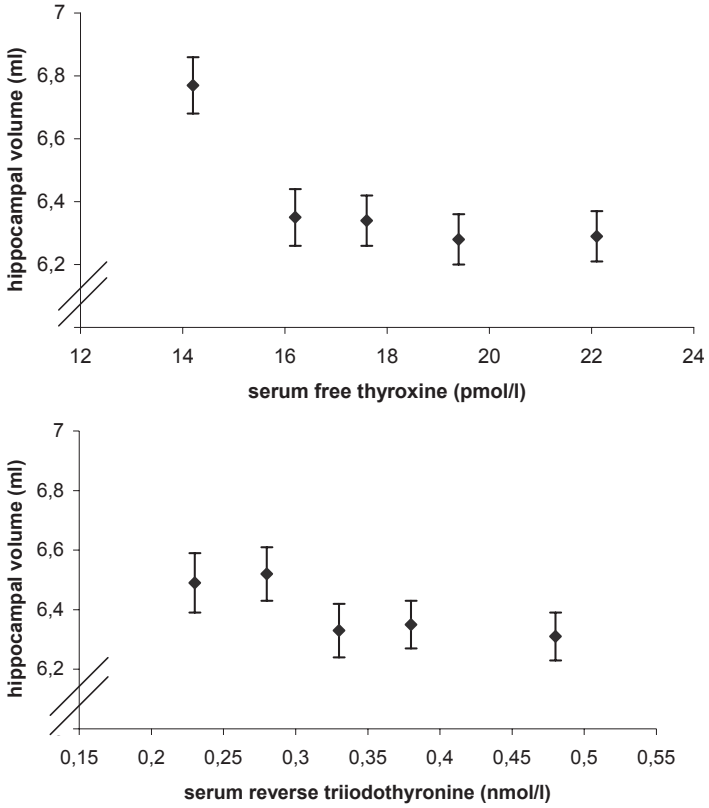

Figure 1. Hippocampal volume according to quintiles of serum free thyroxine and reverse triiodothyronine. The mean hippocampal volume (standard error) is plotted at the median of each quintile and is adjusted for age and sex.

this was not significant (Table 2). Serum levels of TSH were not associated with the risk of dementia, nor were serum levels of the thyroid hormones (Table 3).

TSH was not associated with the extent of atrophy, either when analyzed according to cut-offs in serum TSH values or when analyzed according to quintiles of the distribution of TSH serum levels in our population (Tables 4 and 5). Figure 1 shows the association between serum levels of fT_4 and hippocampal atrophy on MRI. Subjects with higher levels of fT_4 had on average more hippocampal atrophy. Likewise, higher rT_3 levels were also associated with more hippocampal atrophy on MRI (Figure 1). Similar associations were found for amygdalar atrophy. Free T_4, rT_3 and also the ratio of T_3/rT_3 were linearly related to both measures of brain atrophy, yet no such associations were found for both TSH and T_3 levels (Table 5). TPO-Abs did not modify the results between either TSH in categories (Table 4) or thyroid hormone levels and measures of brain atrophy (data not shown).

Additional adjustment for T_3/rT_3 slightly attenuated the associations between fT_4 and brain atrophy (difference in hippocampal volume per standard deviation increase in fT_4 levels –0.07 ml (95% CI –0.15; 0.01) and amygdalar volume –0.09 ml (95% CI –0.16;

Table 4. Thyroid status and atrophy of the medial temporal lobe on MRI (n=489)

	Adjusted Differences for measures of brain atrophy, compared with euthyroid subjects*		
	N†	Hippocampus (ml)	Amygdala (ml)
TSH 0.4-4.3 µU/dL	435	0.0 (ref.)	0.0 (ref.)
TSH > 4.3 µU/dL	10	0.06 (-0.46; 0.59)	-0.11 (-0.53; 0.31)
TSH > 4.3 µU/dL and TPO-Abs positive‡	7	0.40 (-0.22; 1.01)	-0.14 (-0.36; 0.64)
TSH < 0.4 µU/dL	44	0.07 (-0.19; 0.33)	-0.06 (-0.27; 0.16)
TSH < 0.4 µU/dL and TPO-Abs positive	3	0.11 (-0.83; 1.04)	0.22 (-0.54; 0.97)

* Values are age and sex adjusted differences in measures of brain atrophy between subjects with reduced or elevated TSH compared to euthyroid subjects (reference) (95% confidence interval). In the analyses on TPO-Abs, euthyroid subjects without TPO-Abs were reference.

† Number of subjects

‡ TPO-ab level > 35 IU/ml

Table 5. Thyroid hormone levels and atrophy of the medial temporal lobe on MRI (n=489)

	Differences in measures of brain atrophy per SD increase in thyroid hormone levels*	
	Hippocampus (ml)	Amygdala (ml)
TSH (per SD: 1.5 µU/dL)	0.04 (-0.02; 0.11)	0.03 (-0.03; 0.08)
fT_4 (per SD: 0.3 ng/dL)	-0.11 (-0.18; -0.04)	-0.11 (-0.16; -0.05)
T_3 (per SD: 19.5 ng/dL)	0.06 (-0.02; 0.13)	0.04 (-0.02; 0.10)
rT_3 (per SD: 6.5 ng/dL)	-0.09 (-0.16; -0.01)	-0.08 (-0.14; -0.02)
T_3/rT_3 (per SD: 2.7 U)	0.12 (0.05; 0.20)	0.09 (0.02 ; 0.15)

* Values are age and sex adjusted differences in measures of brain atrophy per SD increase in thyroid hormone level (95% confidence interval)

-0.02)). Adjustment for all other confounders, exclusion of subjects with a low or a high TSH concentration, or stratification for *APOE* ε4, changed any of the above-mentioned associations.

DISCUSSION

In this community sample, there was no relation between thyroid function and the risk of dementia or AD. In contrast, we found that persons with higher serum levels of fT_4 and rT_3 had more hippocampal and amygdalar atrophy on MRI. The major strengths of this study are the population-based design, the long follow-up for incident dementia, the large number of volumetric MRI assessments of the hippocampus and amygdala, and the extensive assessment of different indicators of thyroid function. A limitation is the lack of follow-up on the thyroid status of the study participants.

Previously, we showed that subclinical hyperthyroidism increases the risk of dementia and AD over a 2-year follow-up, especially in the presence of TPO-Abs.[7] In the present study, using data from a study sample that was independent of, yet very similar to, the population studied in the previous report, TSH was not related to risk of dementia over a nearly 6-year follow-up. The observation that low TSH in the presence of TPO-Abs was associated with a nearly 4-fold increased risk of dementia is consistent with our previous findings, although this did not reach statistical significance. However, in both studies the number of dementia cases with high or low TSH was relatively small. Moreover, in the previous study, thyroid status was assessed only in a subgroup of the cohort. In addition, due to the small overall number of dementia cases in this subgroup ($n = 25$), thyroid function was not studied within the normal range. The relatively small sample size restricted the power, and together with the short follow-up, this may have minimized precision. In the current report, follow-up for dementia was longer and the number of dementia cases larger ($n = 63$), which enabled us to study thyroid function also within the normal range. TSH was not related to dementia when analyzed continuously according to the distribution in our population. Neither did we find an association between TSH and hippocampal or amygdalar atrophy, which may be indicative of early AD.[10] To our knowledge, low TSH levels have thusfar only been related to AD in only one other study,[35] which used a case-control design. Together with our findings, this suggests that the variation in TSH levels within the normal range is not likely to affect the development of dementia and that the reported associations between low TSH and AD are more likely to result from imminent AD rather than developing Alzheimer pathology.

Interestingly, both higher fT_4 and rT_3 levels were associated with brain atrophy. Since concomitant diseases and associated comorbid conditions are known to change both fT_4 and rT_3 levels,[36] we considered that comorbidity or frailty might explain the associations we found with both fT_4 and rT_3 in this elderly population. Although adjustment for T_3/rT_3 as a measure of non-thyroidal illness and other confounders potentially reflecting comorbidity only slightly attenuated the association between fT_4 and brain atrophy, we cannot exclude residual confounding. As both hippocampal and amygdalar atrophy on MRI in non-demented elderly have been reported to predict risk of AD during a 6-yr follow-up period,[9] it could also be argued that the association of fT_4 and rT_3 with brain atrophy reflects incipient AD. However, exclusion of all subjects who developed AD during nearly 6 yr of follow-up did not change the results. In combination with the absence of a relation between fT_4 or rT_3 levels and risk of AD, this limits the possibility that early AD accounts for the association between thyroid hormone levels and brain atrophy on MRI. We must also keep in mind that not all persons with atrophy develop dementia.

Taken together, these findings argue against an important role of thyroid function in the development of AD. Future studies are needed to elucidate whether the association of higher fT_4 and rT_3 levels with brain atrophy on MRI has functional significance.

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Chapter 2.2

Deiodinase variants and atrophy of the medial temporal lobe

ABSTRACT

Context: *Thyroid function has been related to Alzheimer disease (AD) and neuro-imaging markers thereof. Whether thyroid dysfunction contributes to or results from developing AD remains unclear. Variations in the deiodinase type 1 (DIO1) and type 2 (DIO2) genes that potentially alter thyroid hormone bioactivity may help elucidating the role of thyroid function in AD.*

Objective: *We investigated the association of recently identified polymorphisms in the DIO1 (D1a-C/T, D1b-A/G) and DIO2 (D2-ORFa-Gly3Asp, D2-Thr92Ala) genes with circulating thyroid parameters, and early neuro-imaging markers of AD.*

Design and participants: *The Rotterdam Scan Study, a population-based cohort study among 1,077 elderly individuals aged 60-90 years.*

Main outcome measures: *DIO1 and DIO2 polymorphisms and serum TSH, fT_4, T_3 and rT_3 levels were determined in 995 non-demented elderly, including 473 persons with assessments of hippocampal and amygdalar volume on brain magnetic resonance imaging (MRI).*

Results: *Carriers of the D1a-T allele had higher serum fT_4 and rT_3, lower T_3 and lower T_3/rT_3. The D1b-G allele was associated with higher serum T_3 and T_3/rT_3. The DIO2 variants were not associated with serum thyroid parameters. No associations were found with hippocampal or amygdalar volume.*

Conclusion: *This is the first study to report an association of D1a-C/T and D1b-A/G polymorphisms with iodothyronine levels in the elderly. Polymorphisms in the DIO1 and DIO2 genes are not associated with early MRI markers of AD. This suggests that the previously reported association between iodothyronine levels and brain atrophy reflects comorbidity or non-thyroidal illness rather than thyroid hormones being involved in developing AD.*

INTRODUCTION

Thyroid disorders are associated with cognitive impairment and dementia.[1] Whether thyroid dysfunction also contributes to developing Alzheimer disease (AD) remains unclear. Within the Rotterdam Study we found subclinical hyperthyroidism to increase the risk of dementia and AD over three-fold in a two-year period.[2] Recently, we reported higher serum free T_4 (fT_4) levels within the normal range to be associated with hippocampal and amygdalar atrophy, putative early markers of AD,[3] on brain magnetic resonance imaging (MRI) of non-demented elderly.[4] This suggests that hyperthyroidism contributes to the development of AD. Alternatively, thyroid dysfunction may result from AD as both rT_3 and T_3/rT_3, indicators of non-thyroidal illness,[5] were also associated with brain atrophy on MRI.[4]

Genetic factors could determine up to 65% of the variation in serum thyroid hormone levels in healthy subjects.[6] Genetic variation in thyroid hormone pathway genes may therefore help to elucidate the role of thyroid hormone bioactivity in AD. The peripheral metabolism of thyroid hormone is regulated by three different deiodinases (D1-D3).[7] D1 is present in the thyroid, liver and kidneys and is involved in serum T_3 production and rT_3 clearance. D2 catalyzes local T_3 production in several tissues. In the brain D2 is expressed mainly in astrocytes in various structures including the cerebral cortex, hippocampus and amygdala.[8] D3 regulates T_3 and T_4 clearance. Variants in both the deiodinase type 1 (*DIO1*) and type 2 (*DIO2*) genes were recently reported to alter thyroid hormone levels in healthy blood donors.[9,10] Carriers of the T allele of the D1a-C/T polymorphism had higher serum rT_3 levels and lower T_3/rT_3, whereas the G allele of the D1b-A/G polymorphism was associated with higher T_3/rT_3.[9] Carriers of the D2-ORFa-Asp^3 allele had lower T_4, fT_4 and rT_3 levels, and a higher T_3/T_4 ratio.[10] The D2-Thr92Ala polymorphism has been associated with insulin resistance in different populations,[11,12] but not with serum thyroid levels.[10]

The association of variants in the *DIO1* and *DIO2* genes with serum thyroid hormone levels has not been replicated thus far. Neither the *DIO1* nor the *DIO2* polymorphisms have been studied with respect to hippocampal and amygdalar volume on MRI. The aim of this study was to investigate the association of the D1a-C/T, D1b-A/G, D2-ORFA-Gly3Asp and D2-Thr92Ala polymorphisms with serum thyroid parameters, and putative early MRI markers of AD in a population of non-demented elderly of Caucasian origin.

METHODS

Study population

This study was based on the Rotterdam Scan Study, an ongoing prospective population-based cohort study designed to study causes and consequences of age-related brain changes

on MRI.[13] In 1995 and 1996, subjects were randomly selected in strata of age (5 years, 60 to 90 years) and sex from the population-based Zoetermeer and Rotterdam studies.[14,15] As part of the eligibility criteria subjects who were demented, blind or had MRI contraindications at time of selection were excluded. Complete information including MRI of the brain was obtained in 1,077 subjects (overall response 63%). Participants originating from the Rotterdam Study underwent an additional three-dimensional (3D) MRI sequence during the scanning protocol, which allowed volumetric assessment of the hippocampus and amygdala ($n = 511$). The Rotterdam Scan Study was conducted in accordance with the tenets of the Declaration of Helsinki. The Medical Ethics Committee of the Erasmus University approved the study and written informed consent was obtained from all participants.

Thyroid hormone assessments

Non-fasting blood samples were collected at time of MRI and within 30 minutes serum was separated and stored at –80°C. TSH, fT_4 and T_3 were measured by chemoluminescence assays (Vitros ECI Immunodiagnostic System, Ortho-clinical diagnostics, Amersham, Rochester, NY, USA). Reverse T_3 (rT_3) was measured with an in-house radioimmunoassay.[16] Reference ranges were 0.4-4.3 μU/dL for TSH, 0.85-1.94 ng/dL for fT_4, 92.8-162.9 ng/dL for T_3 and 9.1-22.1 ng/dL for rT_3. Complete thyroid hormone assessments were available for 1,047 persons.

Hippocampal and amygdalar volume

In the 511 participants with a volumetric MRI sequence, coronal brain slices (contiguous 1.5 mm slices) were reformatted from the 3D MRI sequence and aligned to be perpendicular to the long axis of the hippocampus. The procedure of segmenting the hippocampus and amygdala has been described.[17] The left and right hippocampus and amygdala were manually outlined on each slice with a mouse-driven pointer. The areas on each side were multiplied with slice thickness and left and right side were summed to yield estimates of absolute volume (ml). As a proxy for head size, we measured midsagittal area (cm^2) by tracing the inner skull on a reformatted middle sagittal area MRI slice. Head size differences across individuals were corrected for by dividing the uncorrected volumes by the subject's calculated head size area and subsequently multiplying this ratio by the average head size area (men and women separately).[18] Hippocampal and amygdalar volume in this non-demented population ranged from 4.21 to 9.29 ml for hippocampal volume and from 2.17 to 6.77 ml for amygdalar volume.

Genotyping

Genomic DNA was extracted from peripheral leukocytes according to standard procedures. 1-2 ng genomic DNA was dispensed into 384-wells plates using a Caliper Sciclone ALH3000 pipetting robot (Caliper LS, Mountain View, CA, USA). Genotypes were

determined using the Taqman allelic discrimination assay. The Assay-by-Design service (www.appliedbiosystems.com) was used to set up a Taqman allelic discrimination assay for the the D1a-C/T (rs11206244) and D1b-G/T (rs12095080) polymorphisms in the *DIO1* gene[9] and the ORFa-Gly3Asp (rs12885300)[10] and Thr92Ala (rs2250114) polymorphisms in the *DIO2* gene. The PCR reaction mixture included 2 ng of genomic DNA in a 2 µl volume and the following reagents: FAM and VIC probes (200 nM), primers (0.9 uM), 2x Taqman PCR master mix (ABgene, Epsom, UK). Reagents were dispensed in a 384-well plate using the Deerac Equator NS808 (Deerac Fluidics, Dublin, Ireland). PCR cycling reactions were performed in 384 wells PCR plates in an ABI 9700 PCR system (Applied Biosystems Inc., Foster City, CA, USA) and consisted of initial denaturation for 15 minutes at 95°C, and 40 cycles with denaturation of 15 seconds at 95°C and annealing and extension for 60 seconds at 60°C. Results were analysed by the ABI Taqman 7900HT using the sequence detection system 2.22 software (Applied Biosystems Inc., Foster City, CA, USA). Genotyping of the four *DIO1* and *DIO2* polymorphisms succeeded in 1,018 persons in whom also thyroid hormone assessments were available.

Covariates

Several variables may confound an association of *DIO1* and *DIO2* polymorphisms with either thyroid hormone levels or measures of brain atrophy, such as age at time of thyroid assessment, sex, educational level, depressive symptoms, smoking habits, medication use, atrial fibrillation, diabetes mellitus, body mass index (BMI) (calculated as weight in kilograms divided by height in meters squared), cholesterol, creatinine and homocysteine levels. Educational status was defined as the highest education according to UNESCO and dichotomized into primary education only, and more than primary education.[19] Depressive symptoms were assessed with a validated Dutch version of the Center for Epidemiologic Studies Depression (CES-D) scale).[20] Smoking status was categorized into current, former and never smoking. We included reported use of cardiac medication (including amiodarone), β-blocking agents and systemic use of corticosteroids (Anatomical Therapeutical Chemical codes c01, c07 and h02). Presence of atrial fibrillation was based on analysis of digitally stored standard 12-lead electrocardiograms using the Modular Electrocardiogram Analysis System (MEANS).[21] Diabetes mellitus was defined as reported use of oral anti-diabetic treatment or insulin, or a random serum glucose concentration greater than or equal to 202 ng/dL. Serum total and HDL cholesterol, creatinine and glucose levels were determined using an automated enzymatic procedure. Homocysteine levels were determined by a fluorescence polarization immunoassay.[22]

Statistical analysis

Deviation from Hardy-Weinberg proportions was analyzed using a Chi-square test. Analysis of covariance was used to compute age and sex adjusted means of serum TSH

and iodothyronine levels within the D1 and D2 genotype groups. In the subset with MRI measures of brain atrophy, analyses of covariance were used to compute age and sex adjusted means of hippocampal and amygdalar volumes on MRI within genotype. In addition the genotype was entered as a linear term in the models to yield *P*-values of the allele-dose trend. All analyses were performed with exclusion of persons who used thyromimetic or thyrostatic medication: $n = 23$ in the overall analyses and $n = 10$ in the analyses on brain volume, leaving 995 persons for the overall analyses and 473 persons for the analyses on brain volume. All models were additionally adjusted for the other covariates. All statistical analyses were performed using SPSS statistical software version 11 (SPSS Inc., Chicago, Illinois). Linkage disequilibrium for the polymorphisms was quantified by r^2 values calculated with the Haploview Program.[23]

RESULTS

Genotype distributions were similar in the overall study population and the subset with measures of brain atrophy on MRI (Table 1) and were all in Hardy Weinberg equilibrium. Baseline characteristics for the overall study sample and the subset with measures of brain atrophy on MRI are shown in Table 2.

Table 3 presents the associations of the *DIO1* polymorphisms with serum TSH and iodothyronine levels. The D1a-T allele was associated with higher rT_3 and lower T_3 levels and a lower T_3/rT_3 ratio. Free T_4 levels were also higher in carriers of the D1a-T allele. The D1b-G allele of the D1b-A/G variant was related to higher T_3 levels and a higher

Table 1. Polymorphisms in the *DIO1* and *DIO2* genes in the study sample in total and in the subset with hippocampal and amygdalar volumes*

		Total (n=995)	Subset (n=473)
D1a-C/T	CC	409 (41.1)	188 (39.7)
	CT	460 (46.2)	213 (45.0)
	TT	126 (12.7)	72 (15.2)
D1b-A/G	AA	806 (81.0)	382 (80.8)
	AG	182 (18.3)	86 (18.2)
	GG	7 (0.7)	5 (1.0)
D2-ORFa-Gly3Asp	Gly/Gly	425 (42.7)	190 (40.2)
	Gly/Asp	442 (44.4)	211 (44.6)
	Asp/Asp	128 (12.9)	72 (15.2)
D2-Thr92Ala	Thr/Thr	388 (39.0)	181 (38.3)
	Thr/Ala	470 (47.2)	225 (47.6)
	Ala/Ala	137 (13.8)	67 (14.2)

* Values are numbers (percentages)

Table 2. Characteristics of the study sample in total and of the subset with hippocampal and amygdalar volumes*

Characteristic	Total	Subset
Number (n)	995	473
Age (years)	72.2 (7.4)	73.6 (8.0)
Sex (% female)	51.5	49.1
Education (% primary only)	34.9	31.5
Smoking (% current)	17.2	17.5
Atrial fibrillation (%)	1.7	1.3
Diabetes mellitus (%)	7.3	6.7
Body mass index (kg /m^2)	26.6 (3.4)	26.3 (3.6)
Total cholesterol (mg/dL)	227.8 (38.6)	223.9 (42.5)
HDL cholesterol (mg/dL)	50.2 (11.6)	50.2 (15.4)
Serum creatinine (mg/dL)	1.0 (0.2)	1.0 (0.2)
Total homocysteine (mg/dL)	1.6 (0.7)	1.6 (0.6)
Use of cardiac medication (%)	10.4	13.4
Use of β-blocking agents (%)	14.4	18.0
Use of systemic corticosteroids (%)	1.0	0.1
APOE genotype (% ε4 carrier)	26.9	26.5
TSH (μU/dL)†	1.2 (0.7 ; 1.2)	1.2 (0.7 ; 1.2)
fT_4 (ng/dL)	1.4 (0.2)	1.4 (0.3)
T_3 (ng/dL)	129.8 (16.9)	127.8 (17.5)
rT_3 (ng/dL)	22.7 (7.8)	23.4 (17.5)
T_3/rT_3	6.35 (2.06)	5.96 (2.08)
Hippocampal volume (ml)	-	6.38 (0.86)
Amygdalar volume (ml)	-	4.57 (0.73)

* Values are unadjusted means (SD) or percentages,

† Median (interquartile range)

T_3/rT_3 ratio. No effect of the D2-ORFa-Gly3Asp and D2-Thr92Ala polymorphisms was seen on serum thyroid hormone levels (Table 4). Additional adjustment for potential other confounders did not change the associations between the *DIO1* and *DIO2* polymorphisms and TSH or iodothyronine levels. The associations were similar in the subset with measures of brain atrophy on MRI when compared to the overall study sample.

Neither the D1a-C/T, nor the D1b-A/G polymorphism was associated with hippocampal or amygdalar volume on MRI (Table 5). Nor did we find a relation of the D2-ORFa-Gly3Asp and D2-Thr92Ala polymorphisms with brain volume on MRI (Table 6). Additional adjustments did not change the results.

Linkage analysis for the D1a-C/T and the D1b-A/G polymorphisms using the Haploview program showed a r^2 value of 0.06, which is low. In a similar manner, we found a r^2 value of 0.22 for linkage between the D2-ORFa-Gly3Asp and the D2-Thr92Ala

Table 3. Association of serum TSH and iodothyronine levels with the D1a-C/T and D1b-A/G variants (n=995)*

	N	TSH (μU/dL)	fT_4 (ng/dL)	T_3 (ng/dL)	rT_3 (ng/dL)	T_3/rT_3
D1a-C/T						
CC	409	1.53 (0.07)	1.37 (0.01)	131.8 (0.83)	21.8 (0.35)	6.67 (0.10)
CT	460	1.33 (0.07)	1.41 (0.01)	128.9 (0.78)	22.9 (0.33)	6.13 (0.09)
TT	126	1.40 (0.13)	1.41 (0.01)	127.7 (1.50)	23.0 (0.64)	6.00 (0.17)
P-trend		0.13	0.04	0.004	0.03	< 0.001
D1b-A/G						
AA	806	1.44 (0.05)	1.40 (0.01)	129.3 (0.59)	22.6 (0.25)	6.28 (0.07)
AG	182	1.32 (0.11)	1.40 (0.02)	132.9 (1.25)	22.0 (0.53)	6.55 (0.14)
GG	7	1.55 (0.56)	1.37 (0.10)	129.2 (6.36)	20.2 (2.71)	7.03 (0.74)
P-trend		0.40	0.93	0.02	0.17	0.06

* Values are age and sex adjusted means (standard errors)

Table 4. Association of serum TSH and iodothyronine levels with the D2-ORFa-Gly3Asp and D2-Thr92Ala variants (n=995)*

	N	TSH (μU/dL)	fT_4 (ng/dL)	T_3 (ng/dL)	rT_3 (ng/dL)	T_3/rT_3
D2-ORFaGly3Asp						
Gly/Gly	425	1.35 (0.07)	1.40 (0.01)	130.7 (0.82)	22.4 (0.35)	6.38 (0.09)
Gly/Asp	442	1.45 (0.07)	1.41 (0.01)	129.5 (0.81)	22.9 (0.34)	6.19 (0.09)
Asp/Asp	128	1.56 (0.13)	1.37 (0.02)	129.0 (1.49)	21.4 (0.63)	6.65 (0.17)
P-trend		0.14	0.60	0.24	0.54	0.62
D2-Thr92Ala						
Thr/Thr	388	1.46 (0.08)	1.39 (0.01)	130.0 (0.86)	22.3 (0.36)	6.35 (0.10)
Thr/Ala	470	1.36 (0.07)	1.41 (0.01)	130.0 (0.78)	22.6 (0.33)	6.31 (0.09)
Ala/Ala	137	1.52 (0.13)	1.38 (0.02)	129.7 (1.44)	22.5 (0.61)	6.38 (0.17)
P-trend		0.99	0.97	0.86	0.71	0.98

* Values are age and sex adjusted means (standard errors)

polymorphism. Haplotype analyses are not meaningful when the r^2 is this low and were therefore not performed.

DISCUSSION

We found in a population-based study among elderly Caucasian individuals that the D1a-C/T polymorphism was associated with both serum iodothyronine levels and the T_3/rT_3 ratio. The D1b-A/G variant was related to serum T_3 and the T_3/rT_3 ratio. The D2-ORFa-Gly3Asp and D2-Thr92Ala polymorphisms were not related to circulating thyroid parameters. Both the polymorphisms in the *DIO1* and those in the *DIO2* gene were not associated with early MRI markers of Alzheimer pathology.

Table 5. Association of the D1a-C/T and D1b-A/G variants with brain volume on MRI (n=473)*

	N	Hippocampus (ml)	Amygdala (ml)
D1a-C/T			
CC	188	6.33 (0.06)	4.58 (0.05)
CT	213	6.40 (0.06)	4.54 (0.05)
TT	72	6.43 (0.10)	4.62 (0.08)
P-trend		0.32	0.86
D1b-A/G			
AA	382	6.38 (0.04)	4.56 (0.03)
AG	86	6.37 (0.09)	4.65 (0.07)
GG	5	6.31 (0.37)	4.28 (0.30)
P-trend		0.85	0.58

* Values are age and sex adjusted mean volumes (standard errors)

Table 6. Association of the D2-ORFa-Gly3Asp and D2-Thr92Ala variants with brain volume on MRI (n=473)*

	N	Hippocampus (ml)	Amygdala (ml)
D2-ORFaGly3Asp			
Gly/Gly	190	6.41 (0.06)	4.58 (0.05)
Gly/Asp	211	6.36 (0.06)	4.52 (0.05)
Asp/Asp	72	6.34 (0.10)	4.71 (0.08)
P-trend		0.48	0.38
D2-Thr92Ala			
Thr/Thr	181	6.27 (0.06)	4.56 (0.05)
Thr/Ala	225	6.46 (0.06)	4.58 (0.05)
Ala/Ala	67	6.38 (0.10)	4.56 (0.08)
P-trend		0.11	0.85

* Values are age and sex adjusted mean volumes (standard errors)

Strengths of this study are its population-based setting and large number of volumetric assessments on MRI. Potential limitations of genetic association studies are related to population stratification or heterogeneity, which is of particular importance in case-control studies and in persons of mixed racial origin. In our study, this has played no role since all subjects were of Dutch Caucasian origin and can be considered ethnically homogeneous. In addition, allele frequencies of the polymorphisms were in agreement with those reported in other Caucasian subjects.[9,10] Although the polymorphisms were associated with thyroid hormone levels, no association was found with hippocampal and amygdalar volume. Because volumetric measures were only available in half of our sample, this has limited our power to find an association. The 3D sequence necessary to obtain volumetric measures of the hippocampus and amygdala could only be added in participants originating from the Rotterdam Study. As this was due to logistic reasons and not related to clinical characteristics of the participants, this makes selection bias

unlikely. However, larger series with more power are needed to verify and replicate our findings. The relatively small number of subjects that developed dementia during follow-up of the study ($n = 62$, follow-up until January 2005) limited our abilities to investigate the association of variants in the *DIO1* and *DIO2* genes with risk of dementia.

Previous reports suggest that the D1a-T and D1b-G variants in the *DIO1* gene are associated with altered D1 activity.[9-12] As liver D1 plays an important role in the production of serum T_3 from T_4 and in the breakdown of the metabolite rT_3, functionally relevant variants in the *DIO1* gene are expected to affect serum iodothyronine levels, in particular rT_3 and ratios between serum iodothyronines.[7] Peeters et al. analyzed 156 healthy blood donors and reported higher serum levels of rT_3 and a lower T_3/rT_3 ratio in those carrying the D1a-T allele.[9] Both associations were confirmed in this elderly population. In agreement with these findings we found carriers of the D1a-T allele to have lower serum levels of T_3, altogether suggesting a negative effect of the D1a-T variant on total D1 activity. The higher fT_4 levels in carriers of the D1a-T allele may reflect a lower conversion to T_3 by D1 in these subjects.

Since carriers of the D1b-G allele were reported to have a higher serum T_3/rT_3 ratio, the D1b-G allele was suggested to increase total D1 activity,[9] which is strengthened by findings from our study showing D1b-G carriers to have both higher serum levels of T_3 and a higher ratio of serum T_3/rT_3.

No effect of either of the two polymorphisms in the *DIO2* gene was seen on thyroid hormone levels in this elderly population. Whereas the D2-Thr92Ala variant was not associated with serum thyroid hormone levels in previous studies,[9,10,12] the D2ORFa-Gly3Asp variant was, yet only in healthy blood bank donors and not in elderly subjects.[10] Our results are therefore in agreement with previous studies. However, an effect of these variants on local thyroid hormone bioactivity cannot be excluded as D2 is mainly involved in the conversion of T_4 to T_3 at the tissue level.

Recently, we reported hippocampal and amygdalar volume to be associated with higher serum levels of fT_4, rT_3 and a lower T_3/rT_3 ratio in non-demented elderly.[4] Due to the cross-sectional design of the study, it could not be determined whether higher thyroid hormone levels were involved in the development of brain atrophy or resulted from neurodegeneration. Using data from the same study population, we here report that both the D1a-C/T and the D1b-A/G variants were related to iodothyronine levels and the T_3/rT_3 ratio. If thyroid hormone levels were causally related to hippocampal and amygdalar atrophy, one would expect an association of these polymorphisms in the *DIO1* gene, altering life-time exposure to T_3 and rT_3 levels, with these measures of brain atrophy on MRI. The lack of such a relation, suggests that the higher serum levels of rT_3 in those with more marked brain atrophy reflects comorbidity or non-thyroidal illness in the elderly, rather than thyroid function being causally involved in the development of brain atrophy related to AD. The observation that polymorphisms in the *DIO2* gene were

also not related to early brain markers for AD, further argues against an important role of thyroid function in the development of brain atrophy and AD, as D2 is highly expressed in the brain, especially in the hippocampus and amygdala.[8]

Other mechanisms should be considered. First, thyroid hormone concentrations are tightly regulated in the brain.[24] Whereas D2 is important for maintaining adequate levels of T_3, D3 preserves the brain from detrimental T_3 levels by converting T_4 to rT_3. After treating rats for 8 weeks with a high dose of T_4, T_3 concentrations appeared unaltered in cortex, hippocampus and amygdala,[25] and were elevated only in brain areas in which D3 activity was low or absent. Although comparative data based on studies in humans is lacking, this could suggest that the potential effects of D2 polymorphisms may have been counterbalanced by increased D3 activity. Second, somatic alterations of *DIO2* potentially alter the peripheral thyroid hormone milieu, which in turn could contribute to the development of AD. Finally, it is also possible that thyroid hormones contribute to the rate of progression of brain atrophy and subsequently AD, rather than being a cause.

In conclusion, this is the first study to report an association of the D1a-C/T and D1b-A/G variants in the *DIO1* gene with serum iodothyronine levels in the elderly. The absence of a relation between genetically determined bioactivity of thyroid hormones and early brain imaging markers for AD suggests that the previously described association between thyroid hormones and brain atrophy on MRI reflects comorbidity or non-thyroidal illness in the elderly, rather than thyroid hormones being involved in developing AD.

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Chapter 2.3

Thyroid hormones, dementia and neuropathology

ABSTRACT

Thyroid dysfunction is associated with cognitive impairment and dementia, including Alzheimer disease (AD). It remains unclear whether thyroid dysfunction results from, or contributes to, Alzheimer pathology. We determined whether thyroid function is associated with dementia, specifically AD, and Alzheimer-type neuropathology in a prospective population-based cohort of Japanese-American men. Thyrotropin, total and free thyroxine were available in 665 men aged 71-93 years and dementia-free at baseline (1991), including 143 men who participated in an autopsy sub-study. During a mean follow-up of 4.7 (SD: 1.8) years, 106 men developed dementia of whom 74 had AD. Higher total and free thyroxine levels were associated with an increased risk of AD, whereas thyrotropin was not. In the autopsied subsample, higher total thyroxine was associated with higher number of neocortical neuritic plaques and neurofibrillary tangles. As the significant association of thyroxine levels with AD was apparent only when thyroid hormones were measured close in time to dementia diagnosis, higher thyroid hormone levels are more likely to be a consequence rather than contributing to AD.

INTRODUCTION

Clinical thyroid disorders are associated with cognitive impairment and dementia[1] including Alzheimer disease (AD).[2] However, some population-based studies suggest that variation in presumably normal levels of thyroid hormones are also associated with a risk for cognitive-related disorders, but findings are mixed. In the Rotterdam Study sub-clinical hyperthyroidism was associated with an increased risk of dementia and AD[3] and in the Rotterdam Scan Study higher thyroid hormone levels were found to be associated with markers of brain atrophy on MRI scans of non-demented elderly.[4] In contrast, lower thyroxine, rather than thyrotropin, increased risk of cognitive decline in physically impaired elderly women,[5] whereas there was no association of either thyrotropin or thyroxine to cognitive function in an elderly population over 85 years of age.[6]

Findings of an association of AD with sub-clinical levels of thyroid dysregulation are of interest but need replication. There is also a question as to whether the association differs according to time-to-event; shorter time-to-event might suggest the association reflects the effect of dementia on thyroid function and not vice versa.[4] Here we investigate the association of thyroid hormone levels to the risk for AD and its neuropathologic markers. Data are from the Honolulu-Asia Aging Study (HAAS), a longitudinal study that includes assessment of clinical dementia, as well as an autopsy sub-study of the cohort.

METHODS

Design

The baseline sample consisted of participants of the Honolulu Heart Program, a prospective cohort study carried out among Japanese-American men living on the Island of Oahu, Hawaii from 1965 onwards.[7] Participants were examined on three occasions between 1965 and 1971. Of the 4,676 survivors, 3,734 (80%) participated in a fourth examination between 1991 and 1993 as part of the HAAS. Between 1994-1996 and 1997-1999 two additional examinations were carried out (participation rates 84 and 75% respectively). Prevalent dementia was ascertained at examination 4 and incident dementia at examinations 5 and 6. In 1991, an autopsy program was instituted to study risk factors for, and disease correlates of, neuropathologic markers of brain disease. All participants gave written, informed consent at each examination. Family members gave permission for cases of dementia. The study protocol was approved by the Kuakini Medical Center institutional review board.

Dementia case finding procedures

Dementia and its subtypes were identified in a multi-step case-finding procedure, described in detail elsewhere.[8,9] In brief, all participants underwent neuropsychological screening with the 100-point Cognitive Abilities Screening Instrument (CASI), a measure of global function that has been validated in English and Japanese.[10] Diagnosis was based on neuropsychologic testing using the Consortium to Establish a Registry for Alzheimer Disease (CERAD) battery, a neurologic exam and an informant interview. Those with dementia received work-up with neuroimaging (in 86%) and blood tests. All recognized subtypes of dementia were considered in the diagnostic consensus conference that included a neurologist and at least two other study investigators. Dementia was diagnosed according to DSM-III-R criteria,[11] probable and possible AD according to National Institute of Neurological and Communicative Disorders and Stroke-Alzheimer Disease and related Disorders Association criteria,[12] and vascular dementia according to California Alzheimer Disease and Treatment Centers criteria.[13] The remaining subtypes included subdural hematoma, Parkinson disease, cortical Lewy body disease, Pick disease, and cause not determined. Among participants who received autopsy evaluation, approximately two-thirds of the clinical Alzheimer cases met CERAD neuropathologic criteria[14] for AD.[15]

Autopsy sub-study

Procedures for autopsy and neuropathological examination have been described elsewhere.[15] At death, brains were fixed in formalin for a minimum of 10 days. After fixation, brains were weighed, the cerebellum and brainstem were removed from the cerebral hemispheres, and all were cut serially in the coronal plane at 1-cm thickness. Slices were examined for grossly apparent neuropathologic lesions, and the whole brain and slices were photographed. Tissue from four areas of neocortex (middle frontal gyrus, inferior parietal lobule, middle temporal gyrus, and occipital cortex) and two areas of hippocampus (CA1 and subiculum) was used to prepare Bielschowsky silver-stained sections. Samples were evaluated by one of three neuropathologists who were blinded to clinical information. Senile plaques (SP) (diffuse and neuritic plaques), neuritic plaques (NP) and neurofibrillary tangles (NFT) were counted in five fields from the CA1 and subiculum of the hippocampus and five fields each from the four areas of neocortex. Counts were standardized to 1-mm^2 field areas.[16] Fields were selected for counting from areas with the highest numbers of lesions, and the field with the highest count was taken to represent the cortical or hippocampal area. A neuropathological diagnosis of AD was based on CERAD criteria.[14] These criteria included a maximum NP count of at least 4 per mm^2 for probable AD and at least 17 per mm^2 for definite AD.

Thyroid status

At time of examination 4, fasting blood samples were collected and put on ice immediately. Within 30 minutes, serum was separated by centrifugation and stored at –70°C. Thyroid hormones were assessed in a random sub-sample of 1,001 men who participated in examination 4. Several biochemical markers of thyroid function were assayed, including thyrotropin, free thyroxine (fT_4) and total thyroxine (T_4). Thyrotropin, fT_4 and T_4 were all measured by chemiluminescence assays on a DPC2000 analyzer (Diagnostic Product Co., Los Angeles, CA). The thyrotropin assay had an analytical sensitivity of 0.004 μU/dL and an inter-assay precision of 3.8% at 1.3 μU/dL. The fT_4 assay had an analytical sensitivity of 0.30 ng/dL and an intra-assay precision of 4.8% at 2.10 ng/dL. The T_4 assay had an analytical sensitivity of 0.30 μg/dL and an intra-assay precision of 4.6% at 8.23 μg/dL.

Thyrotropin and fT_4 serum levels were assessed to define thyroid status. Serum thyrotropin concentrations above the reference range (0.4 – 4.3 μU/dL) may indicate hypothyroidism and concentrations below the reference range may indicate hyperthyroidism. However, in these instances fT_4 concentrations are usually within the reference range (0.85 -1.94 ng/dL). Whereas an isolated high thyrotropin level indicates subclinical hypothyroidism, isolated low thyrotropin levels may indicate subclinical hyperthyroidism but may be also due to non-thyroidal illness or drug effects.[17] Clinical hypothyroidism was defined as a concentration of serum thyrotropin above the upper limit of the reference range and fT_4 concentrations below the lower limit of the reference range.[18] Clinical hyperthyroidism was defined as a concentration of serum thyrotropin below the reference range and fT_4 concentrations above the reference range.[18]

Covariates

The association between thyroid hormones and dementia or neuropathological markers thereof may potentially be confounded by a number of variables affecting health status; further, vascular risk factors may play an important role in the etiology of AD,[19] and are also related to thyroid function.[20] Therefore, we adjusted for the following variables: age, age at death (for the autopsy sub-study), educational level and depressive symptoms, albumin levels, body mass index (kg/m^2) (BMI), total and HDL cholesterol, diabetes mellitus, smoking status (never, former, current) systolic and diastolic blood pressure. Use of thyroid medication and other drugs potentially changing thyroid hormone levels including beta-blocking agents and use of anti-arhythmics at time of blood draw was also entered in the statistical models. *APOE* genotyping was performed at the Joseph and Kathleen Bryan Alzheimer Disease Research Center with restriction isotyping using a polymerase chain reaction protocol.[21]

STATISTICAL ANALYSIS

Incident Dementia

After exclusion of one participant with an unusually high level of fT_4 (4.35 ng/dL), with other thyroid hormones being normal, the analytical sample consisted of 1,000 participants. Of 1,000 participants with thyroid hormone assessments, 131 were demented at exam 4, and 205 participants died or refused further participation, leaving 665 participants at risk for dementia. After a duration of 3,204 person years of follow-up (mean: 4.7 years, SD: 1.8 years), 106 participants developed dementia, of whom 74 had AD (including AD cases with contributing cerebrovascular disease). Of those 106 dementia cases, 71 were diagnosed at exam 5 and 34 at exam 6. Analysis of covariance adjusted for age was used to compare characteristics of participants with and without thyroid hormone assessments at examination 4. No statistically significant differences in socio-demographic variables or cardiovascular risk factors were observed between participants in the sub-sample with thyroid hormone assessments and those without (data not shown).

In the analyses, thyroid hormone levels were expressed in two ways. First, thyroid status was classified as high or low levels of thyrotropin; the reference group consisted of subjects with thyrotropin levels within the reference range. Second, we expressed the continuous measures as unit of a standard deviation increase if the observed association was not obviously non-linear.

Cox proportional hazards regression analysis was used to calculate hazard ratios (HR) with 95% confidence intervals (95% CI) for total dementia and AD (with and without cerebrovascular disease). End of study was defined as date of diagnosis for dementia cases and either date of last study center visit or date of death for the other participants. To see whether time between assessment of thyroid hormones and clinical onset of dementia modified the association between thyroid function and dementia, the analyses were repeated separately for participants diagnosed with dementia at exam 5 (average of 1.5 yrs since blood draw) and at exam 6 (average of 4.6 yrs since blood draw).

Autopsy sub-sample

The analytical sample for the autopsy study is 143, only five of whom had high and four of whom had low thyrotropin levels. Therefore we did not examine differences in pathology among these sub-groups. Analysis of covariance adjusted for age was used to compare characteristics of the autopsy cases with participants who dropped out after exam 4 within the thyroid sample. Baseline characteristics of the included autopsy cases did not differ from those in the thyroid sample who dropped out after exam 4 (data not shown).

Multiple regression models with a negative binomial distribution were used to assess the association of thyroid hormones with NFT and NP. NP were only counted up to 17, which conforms to a definite diagnosis of AD.[14] Goodness of fit statistics showed a model

based on negative binomial distribution was the best fit to the data. To see whether an association between thyroid hormones and neuropathologic markers was specific for AD, and whether time between assessment of thyroid hormones and diagnosis of dementia was of influence, analyses in this sub-study were stratified into participants who had dementia at exam 4 ($n = 51$), those who developed dementia at exams 5 and 6 ($n = 26$), and those who had not been diagnosed with dementia before death ($n = 66$).

Adjusted analyses

All analyses were adjusted for age. To account for changes in thyroid hormone levels due to concomitant disease, additional adjustments were made by including the socio-demographic, medical history and biochemical markers described above. All analyses were repeated after exclusion of participants with clinical hypo- ($n = 5$) or hyperthyroidism ($n = 1$) and thyroid medication ($n = 5$). Finally, for dementia we repeated the analyses in strata of *APOE* genotype. We classified participants into those with and without an ε4 allele. Due to small numbers in the autopsy sub-study, these analyses were not stratified according to *APOE* status. All statistical analyses were performed using SPSS statistical software version 11 (SPSS Inc., Chicago, Illinois)[22] and SAS version 8 (SAS, Cary, NC).[23]

RESULTS

Thyroid status

Mean thyrotropin and thyroid hormone levels for prevalent dementia cases, incident dementia cases at exam 5, incident dementia cases at exam 6 and non-demented participants during follow-up are presented in Table 1.

Table 1. Thyroid hormone levels by dementia status at blood draw, the Honolulu-Asia Aging Study*

Age adjusted mean	*p*-value for difference‡	Dementia			No dementia during follow-up
		Prevalent	Incident		
			Exam 5	Exam 6	
TSH (mU/L) †	0.8	1.20	1.60	1.72	2.18
Total T_4 (nmol/L)	0.01	98.2	106.7	96.3	99.6
Free T_4 (pmol/L)	0.04	16.1	17.4	15.7	16.4

* Values are age adjusted means

† Median

‡ p for any difference in mean hormone level across groups defined by dementia status

Table 2. Baseline characteristics of the study sample at risk for dementia (n=665), The Honolulu-Asia Aging Study*

Characteristics	Sample at risk for dementia
Number (n)	665
Age at baseline (years)	77.0 (4.0)
Education (years)	10.6 (3.1)
Late-life total cholesterol (mg/dL)	190.6 (32.3)
Late-life HDL cholesterol (mg/dL)	50.4 (13.0)
Late-life body mass index (kg/m^2)	23.8 (3.0)
Diabetes mellitus (%)	34.5
Late-life smoking (% current)	39.2
Late-life diastolic blood pressure (mmHg)	80.5 (10.3)
Late-life systolic blood pressure (mmHg)	147.8 (21.9)
Late-life beta-blocker use (%)	9.7
Late-life anti-arrhythmic use (%)	13.7
Depressive symptoms (%)†	20.6
APOE genotype (% ε4 carrier)	19.4

* Values are means (standard deviation) or percentages

† Center for Epidemiologic Studies of Depression (CES-D) > 9

Characteristics of the 665 participants at risk for dementia are shown in Table 2. There were 615 (92.5%) participants with normal thyrotropin levels. Of these, 596 also had normal fT_4 levels, 14 had low and 5 had high fT_4 levels. In those with low or high fT_4, the levels deviated only slightly from the reference range, therefore all 615 participants were considered euthyroid. Twenty-six participants had an abnormally high thyrotropin level: 23 of these had a subclinical and 3 a clinical hypothyroidism according to biochemical criteria. Twenty-four participants had an abnormally low thyrotropin level: all of these had a subclinical and none had clinical hyperthyroidism according to biochemical criteria. Plasma levels of thyrotropin were significantly and inversely correlated with both T_4 ($r = -0.20$) and fT_4 ($r = -0.22$).

Thyroid hormones and dementia

Thyrotropin was not associated with the risk of dementia and AD. Yet, with each standard deviation increase in fT_4 the risk of dementia increased over 20% and the risk of AD increased over 30% (Table 3). Per standard deviation increase in T_4, the risk of dementia increased 19% and the risk of AD increased 22%; however this was not statistically significant. Results did not markedly change after additional adjustment for potential confounders, stratification by ApoE ε4 status or exclusion of participants with hyper- or hypothyroidism or on thyroid medication.

Table 3. Thyroid hormone levels and the risk of dementia and Alzheimer disease, the Honolulu-Asia Aging Study

	Dementia (n=106)	Alzheimer disease (n=74)
	Hazard ratio (95% CI)*	Hazard ratio (95% CI)*
Model 1†		
TSH (per SD)	0.93 (0.82; 1.06)	0.92 (0.78; 1.09)
fT4 (per SD)	1.21 (1.04; 1.40)	1.31 (1.14; 1.51)
T4 (per SD)	1.19 (0.99; 1.43)	1.22 (0.98; 1.52)
Model 2‡		
TSH (per SD)	0.89 (0.74; 1.07)	0.90 (0.71; 1.13)
fT4 (per SD)	1.20 (1.05; 1.37)	1.30 (1.14; 1.47)
T4 (per SD)	1.10 (0.87; 1.39)	1.14 (0.86; 1.52)

* Values are hazard ratios for dementia and Alzheimer disease per SD increase in thyroid hormone level (95% confidence interval)

† Model 1: age adjusted

‡ Model 2: age, albumin, educational level, depressive symptom score, body mass index, systolic and diastolic blood pressure, anti-arrhythmic and beta-blocking agent use adjusted

Stratification on follow-up duration showed the association was confined to participants who were diagnosed with dementia during exam 5 ($n = 71$) and absent in participants who were diagnosed with dementia during exam 6 ($n = 35$): the hazard ratio (95% CI) per SD increase in fT_4 for dementia detected during exam 5 was 1.29 (95% CI, 1.13;1.47) and for dementia detected during exam 6 was 0.89 (95% CI, 0.66; 1.22). Results were similar for T_4 and for AD.

The risk of dementia did not differ between participants with normal thyrotropin levels and those with an abnormally high or low thyrotropin level, but these analyses were limited by low numbers: three of the 26 participants with an abnormally high thyrotropin level at baseline developed dementia, whereas six of the 24 participants with an abnormally low thyrotropin level developed dementia.

Thyroid hormones and neuropathology

Thyrotropin and fT_4 were not associated with neuropathologic markers of dementia (Table 4). Higher levels of T_4 were associated with more neocortical NFT and NP. Per SD increase in T_4, the neocortical NFT count was 0.25 (95% CI, 0.05; 0.46) higher and the neocortical NP count was 0.22 (95% CI, -0.01; 0.44) higher, although the latter was non-significant. T_4 was not associated with hippocampal NFT and NP. These results were slightly strengthened after adjusting for potential confounders. Stratifying into participants who developed dementia and those who remained dementia-free did not change the results.

Table 4. Association of thyroid hormone levels with neuropathologic markers of dementia, the Honolulu-Asia Aging Study

	Neuropathologic markers Differences in autopsy measures per SD increase in thyroid hormone level (95% confidence interval)*			
	Hippocampal NFT	Neocortical NFT	Hippocampal NP	Neocortical NP
Model 1†				
TSH (per SD)	0.01 (-0.02 ; 0.04)	- 0.01 (-0.07 ; 0.04)	0.02 (-0.03 ; 0.06)	-0.01 (-0.06 ; 0.05)
FT4 (per SD)	0.11 (-0.05 ; 0.28)	0.15 (-0.18 ; 0.47)	-0.10 (–0.36 ; 0.15)	0.01 (-0.28 ; 0.29)
T4 (per SD)	0.10 (-0.03 ; 0.23)	0.25 (0.05 ; 0.46)	0.15 (-0.04 ; 0.34)	0.22 (-0.01 ; 0.44)
Model 2‡				
TSH (per SD)	0.01 (-0.02 ; 0.04)	-0.02 (-0.07 ; 0.03)	-0.02 (-0.07 ; 0.02)	0.00 (-0.06 ; 0.05)
FT4 (per SD)	0.08 (-0.09 ; 0.26)	0.19 (-0.16 ; 0.54)	-0.14 (-0.41 ; 0.14)	-0.06 (-0.36 ; 0.24)
T4 (per SD)	0.09 (-0.05 ; 0.22)	0.36 (0.15 ; 0.57)	0.16 (-0.05 ; 0.36)	0.31 (0.08 ; 0.54)

* Values represent adjusted differences in autopsy measures per SD increase in thyroid hormone level (95% confidence interval). NFT: neurofibrillary tangles, NP: neuritic plaques, CI: confidence interval.

† Model 1: adjusted for age at death

‡ Model 2: additionally adjusted for albumin, educational level, depressive symptom score, body mass index, systolic and diastolic blood pressure, anti-arrhythmic and beta-blocking agent use

DISCUSSION

In this population-based study of elderly men, higher levels of fT_4 and T_4 were associated with an increased risk for both dementia and AD. T_4 was also associated with more neurofibrillary tangles and neuritic plaques in the cerebral cortex, whereas fT_4 was not. Adjustment for potential confounding factors did not change the results.

Strengths of this study are its prospective population-based design, the six years of follow-up and the extensive diagnostic work-up for dementia including neuroimaging in most cases. In addition to the diagnosis of a clinical dementia syndrome, neuropathologic markers of AD were available in an autopsy series of the cohort. It should be noted, thyroid hormones were assayed in only one third of all study participants. However, we randomly selected participants for the assessments of thyroid hormones, and did not find any differences between the subgroups with and without thyroid hormone assessments. Further, the number of participants with (subclinical) hypo- and hyperthyroidism were thus quite small, limiting our analyses on participants with abnormal thyroid function.

Whereas in the Rotterdam Study an association between subclinical hyperthyroidism and dementia was found,[3] in this study thyrotropin was not related to clinically diagnosed AD or Alzheimer-type neuropathology. This was the case when analyzed continuously or when analyzed in strata of normal or abnormal thyroid function, although the analyses on high and low thyrotropin were limited due to low numbers. Thyrotropin values may however be altered by as much as 30% depending on time of day of phlebotomy, and the fasting or non-fasting status of the participant.[24] The absence of an effect of thyrotropin in

our study could thus in part be due to differences in time of blood collection or in certain characteristics of the population studied. Moreover, a blunted response of thyrotropin to thyrotropin-releasing hormone has been reported in elderly with major depression or AD,[25] indicating that in these conditions thyrotropin does not always adequately reflect thyroid function.[26] Alzheimer cases with a blunted thyrotropin response however did have higher mean T_4 levels than cases with a normal thyrotropin response.[25] Although thyrotropin levels were somewhat lower in the prevalent dementia cases compared to non-demented participants, this was not significant.

Whereas thyrotropin was not associated with AD in our study, both T_4 and fT_4 were associated with an increased risk. This association was restricted to cases detected during exam 5 in whom thyroid hormones were assessed up to only 3 years before the clinical threshold of dementia. This is in line with findings from the Rotterdam Study and the Rotterdam Scan Study where thyroid function was also only associated with the short-term risk of dementia but not with longer-term risk. The absence of an association at longer follow-up duration suggests that higher thyroid hormone levels reflect subclinical dementia rather than a contributing factor.

Subclinical dementia might lead to higher thyroid hormone levels through several mechanisms. First, higher T_4 levels may be due to neurodegeneration in participants with subclinical dementia. The hippocampus, a structure in the medial temporal lobe of the brain, is involved early in Alzheimer pathogenesis and has been shown to be reduced in volume on brain imaging up to six years before clinical detection of AD.[27] The hippocampus is involved in the setting of the basal activity of the thyroid axis through hippocampal-hypothalamic connections. By decreasing thyroid-hormone-releasing hormone gene expression in the hypothalamus, the hippocampus exerts a negative effect on this axis.[28] If the affected hippocampus in AD leads to less feedback on the hypothalamo-pituitary-thyroid axis, higher levels of fT_4 could be a consequence. The finding that higher serum fT_4 levels are associated with smaller hippocampal volumes on MRI scans of non-demented elderly,[4] may offer support for this hypothesis, although in the same study thyroid hormone levels were not associated with risk of dementia during six years of follow-up.

Second, higher T_4 levels may result from dementia through concomitant non-thyroidal illness. Evaluation of thyroid function in the elderly is complicated by an increased prevalence of non-thyroidal illness,[29] in which thyroid hormone and thyrotropin concentrations are altered, without overt thyroid dysfunction being present. Several conditions including malnutrition, starvation, and inflammatory processes accompanying disease are associated with non-thyroidal illness. In these situations, T_4 is converted preferentially to reverse T_3 instead of T_3.[30] The finding that not only fT_4 but also higher levels of reverse T_3 were found to be associated with smaller hippocampal volume on MRI of non-demented elderly[4] indeed suggests that this may be an important mechanism. Since

both T_3 and reverse T_3 were not measured in our study, we were not able to adjust for non-thyroidal illness. The fact that results remained unaltered after adjusting for potential other confounders, argues at least in part against an effect of comorbidity, although residual confounding by other measures influencing both thyroid hormone levels and our outcome measures cannot be excluded.

The autopsy results support the association between T_4 and AD, given the higher count of neocortical NFT and NP in participants with higher T_4. Yet the absence of an association of T_4 with hippocampal NFT and NP, together with the absence of an association between the other measures of thyroid function and Alzheimer-type neuropathology, argues against an important role of thyroid hormones in developing AD. These findings provide further evidence for the association between thyroid hormones and dementia being the result rather than a cause of Alzheimer pathology.

To conclude, in our study of elderly Japanese-American men, higher thyroid function as indicated by increased levels of fT_4 and T_4 levels within the normal range was associated with an increased risk of dementia and AD within the first 3 years of follow-up but not with longer follow-up. In addition, higher levels of total T_4 were associated with Alzheimer-type neuropathology. Together our findings suggest that higher fT_4 and T_4 levels are more likely to be a consequence rather than contributing to AD.

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Chapter 3

Steroid hormones and risk of dementia

Chapter 3.1

Endogenous testosterone and risk of dementia

ABSTRACT

Context: *Low testosterone levels have been associated with Alzheimer disease (AD) in elderly men, but may result from rather than contribute to developing AD.*
Objective: *To determine whether endogenous testosterone levels are related to risk of AD in elderly men and women.*
Design and participants: *Within the Rotterdam Study, a population-based prospective cohort study among 7,983 elderly, we performed a case-cohort study.*
Main outcome measures: *Serum levels of total and bioavailable testosterone were measured in a random subcohort of 634 men and 718 women and in an additional 46 men and 85 women diagnosed with dementia during follow-up (mean 9.3 years). In a subset of 455 non-demented elderly (234 men, 221 women) we assessed hippocampal and amygdalar atrophy, markers of early AD, on brain magnetic resonance imaging (MRI).*
Results: *Cox proportional hazards models showed that in men, total testosterone level was not related to the risk of AD. Higher levels of bioavailable testosterone were associated with a decreased risk of AD within 2 years from baseline, whereas no relation was found during longer follow-up (maximum 14.1 years). In line, total and bioavailable testosterone levels were not related to hippocampal or amygdalar atrophy on MRI. No associations were found in women. Additional adjustment or stratification by apolipoprotein E genotype or smoking did not change results.*
Conclusion: *Higher bioavailable testosterone levels are associated with a decreased short-term risk of AD in elderly men, but not with the longer-term risk. This suggests that low testosterone levels result from rather than contribute to AD.*

INTRODUCTION

Endogenous testosterone levels decrease in older men.[1] Low levels of testosterone are related to loss of muscle strength and lean body mass, and possibly also to decreased bone density, loss of insulin sensitivity and reduced sexual function.[2] Low levels of testosterone have been suggested to increase also the risk of cognitive decline and dementia.

A relation between testosterone and the risk of dementia is biologically plausible. Androgen receptors, which mediate the effects of testosterone, are present in multiple brain areas and their density is particularly high in the medial temporal lobe,[3] the region that is affected by Alzheimer pathology early in the disease course.[4] Laboratory studies suggest that testosterone reduces β-amyloid formation from the amyloid precursor protein,[5] β-amyloid neurotoxicity[6] and hyperphosphorylation of tau protein,[7] that are considered pathophysiological hallmarks of Alzheimer disease (AD). Observational studies reported lower levels of bioavailable or free testosterone both in men[8-11] and women[10] with AD compared with age-matched controls. In addition, prospective findings from the Baltimore Longitudinal Study of Aging showed lower free testosterone to be associated with an increased risk of AD among elderly men.[12]

However, low testosterone may also result from co-morbidity,[13] which might explain the association with dementia.[14] Bioavailable and free testosterone levels are lower in conditions that increase sex hormone-binding globulin (SHBG) levels, including wasting and subclinical hyperthyroidism.[15] Levels of SHBG were reported to be increased both in men[8,10] and women[16] with AD and could therefore explain associations between levels of testosterone and AD.[10]

At present, it is unclear whether low testosterone results from co-morbidity associated with dementia, or contributes to Alzheimer pathology. Brain atrophy, particularly of medial temporal lobe structures including the hippocampus and amygdala, is visible on magnetic resonance imaging (MRI) of the brain years before a clinical diagnosis of AD[17] and specifically reflects neuronal loss and neurofibrillary tangles.[18] We therefore investigated the association of testosterone and SHBG with risk of dementia, including AD, as well as severity of hippocampal and amygdalar atrophy on brain MRI as putative early pathological markers of AD in older men and women. We used data from the Rotterdam Study, a large population-based prospective cohort study in the Netherlands.

METHODS

Study Population

This study was based on the Rotterdam Study, a large prospective population-based study conducted among 7,983 elderly aged 55 years or older, that aims to assess determinants

of diseases in the elderly.[19] The study was conducted in accordance with the tenets of the Declaration of Helsinki. The Medical Ethics Committee of Erasmus Medical Center, The Netherlands, approved the study. Baseline examinations took place between 1990 and 1993. A research assistant visited participants at home and obtained information on medical history, medication use and determinants of diseases. In addition participants were invited to visit the study center for clinical examinations. At the baseline clinical examination, blood samples were drawn from 7,050 participants of whom 7,047 underwent screening for dementia. Prevalent dementia was diagnosed in 334 of these, who were excluded from the analyses in our current study. This resulted in a cohort of 6,713 subjects who had blood samples taken and were at risk for incident dementia. Follow-up examinations were conducted between 1993-1994, 1997-1999 and 2002-2004. In addition, through linkage with records of general practitioners, the total cohort was continuously monitored for major disease outcome. This resulted in a virtually complete follow-up until January 1, 2005.

Between 1995 and 1996, we randomly selected 965 living members of the Rotterdam Study for participation in the Rotterdam Scan Study, which was designed to investigate determinants and consequences of age-related brain changes in the elderly.[20] Individuals who were demented, blind, or had brain Magnetic Resonance Imaging (MRI) contraindications were excluded. Among 832 eligible, 563 participants gave their written informed consent to participate (response rate 68%). Complete MRI data, including a three-dimensional (3D) volumetric MRI sequence, was obtained in 511 non-demented participants.

Study design

For reasons of efficiency, a case-cohort design[21] was used. In this design, a random sample, or 'subcohort', is drawn from the source population that is followed up for disease outcome. Subjects from the source population who develop the disease outside the subcohort are selected as additional cases and added to the analyses. Baseline exposure (in our study, testosterone) is measured in the cases and controls included in the subcohort and in the additional cases. In 2000, a random subcohort of 1,352 subjects was drawn from our cohort at risk (Fig.). In the subcohort, we identified 125 patients (43 men, 82 women) who developed dementia during follow-up until January 1, 2005. In addition, we added 131 patients (46 men, 85 women) who had developed dementia during follow-up outside the subcohort. Of the total of 256 incident dementia patients (89 men, 167 women), AD was diagnosed in 191 subjects (55 men, 136 women), vascular dementia in 40 subjects (20 men, 20 women) and 25 patients were diagnosed with dementia due to other causes including dementia in Parkinson disease, multisystem atrophy, frontotemporal dementia, and Lewy body dementia.

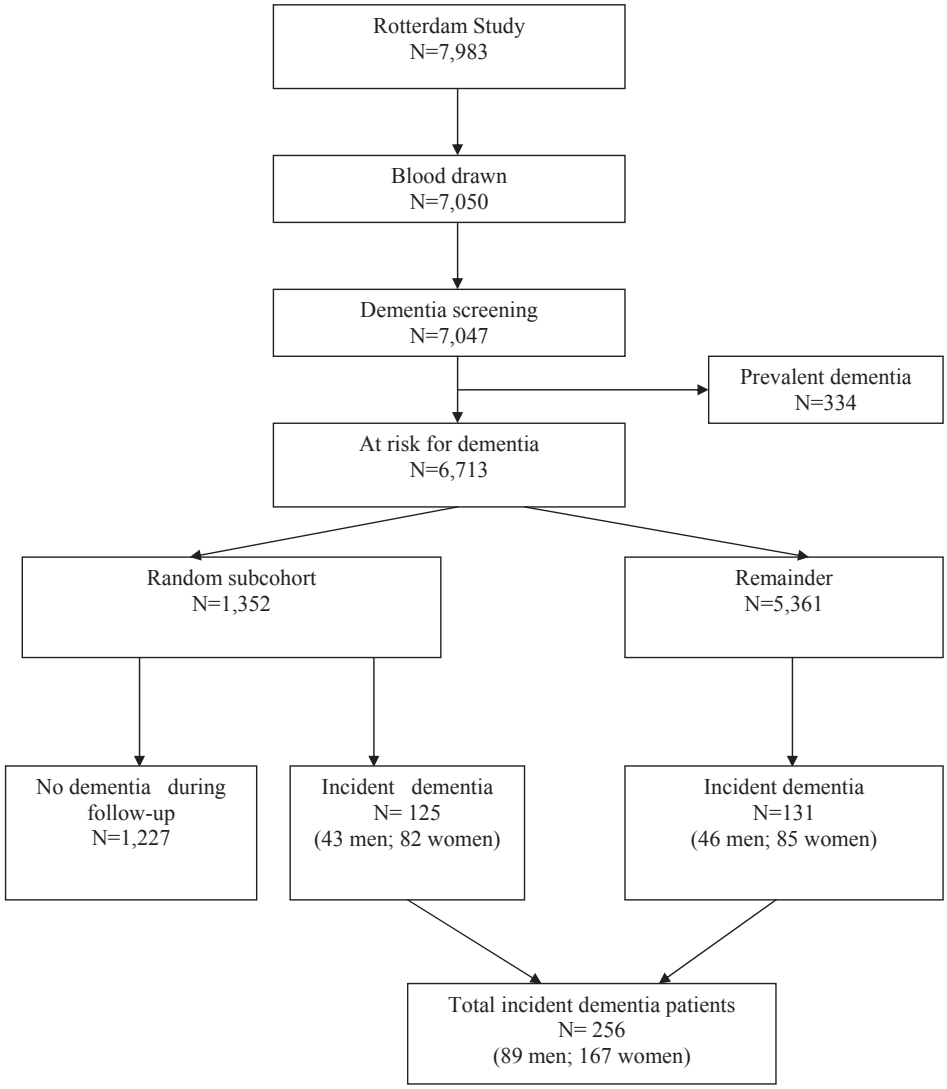

Figure. Description of the study population

In addition, testosterone levels were available in 455 of the 511 participants with complete MRI data.

Ascertainment of incident dementia

All participants were free of dementia at baseline and the cohort was followed up for incident dementia. The diagnosis of dementia was made following a three-step protocol.[22] Two brief tests of cognitive function (Mini-Mental State Examination (MMSE)) [23] and Geriatric Mental State schedule (GMS)[24] organic level were used to screen all

participants. Screen-positives (MMSE score < 26 or GMS organic level > 0) underwent the Cambridge examination for mental disorders of the elderly (Camdex).[25] Participants who were suspected of having dementia were examined by a neuropsychologist if additional neuropsychological testing was required for diagnosis. When available, imaging data were used to assess subtype of dementia. In addition, the total cohort was continuously monitored for incident dementia through computerized linkage between the study database and digitalized medical records from general practitioners and the Regional Institute for Outpatient Mental Health Care.[22] The diagnosis of dementia was made in accordance with internationally accepted criteria for dementia (DSM-III-R),[26] AD (NINCDS-ADRDA)[27] and vascular dementia (NINDS-AIREN),[28] by a panel of a neurologist, neuropsychologist and research physician.

MRI acquisition

Brain scans were performed using a 1.5-Tesla MRI scanner (VISION MR, Siemens AG, Erlangen, Germany). The scanning protocol included a sequence of proton-density images, a sequence of T2-weighted images, and a sequence of T1-weighted images. For volumetric measurement of the hippocampus and amygdala, a custom-made, inversion-recovery double-contrast 3D half-Fourier acquisition single-shot turbo spin echo (HASTE) sequence was included (inversion time 440 ms, [TR] 2800 contiguous sagittal slices of 1.2-mm, matrix 192x256, field of view 256x256). Two HASTE modules were sequentially acquired after the inversion pulse (effective TEs of 29 ms and 440 ms), of which the first was used for volume measurements.

Hippocampal and amygdalar volume measurement

Coronal brain slices (contiguous 1.5 mm slices) were reformatted from the 3D MRI sequence and aligned to be perpendicular to the long axis of the hippocampus. The procedure of segmenting the hippocampus and amygdala has been described.[17] The left and right hippocampus and amygdala were manually outlined on each slice with a mouse-driven pointer. The areas on each side were multiplied with slice thickness and left and right side were summed to yield estimates of absolute volume (ml). As a proxy for head size, we measured midsagittal area (cm^2) by tracing the inner skull on a reformatted middle sagittal area MRI slice. Head size differences across individuals were corrected for by dividing the uncorrected volumes by the subject's calculated head size area and subsequently multiplying this ratio by the average head size area (men and women separately).

Hormone assessments

Non-fasting blood samples were drawn at the baseline examination (1990-1993) in the research center between 8.30 and 16.00 h. Platelet poor plasma was frozen in liquid

nitrogen and stored at –80 C until the hormone measurements in 2000. Testosterone and SHBG were determined by direct immunoassays. Testosterone was estimated in single measurements by radio-immuno assay using coated tubes (detection limit 2.8 ng/dL). This assay has been regularly checked in external quality control schemes, using liquid chromatography/dual mass spectroscopy (LC/MS-MS). Testosterone values obtained with our assay are highly comparable to those obtained with LC/MS-MS. For 31 samples with testosterone concentrations between 4 and 25 nmol/l we obtained the following regression line: immunoassay = 0.92 * LC/MS-MS + 0.17, with a correlation coefficient of 0.959. SHBG was measured in duplicate using double antibody RIA (both from Diagnostic Systems Laboratories, Webster, TX). As measures of biologically active testosterone, the free fraction of testosterone and non-SHBG-bound (bioavailable) testosterone were calculated according to the method described by Södergård et al.[29] Calculated levels of free or bioavailable testosterone levels are considered most reliable, when compared to the free androgen index in which total testosterone is divided by SHBG or direct measurement of free levels by analog assays.[30] Albumin was measured by photometry (Roche, Mannheim, Germany).

Intra-assay coefficients of variation, determined on the basis of duplicate results of internal quality control serum pools with three different levels of analyte were 9.4% for testosterone and 4.5% for SHBG. Inter-assay coefficients of variation were 19% for testosterone and 14% for SHBG. Results of all batches were therefore normalized by multiplying all concentrations within a batch by a factor, a method which equalized results for the internal quality-control pools. This was considered justified because the results of these pools and the mean results for male and female sera in each assay batch showed very similar patterns, and has been described previously.[31] This resulted in inter-assay variation coefficients in the same order of magnitude compared to the intra-assay variation.

The evaluation of testosterone in older men and women is complicated, because the decrease in testosterone levels at higher age is paralleled by an increase of SHBG.[1] SHBG is also increased in thyrotoxicosis, in smokers, and liver cirrhosis. In contrast, low SHBG levels have been associated with markers of cardiovascular disease, hyperinsulinism and metabolic syndrome, and an increased risk of diabetes and cardiovascular mortality. The measurement of SHBG in clinical practice has therefore been suggested to be useful not only for the correct interpretation of serum levels of testosterone, but also for the assessment of peripheral effects of hormones regulating SHBG production, and the evaluation of insulin resistance and cardiovascular risk.[32]

Covariates

Educational level, smoking habits, use of systemic glucocorticoids and hormone replacement therapy were assessed during the baseline interview. Level of education was dichotomised into primary education (with or without a higher not completed education)

versus lower vocational to university education. Smoking was categorized as current, former and never smoking. Height and weight were measured at the research center and the body mass index (BMI) was calculated by dividing body weight (kg) by height squared (m^2). Diabetes mellitus was considered present if participants reported use of antidiabetic medication or when the random or a post-load serum glucose level of 202 ng/dl or greater. Plasma creatinine levels were assessed using an automated enzymatic procedure (Roche, Mannheim, Germany). Apolipoprotein E (*APOE*) genotype was assessed on coded DNA samples using polymerase chain reaction without knowledge of the dementia diagnosis.[33] Participants were categorized on the basis of the presence or absence of the apolipoprotein ε4 (*APOE* ε4) allele.

Statistical analysis

Baseline characteristics of the men and women in the subcohort and the subset with MRI were compared with the total cohort at risk by means of analysis of variance for continuous variables (one-way ANOVA) and χ^2 statistics for categorical variables. The association of testosterone and SHBG with dementia and subtypes of dementia was evaluated in a case-cohort design using Cox proportional hazards models with modification of the standard errors based on robust variance estimates. In the case-cohort analysis, only subjects from the random cohort contribute to the follow-up time. All dementia-cases are counted in the Cox model, yet only the dementia cases that developed within the subcohort contribute time at risk. We used the method according to Barlow in which the random subcohort is weighted by the inverse of the sampling fraction.[21] The sampling fraction consists of the number of persons in the subcohort with measurements of testosterone (or SHBG) divided by the number of people in the source population. The size of the sampling fraction differed for men and women and the type of testosterone (total or bioavailable) or SHBG. All analyses were performed in men and women separately. First, the analyses were performed with adjustment for age (used as the time scale) and second with additional adjustment for education, BMI, smoking, diabetes, creatinine and albumin. Testosterone or SHBG concentration was represented in the model either by two dummy variables for the middle and highest tertiles or by a linear term (per standard deviation (SD)). The linear term was expressed per SD increase to compare the associations across different measures of testosterone. In the proportional hazards models, entry time was defined as age at study entry. Participants were followed until age at diagnosis of dementia, age at death or age at end of study, whichever came first. To see whether hormone levels might have changed due to subclinical dementia, subsequent analyses were performed separately on cases identified within the first 2 years and those identified after the first 2 years of follow-up duration. In the subset with MRI examinations, analyses of covariance were used to compute adjusted means of hippocampal and amygdalar volumes on MRI.

In additional analyses we excluded subjects using systemic glucocorticoids (8 men, 13 women) and hormonal replacement therapy, that was used by women only ($n = 16$). Smoking has been associated with an increased risk of dementia,[34] and has been related to higher testosterone and SHBG concentrations in elderly men.[35] We therefore also performed the analyses separately in smokers and those who never smoked or were former smokers. The ε4 allele of apolipoprotein E is an important genetic risk factor for AD[36] and testosterone levels have been reported to be lower in non-demented elderly men who are carrier of the *APOE* ε4 allele.[37] In addition, the observed association between testosterone and cognitive function or AD in elderly men may be dependent on *APOE* ε4.[38] We therefore also performed the analyses within strata of *APOE* ε4 allele. Analyses were performed using SAS 8.2 (SAS, Cary, NC) and SPSS 11.0 (SPSS Inc., Chicago, Ill) software.

RESULTS

Baseline characteristics of men and women in the cohort at risk, the random subcohort and the subset with MRI examinations are shown in Table 1. Both for men and women

Table 1. Baseline characteristics of the total cohort at risk, the random cohort and the subset with MRI*

	Men			Women		
	Total cohort at risk	Random subcohort	Subset with MRI	Total cohort at risk	Random subcohort	Subset with MRI
Number (n)	2,743	556	234	4,000	669	221
Age (years)	68.0 (8.0)	68.7 (8.2)	68.5 (7.7)	69.6 (9.2)	70.4 (9.3)	68.7 (8.2)
Education (% primary education only)	26	28	22	45	43	38
Body mass index (kg/m^2)	25.7 (3.7)	25.6 (3.0)	25.9 (2.8)	26.8 (4.1)	26.4 (3.8)	26.5 (4.0)
Total cholesterol (mg/dL)	243.2 (46.3)	243.2 (42.5)	247.1 (42.5)	262.5 (46.3)	266.4 (46.3)	266.4 (46.3)
HDL cholesterol (mg/dL)	46.3 (11.6)	46.3 (11.6)	46.3 (11.6)	50.1 (15.4)	50.1 (15.4)	57.9 (11.6)
Diabetes (%)	10	9	6	10	7	5
Smoking (% current)	30	29	23	19	19	18
Creatinine (mg/dL)	0.99 (0.21)	0.99 (0.16)	0.99 (0.16)	0.86 (0.23)	0.85 (0.19)	0.70 (0.12)
Albumin (g/dL)	0.43 (0.28)	0.43 (0.27)	0.43 (0.27)	0.43 (0.26)	0.43 (0.26)	0.43 (0.26)
Use of hormone replacement therapy (%)	-	-	-	2	3	3
Use of systemic corticosteroids (%)	2	1	1	2	2	2
Total testosterone (ng/dL)	-	331.2 (115.3)	334.1 (118.1)	-	40.3 (23.0)	37.4 (25.6)
Bioavailable testosterone (ng/dL)	-	195.8 (80.6)	201.6 (86.4)	-	20.2 (11.5)	20.2 (11.5)
SHBG (mg/dL)†	-	13.8 (5.5)	13.7 (5.5)	-	17.6 (7.3)	17.5 (6.9)

* Values are means (standard deviations) or percentages

† Sex hormone-binding globulin

the random subcohort with testosterone measurements was not different from the total cohort at risk. The groups with MRI examinations only differed from the overall cohort, in that diabetes was significantly less frequent among both men and women. Total and bioavailable testosterone decreased with age, whereas SHBG showed an increase with age, both in men and women.

The associations of total and bioavailable testosterone and SHBG with dementia and its subtypes in men are presented in Table 2. In the overall analyses, neither total, nor bioavailable testosterone was associated with the risk of dementia and its major subtypes. Men in the upper tertile of SHBG had a higher risk of overall dementia compared with men in the lowest tertile, although this was non-significant. When we entered SHBG concentration as a continuous variable, per SD increase in SHBG the risk of dementia increased by 35%. Subtype analyses showed that this increase was confined to AD, where each SD increase in SHBG increased the risk by 54%, whereas no relation was found with vascular dementia. After additional adjustment for education, BMI, total and HDL cholesterol, smoking, diabetes, creatinine and albumin the association between SHBG and dementia attenuated and became non-significant, whereas the association with AD also attenuated but remained significant. Because of the small number of cases, we could

Table 2. Hazard ratios (with corresponding 95% Confidence Intervals) of the association of total and bioavailable testosterone and sex-hormone binding globulin with risk of dementia and its subtypes in men

	Dementia			Alzheimer disease			Vascular dementia	
Testosterone	No. of cases	HR*	HR†	No. of cases	HR*	HR†	No. of cases	HR*
Total								
Lowest tertile	35	1.00 (ref.)	1.00 (ref.)	19	1.00 (ref.)	1.00 (ref.)	11	1.00 (ref.)
Middle tertile	29	0.98 (0.78; 1.35)	0.92 (0.52; 1.65)	17	0.99 (0.48; 2.05)	0.91 (0.43; 1.94)	7	0.82 (0.30; 2.27)
Highest tertile	25	1.32 (0.72; 1.41)	1.20 (0.64; 2.26)	19	1.81 (0.89; 3.72)	1.51 (0.70; 3.24)	2	0.43 (0.09; 2.00)
Per SD	89	1.03 (0.78; 1.35)	0.98 (0.73; 1.32)	55	1.13 (0.38; 1.63)	1.04 (0.68; 1.59)	20	0.69 (0.45; 1.06)
Bioavailable								
Lowest tertile	30	1.00 (ref.)	1.00 (ref.)	16	1.00 (ref.)	1.00 (ref.)	10	1.00 (ref.)
Middle tertile	25	0.73 (0.40; 1.34)	0.75 (0.40; 1.39)	15	0.80 (0.32; 1.97)	0.83 (0.33; 2.09)	6	0.96 (0.32; 2.90)
Highest tertile	18	0.84 (0.38; 1.86)	0.89 (0.39; 2.00)	13	0.49 (0.22; 1.06)	0.47 (0.20; 1.08)	2	0.59 (0.10; 3.57)
Per SD	73	0.85 (0.61; 1.17)	0.84 (0.59; 1.18)	44	0.82 (0.54; 1.25)	0.79 (0.49; 1.27)	18	0.77 (0.40; 1.48)
SHBG‡								
Lowest tertile	30	1.00 (ref.)	1.00 (ref.)	16	1.00 (ref.)	1.00 (ref.)	10	1.00 (ref.)
Middle tertile	25	0.83 (0.38; 1.79)	0.82 (0.36; 1.83)	15	0.85 (0.33; 2.20)	0.83 (0.31; 2.22)	6	0.53 (0.12; 2.39)
Highest tertile	18	1.47 (0.71; 3.04)	1.22 (0.57; 2.62)	13	1.69 (0.72; 3.97)	1.25 (0.51; 3.12)	2	0.81 (0.22; 2.92)
Per SD	73	1.35 (1.01; 1.81)	1.30 (0.96; 1.77)	44	1.54 (1.12; 2.11)	1.47 (1.02; 2.09)	18	0.83 (0.44; 1.56)

* Adjusted for age

† Adjusted for age, education, body mass index, smoking, diabetes, albumin and creatinine

‡ SHBG: Sex hormone-binding globulin

not examine the associations of hormones with vascular dementia in the fully adjusted model. Among men, separate analyses on short-term and longer-term follow-up duration showed that bioavailable testosterone levels were associated with a diagnosis of dementia ($n = 19$) and AD ($n = 11$) during short-term follow-up (maximum follow-up 2 years), whereas no associations were found during longer-term follow-up (maximum follow-up 14.1 years). Results for AD are presented in Table 3. Per SD increase in bio-

Table 3. Hazard ratios (with corresponding 95% Confidence Intervals) of the association of total and bioavailable testosterone and sex-hormone binding globulin with risk of Alzheimer disease in men according to time-to-event

	Short follow-up (median 1.0, range 0.1-2.0 years)			Long follow-up (median 10.7, range 0.1-14.1 years)		
	No. of cases	HR*	HR†	No. of cases	HR*	HR†
Total testosterone (per SD)	16	0.85 (0.43; 1.71)	0.56 (0.20; 1.58)	39	1.26 (0.82; 1.93)	1.23 (0.75; 2.05)
Bioavailable testosterone (per SD)	11	0.38 (0.17; 0.86)	0.22 (0.08; 0.63)	33	1.03 (0.65; 1.61)	0.98 (0.59; 1.62)
Sex hormone-binding globulin (per SD)	11	1.67 (0.94; 2.95)	1.62 (0.62; 4.20)	33	1.52 (1.05; 2.20)	1.51 (0.84; 2.10)

* Adjusted for age

† Adjusted for age, education, body mass index, smoking, diabetes, albumin and creatinine

Table 4. Hazard ratios (with corresponding 95% Confidence Intervals) of the association of total and bioavailable testosterone and sex-hormone binding globulin with risk of dementia and its subtypes in women

	Dementia			Alzheimer disease			Vascular dementia	
Testosterone	No. of cases	HR*	HR†	No. of cases	HR*	HR†	No. of cases	HR*
Total								
Lowest tertile	46	1.00 (ref.)	1.00 (ref.)	43	1.00 (ref.)	1.00 (ref.)	9	1.00 (ref.)
Middle tertile	54	1.01 (0.64; 1.63)	0.95 (0.78;1.55)	43	1.07 (0.65; 1.76)	0.98 (0.58; 1.67)	6	0.91 (0.29; 2.84)
Highest tertile	57	1.17 (0.76; 1.83)	1.13 (0.71-1.82)	50	1.31 (0.82; 2.10)	1.31 (0.79; 2.19)	5	0.66 (0.19; 2.14)
Per SD	167	1.10 (0.94; 1.30)	1.09 (0.92-1.30)	136	1.11 (0.94; 1.32)	1.11 (0.93; 1.32)	20	0.98 (0.62; 1.67)
Bioavailable								
Lowest tertile	49	1.00 (ref.)	1.00 (ref.)	38	1.00 (ref.)	1.00 (ref.)	8	1.00 (ref.)
Middle tertile	45	0.92 (0.57; 1.49)	0.89 (0.52; 1.50)	36	1.01 (0.60; 1.69)	0.96 (0.54; 1.70)	5	0.82 (0.26; 2.61)
Highest tertile	40	1.09 (0.66; 1.81)	1.08 (0.62; 1.90)	33	1.13 (0.65; 1.97)	1.15 (0.62; 2.15)	5	0.96 (0.28; 3.31)
Per SD	134	1.18 (0.98; 1.41)	1.19 (0.97; 1.46)	107	1.15 (0.95; 1.40)	1.18 (0.95; 1.47)	18	1.20 (0.79; 1.91)
SHBG‡								
Lowest tertile	49	1.00 (ref.)	1.00 (ref.)	38	1.00 (ref.)	1.00 (ref.)	8	1.00 (ref.)
Middle tertile	45	1.06 (0.61; 1.86)	1.11 (0.63; 1.96)	36	0.93 (0.51; 1.70)	0.99 (0.53; 1.84)	5	2.30 (0.47; 11.1)
Highest tertile	40	1.13 (0.66; 1.97)	1.32 (0.75; 2.34)	33	1.04 (0.59; 1.86)	1.17 (0.63; 2.17)	5	1.68 (0.35; 8.27)
Per SD	134	0.96 (0.76; 1.32)	1.04 (0.81; 1.33)	107	0.97 (0.76; 1.25)	1.02 (0.78; 1.34)	18	0.92 (0.49; 1.70)

* Adjusted for age

† Adjusted for age, education, body mass index, smoking, diabetes, albumin and creatinine

‡ SHBG: Sex hormone-binding globulin

Table 5. Associations between total and bioavailable testosterone levels, sex hormone-binding globulin and hippocampal and amygdalar volumes in men and women*

	Hippocampal volume (ml)		Amygdalar volume (ml)	
	Men	Women	Men	Women
Total testosterone				
Lowest tertile	0.0 (ref.)	0.0 (ref.)	0.0 (ref.)	0.0 (ref.)
Middle tertile	-0.13 (-0.41; 0.15)	-0.09 (-0.35; 0.17)	0.03 (-0.20; 0.27)	-0.08 (-0.29; 0.14)
Highest tertile	-0.15 (-0.43; 0.13)	-0.16 (-0.42; 0.10)	0.10 (-0.14; 0.34)	-0.14 (-0.35; 0.07)
Per SD increase	-0.01 (-0.14; 0.12)	-0.03 (-0.17; 0.10)	-0.002 (-0.12; 0.11)	-0.05 (-0.17; 0.06)
Bioavailable testosterone				
Lowest tertile	0.0 (ref.)	0.0 (ref.)	0.0 (ref.)	0.0 (ref.)
Middle tertile	-0.16 (-0.47; 0.15)	-0.16 (-0.44; 0.12)	0.03 (-0.23; 0.29)	-0.09 (-0.32; 0.14)
Highest tertile	-0.22 (-0.55; 0.10)	-0.17 (-0.45; 0.11)	-0.007 (-0.28; 0.26)	-0.10 (-0.33; 0.13)
Per SD increase	-0.02 (-0.17; 0.13)	-0.06 (-0.18; 0.07)	0.005 (-0.12; 0.13)	-0.09 (-0.19; 0.01)
Sex hormone-binding globulin				
Lowest tertile	0.0 (ref.)	0.0 (ref.)	0.0 (ref.)	0.0 (ref.)
Middle tertile	-0.22 (-0.54; 0.08)	-0.13 (-0.41; 0.15)	-0.03 (-0.29; 0.23)	-0.08 (-0.31; 0.15)
Highest tertile	-0.27 (-0.59; 0.05)	-0.12 (-0.40; 0.16)	-0.07 (-0.34; 0.21)	-0.10 (-0.34; 0.13)
Per SD increase	0.06 (-0.06; 0.18)	0.05 (-0.07; 0.17)	-0.09 (-0.11; 0.09)	0.09 (-0.03; 0.20)

* Values are age-adjusted mean differences (95% Confidence Intervals)

available testosterone we observed a 60% decreased risk for AD during short-term follow up. The observed increased risk of AD among men with higher SHBG levels was highest at shorter follow-up, although this was statistically not significant. Total testosterone was not related to the risk of dementia, regardless of follow-up duration. The associations of total and bioavailable testosterone and SHBG with dementia and subtypes of dementia in women are presented in Table 4. No associations between either testosterone or SHBG levels with risk of dementia were found in women, regardless of follow-up duration.

The age-adjusted associations of total and bioavailable testosterone and SHBG with atrophy of the medial temporal lobe on MRI are shown in Table 5. Neither testosterone nor SHBG levels were associated with hippocampal and amygdalar atrophy on MRI in non-demented elderly men. Similarly, no associations were found in non-demented elderly women. Additional adjustment for other confounders did not change the results.

Exclusion of subjects using systemic glucocorticoids and hormonal replacement therapy, or stratification by smoking or apolipoprotein E genotype did not markedly change the results, neither for men nor for women. The interaction terms between smoking or *APOE* genotype and concentrations of testosterone and SHBG levels were all not significant ($p>0.3$).

DISCUSSION

In our study, lower bioavailable testosterone levels were associated with an increased short-term risk of dementia and AD in elderly men, but not with longer-term risk. Higher SHBG levels were also most strongly associated with an increased risk of dementia and AD during short-term follow-up in elderly men. Total testosterone was not associated with dementia, and neither testosterone nor SHBG were related to hippocampal and amygdalar atrophy on MRI in non-demented elderly men. No associations were observed in women.

The major strengths of this study are the population-based design, the long follow-up for incident dementia and the large number of volumetric MRI assessments of the hippocampus and amygdala. Furthermore, the follow-up with respect to dementia was virtually complete and selection bias unlikely. Unfortunately, because of the small volumes of plasma available, we were not able to run the hormone assays in duplicate. Single-sample measurements yield less precise estimations than duplicate measurements, which may have resulted in an underestimation of effects. In addition, blood samples were stored for almost 10 years before the hormone assays were performed. This may have affected the stability of the samples and the reliability of the results. Because storage of plasma at –80°C over a longer period of time has been shown to minimally affect serum levels of testosterone as well as the ranking order of individuals based on their hormone levels over time,[39] it is unlikely that the long period of storage biased our results. Finally, our testosterone assessments were based on a single blood sample drawn at the study baseline between 1990 and 1993, which is a limitation of our study. Multiple testosterone assessments would have more precisely characterized the prevailing androgenic state of the study participants. Although this will not have introduced a systematic bias, it may have increased the random error and thereby decreased the power to find an association should one exist. The absence of multiple prospective measures of testosterone also excluded the ability to examine testosterone at different points in time prior to dementia diagnosis for each case separately.

The observation that lower bioavailable testosterone levels are associated with short-term risk of AD in elderly men, is in keeping with previous reports that showed bioavailable or free testosterone levels to be lower in male Alzheimer cases when compared to controls.[10,11] The absence of an association between testosterone levels and longer-term risk of AD in our study is in agreement with findings from the Honolulu-Asia Study showing no relation between bioavailable testosterone and risk of AD among elderly men during 6 years of follow-up.[40] Yet, these results contradict findings from the Baltimore Longitudinal Study of Aging, in which lower biovailable testosterone was associated with a decreased risk of AD among elderly men during a mean follow-up of 19 years.[12] The latter finding may indicate that hypogonadism precedes the onset of dementia, yet

total testosterone was normal in those who developed AD.[12] Moreover, in the Baltimore Longitudinal Study of Aging bioavailable testosterone was estimated by means of the free androgen index, which is expressed as the ratio of total testosterone over SHBG. Therefore, both total testosterone and SHBG may have contributed to the significant findings for the free androgen index. The observation of low bioavailable rather than total testosterone preceding dementia suggests an important role of SHBG. It has therefore been suggested that increased SHBG rather than decreased testosterone contributed to the association between free testosterone and AD.[14] In our study, indeed higher SHBG rather than lower testosterone was associated with an increased risk of dementia and AD during overall follow-up in elderly men. Additional adjustment for factors increasing SHBG levels, including smoking and low BMI, attenuated these findings. Although the increased risk for AD remained significant, residual confounding cannot be excluded. Adjustment for in particular thyroid function may be relevant as subclinical hyperthyroidism has been related to both higher SHBG levels[11] and risk of AD.[41] Thyroid function was, however, not routinely assessed in all participants. In addition, the increased risk of dementia associated with higher SHBG levels was particularly high during the first years of follow-up. Although this was statistically not significant, this may be due to the relatively low number of dementia cases. Our finding is consistent with cross-sectional studies reporting increased levels of SHBG, in men with AD[8,16] and suggests that the associations between low testosterone levels and dementia could result from co-morbidity.[14] In line with this hypothesis, we found no relation between either testosterone or SHBG and atrophy of the medial temporal lobe on MRI in non-demented elderly men. Within the Honolulu-Asia Aging Study, testosterone and SHBG were also not associated with hippocampal atrophy on MRI[42] whereas higher SHBG and not testosterone was related to neurofibrillary tangles in an autopsy sub-sample.[43]

The lack of an association of testosterone with risk of dementia among women in our study might be explained by their lower testosterone values and greater variability thereof when compared to men, thus reducing the possibility to observe an association in women.

To summarize, both lower bioavailable testosterone levels and higher SHBG levels were associated with an increased short-term risk of dementia, including AD, yet not with the longer-term risk in elderly men. The absence of an association of total testosterone with dementia, suggests that higher SHBG levels rather than lower testosterone levels contribute to the observed association, which may reflect comordity in imminent dementia. In line with these findings, neither testosterone nor SHBG levels were related to medial temporal lobe atrophy on MRI in non-demented elderly that may mark early AD. Altogether, our findings suggest that low testosterone levels result from comorbidity associated with AD rather than being involved in Alzheimer pathology in elderly men.

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Chapter 3.2

Glucocorticoid receptor variant and risk of dementia

ABSTRACT

Objective: *Elevated glucocorticoid levels are associated with dementia. A glucocorticoid receptor gene variant (ER22/23EK) is related to relative glucocorticoid resistance. We investigated whether the ER22/23EK allele is associated with dementia and structural brain abnormalities.*

Methods: *This study was performed in two prospective population-based cohort studies among elderly. The first study included 6,034 participants who were screened for dementia (mean follow-up 5.8 years). The second study included 1,011 elderly subjects with an MRI at baseline and follow-up. The ER22/23EK allele was assessed for association with dementia, cognitive function and white matter lesions.*

Results: *The ER22/23EK allele was associated with a decreased risk of dementia. Among non-demented participants, ER22/23EK-carriers had a better performance on psychomotor speed tests than non-carriers. No differences were found in memory function between genotypes. In addition, both presence and progression of white matter lesions was lower in ER22/23EK-carriers. No association was found with brain atrophy on MRI.*

Conclusions: *Our findings suggest a protective effect of the ER22/23EK allele on the risk of dementia and white matter lesions.*

INTRODUCTION

Glucocorticoids have a wide variety of effects on peripheral organs, as well as on brain physiology.[1] The glucocorticoid receptor (GR) is the major factor in the mediation of the effects of cortisol. Sensitivity to glucocorticoids between individuals is highly variable, whereas the intra-individual sensitivity is rather stable, suggesting that glucocorticoid sensitivity is determined by genetic factors.[2]

Previously, within the Rotterdam Study we reported several polymorphisms of the *GR* gene (NR3C1).[3] One of these polymorphisms consists of two linked single nucleotide mutations (GAGAGG→GAAAAG) in codons 22 and 23 in exon 2. The first mutation is silent, both codons code for glutamic acid (E). The second mutation, results in a change from arginine (R) to lysine (K).[3] Carriers of this ER22/23EK allele were found to be significantly more resistant to the effects of glucocorticoids than non-carriers.[4] We also found these ER22/23EK-carriers to have a better insulin sensitivity and lower cholesterol levels.[4] In a separate population of elderly men we reported the ER22/23EK allele to be associated with longevity, as well as lower C-reactive protein levels, possibly reflecting a beneficial cardiovascular profile.[5] Recently, the molecular mechanism by which the ER22/23EK allele led to decreased sensitivity to glucocorticoids was elucidated.[6]

No data have been reported concerning the role of the ER22/23EK allele in relation to cognitive function. HPA-axis overactivity, which is related to stress and leads to increased levels of cortisol,[7] has been associated with cognitive impairment and dementia.[8] In prospective studies among both Alzheimer disease (AD) patients and healthy elderly, higher plasma cortisol levels have been associated with decline in cognitive function over time.[9-11] Furthermore, HPA-axis overactivity is related to an increased vascular risk, including hypertension and obesity.[12,13] Increasing evidence suggests that cerebrovascular pathology is important in the etiology and clinical course of dementia and AD.[14] In this context, white matter lesions and brain infarctions on MRI have also been associated with cognitive function.[15,16]

We hypothesized a protective effect of the ER22/23EK allele with respect to risk of dementia. Therefore, we investigated the relationship between this allele and the risk of dementia and cognitive performance in the Rotterdam Study. In addition, we studied the relationship between this allele and both cognitive function and structural brain abnormalities on MRI in the Rotterdam Scan Study. The effects on brain structures could be either direct (less harmful cortisol effects due to relative glucocorticoid resistance), or indirect (due to a better metabolic status). In order to study direct cortisol effects on the brain, we tested memory function and measured hippocampal and amygdalar volumes on MRI, which have been shown to be directly affected by increased cortisol levels.[17] Indirect effects of a relative cortisol resistance were studied by psychomotor speed tests, as well as cerebral white matter lesions on MRI, which are related to vascular disease.[18]

METHODS

Study design

The Rotterdam Study is a population-based, prospective cohort study designed to study the frequency and determinants of chronic diseases in the elderly.[19]

All inhabitants of Ommoord, a district of Rotterdam, the Netherlands, aged 55 years and over including those living in institutions were invited, of whom 7,983 gave their written informed consent and participated in the study (response 78%). At baseline, 7,528 subjects were screened for dementia.[20] Of these, 483 were diagnosed to be demented. The cohort at risk of dementia thus comprised 7,045 subjects. Two follow-up examinations took place in 1993-1994 and 1997-1999. The total cohort was further continuously monitored for mortality and major morbidity. Follow-up for dementia was virtually complete (99.9%).

The Rotterdam Scan Study is a separate, prospective cohort study designed to investigate determinants and consequences of brain abnormalities on MRI in the elderly.[14] In 1995-1996 participants were randomly selected from the Rotterdam Study and the Zoetermeer study, another ongoing prospective cohort study in The Netherlands, after stratification by sex and age in 5-year age groups. Elderly with MRI contraindications or dementia at baseline were excluded. Complete information including a cerebral MRI scan was obtained in 1,077 participants (response 63%). A total of 951 participants, who were eligible for a second MRI examination, were re-invited in 1999-2000 of whom 668 participated (response rate 70%).

Both studies have been approved by the Medical Ethics Committee of Erasmus Medical Center, The Netherlands.

Dementia diagnosis

Case-finding and diagnostic procedures for dementia and AD have been described[21] and were equal for both studies. Both at baseline and follow-up examinations, a stepwise procedure was used. First, subjects were cognitively screened with the Mini-Mental State Examination (MMSE)[22] and the Geriatric Mental State (GMS) schedule organic level.[23] Second, if subjects scored below 26 on the MMSE or above 0 on the GMS organic level, the Cambridge Examination of Mental Disorders in the Elderly (CAMDEX),[24] including an informant interview, was administered. Finally, subjects suspected of having dementia were further examined by a neurologist, a neuropsychologist and, if possible, had an MRI of the brain. In addition, continuous monitoring of the cohort for incident dementia cases took place through computerized linkage between the study database and computerized medical records from general practitioners and through surveillance of Regional Institute for Outpatient Mental Health Care reports.[21] Dementia diagnoses were based on DSM-III-R criteria, AD and vascular dementia diagnoses were subsequently based on

the NINCDS-ADRDA and the NINDS-AIREN criteria respectively.[25-27] Final diagnoses were made based on all existing information by an expert panel including the neurologist, neuropsychologist and research physician.

Neuropsychological testing

In addition to the MMSE, which was administered in both studies, participants in the Rotterdam Scan Study underwent more detailed neuropsychological testing at baseline (1995-1996) including an abbreviated Stroop test, the Letter-Digit Substitution task (a modified version of the Symbol Digit Modalities Test), a verbal fluency test, a Paper-and-Pencil Memory Scanning Task and a 15-word verbal learning test (based on Rey's recall of words).[15] From these tests we constructed compound scores for psychomotor speed, memory performance, and global cognitive function by transforming individual test scores into standardized Z-scores.[15]

MRI procedures

Within the Rotterdam Scan Study, cranial MRI scanning was performed in all participants with 1.5-Tesla scanners at two study centers (Gyroscan, Philips NT, Best, The Netherlands or VISION MR, Siemens, Erlangen, Germany) using standard T1, T2 and proton-density weighted MR sequences. MRI acquisition parameters have been described.[15] For the 563 participants of the Rotterdam Study a custom-made double contrast 3D half-Fourier acquisition single-shot turbo spin echo (HASTE) sequence was added for volumetric assessments of the hippocampus and amygdala. In 1999-2000 all second MRI scans were made with the VISION MR scanner using the same sequences. All scan assessments were done by raters blinded to any clinical information related to the participants.

Hippocampal and amygdalar volumes. Hippocampal and amygdalar volumes were measured on coronal slices (1.5 mm, no interslice gap) reconstructed from the HASTE sequence to be perpendicular to the long axis of the hippocampus, as previously described.[28] Briefly, the left and right hippocampus and amygdala were manually traced on each slice by means of a mouse driven pointer and volumes (ml) were calculated by summing the areas multiplied by slice thickness. Total hippocampal or amygdalar volume was calculated by summing the left and right hippocampal or amygdalar volume. Midsagittal area (cm^2) was measured by tracing the inner skull to obtain a proxy for intracranial volume. Head size differences across individuals were corrected for by dividing the uncorrected volumes by the subject's calculated head size area and subsequently multiplying this ratio by the average head size area (men and women separately).

White matter lesions. At baseline, white matter lesions were assessed in all participants of the Rotterdam Scan Study ($n = 1,077$) and were considered present if visible as hyperintense on proton density and T2-weighted images, without prominent hypointensity on T1-weighted images and scored in periventricular (range 0-9) and subcortical white

matter regions (approximated volume, range 0 to 29.5 ml) on the proton density scans.[15] After 3 years, 668 participants underwent repeated MRI scanning. Change in periventricular and subcortical white matter lesion severity was rated with a semiquantitative scale, and progression was rated as no, minor or marked progression.[29]

Brain infarctions. We defined brain infarcts as focal hyperintensities on T2-weighted images, 3 mm in size or larger. Proton-density scans were used to distinguish infarcts from dilated perivascular spaces. Hyperintensities in the white matter also had to have corresponding prominent hypointensities on T1-weighted images, in order to distinguish them from cerebral white matter lesions.[16]

Genetic analysis

At baseline peripheral venous blood samples were drawn and genomic DNA was isolated from whole blood using standard techniques. Genotyping was performed by allelic discrimination using TaqMan Universal PCR master mix (Applied Biosystems, Nieuwerkerk aan den IJssel, The Netherlands), primers (forward: 5'-TCCAAAGAAT CATTAACTCCTGGTAGA-3'and reverse:5'-GCTCCTCCTCTTAGGGTTTTATAGAA G-3') and probes (Applied Biosystems) and a Taqman ABI Prism 7700 Sequence Detection System (Applied Biosystems). Used probes were 5'-FAM-ACATCTCCCCTCTCCT-GAGCAAGC-3' and 5'-VIC-ACATCTCCCTTTTCCTGA GCAAGCA-3' (Applied Biosystems). Reaction components and amplification parameters were based on the manufacturer's instructions using an annealing temperature of 60°C and optimized concentrations for primers and probes of 400 nmol/L and 100 nmol/L, respectively. We re-analyzed genotypes in 100 samples by PCR-RFLP analysis using the *Mnl*I restriction enzyme (New England Biolabs, Leusden, The Netherlands) and a digestion of 1 hour at 37°C and found identical genotypes.

Assessment of covariates

Covariates were assessed at the baseline examinations and were similar in both studies. Blood pressure was measured twice on the right arm with a random zero sphygmomanometer. We used the average of these two measurements. Diabetes mellitus was defined present if participants reported use of oral antidiabetic treatment or insulin, or if a random serum glucose level was greater than or equal to 202 ng/dL at baseline. Serum total cholesterol levels were determined using an automated enzymatic procedure. Body mass index (BMI) was calculated as weight divided by the square of height. Smoking habits were assessed with a structured questionnaire. Apolipoprotein-E (*APOE*) genotyping was performed on coded DNA samples without knowledge of the diagnosis. The PCR product was digested with the restriction enzyme *HhaI*, and fragments were separated by electrophoresis.[30]

Data analysis

First, we examined the relation between the ER22/23EK allele and dementia within the Rotterdam Study. The likelihood for ER22/23EK-carriers of being demented at baseline was assessed by means of logistic regression. The prospective relation with incident dementia was assessed with Cox proportional hazard models. Follow-up time was calculated from baseline until death, diagnosis of dementia, or end of follow-up, whichever came first. Age at onset of dementia was determined as age at diagnosis. Linear regression analysis was used to study the association between the allele and MMSE in both the Rotterdam Study and the Rotterdam Scan Study, and to analyze cognitive functioning in more detail using compound scores for neuropsychological tests in the Rotterdam Scan Study. Since the ER22/23EK allele has been associated with absence, rather than presence of disease, compound scores were further analyzed using logistic regression after dichotomization at the median level to compare relatively bad performers (below the median) with good performers (above the median).

Differences between the distribution of the ER22/23EK allele and structural abnormalities on MRI were studied in the Rotterdam Scan Study. Both measures of brain atrophy and white matter lesions were analyzed using analysis of covariance (ANCOVA). We used logistic regression to investigate the possible association with the presence of a brain infarct on the baseline scan, and - since we hypothesized that the ER22/23EK allele is associated with absence rather than presence of white matter lesions - with white matter lesions dichotomized according to presence or absence of these lesions at baseline. Progression of white matter lesions at follow-up was also analyzed according to presence or absence of progression of lesions (no versus any progression). Due to the low number of participants with marked progression (approximately 10% in both periventricular and subcortical regions), minor and marked progression were not analyzed separately. The limited number of incident infarcts also precluded a separate analysis on incident infarcts on MRI.

All analyses were adjusted for age and sex. To elucidate whether associations might be explained by vascular intermediates, analyses were repeated after additional adjustments for hypertension, BMI, diabetes, cholesterol levels, as well as smoking status and exclusion of subjects with a history of stroke at baseline. In addition, subjects with a stroke preceding a dementia diagnosis were censored at the date of stroke diagnosis in the analyses on dementia incidence. In all analyses, heterozygous and homozygous carriers were analyzed together as carriers of the ER22/23EK allele. All analyses were performed using SPSS statistical software version 11 (SPSS Inc., Chicago, Illinois).

RESULTS

Baseline characteristics for both study samples are shown in Table 1. GR genotypes were present for 6,034 participants in the Rotterdam Study. We identified 389 heterozygous ER22/23EK-carriers (6.5%) and 7 homozygous ER22/23EK-carriers (0.1%) in this population. At baseline, data on dementia were present in a total of 5,990 participants of whom 395 carried the ER22/23EK allele. In the Rotterdam Scan Study, GR genotypes were present for 1,011 participants (78 (7.7%) heterozygous and 1 (0.1%) homozygous ER22/23EK-carriers). Genotype frequencies of both study populations were in Hardy-Weinberg equilibrium.

Table 1. Baseline characteristics of the study populations*

	Rotterdam Study	Rotterdam Scan Study†
Number (n)	6,034	1,011
Age (years)	69.3 (9.0)	72.3 (7.4)
Sex (% female)	59.7	51.4
Smoking (% current)	22.2	17.2
Diabetes Mellitus (%)	10.1	7.1
Systolic blood pressure (mmHg)	139.3 (22.2)	147.6 (21.6)
Diastolic blood pressure (mmHg)	73.7 (11.4)	78.9 (11.8)
Body mass index (kg/m^2)	26.3 (3.7)	26.6 (3.6)
Total cholesterol (mg/dL)	254.8 (46.3)	227.8 (38.6)
ER22/23EK (% carrier)	6.6	7.8

* Values represent means (standard deviation) or percentages (%)

† Overlap with Rotterdam Study n=515 participants

Dementia

In the Rotterdam Study genotype frequencies of the ER22/23EK allele were 6.8% in the non-demented and 1.2% in the demented at baseline. Two out of 172 participants with dementia (both AD) had one ER22/23EK allele. After adjustment for age and sex, the frequency of the ER22/23EK allele was significantly (more than 80%) lower, in both dementia and AD patients compared to non-demented subjects (Table 2).

After exclusion of participants demented at baseline, the cohort was followed for incident dementia. During 38,763 person-years of follow-up (mean (SD) 5.8 (1.6) years) 329 participants developed dementia, of which 243 had AD. Sixteen participants with one ER22/23EK allele developed dementia during follow-up (of whom 12 had AD and 3 vascular dementia), whereas none of the homozygous carriers did). Genotype frequency of the ER22/23EK allele was 6.9% in the non-demented and 4.9% in the demented participants. The ER22/23EK allele was associated with a decreased risk of developing dementia. Risk for both overall dementia and AD was nearly 40% lower in carriers of

Table 2. Frequencies of the ER22/23EK allele and risk of dementia*

	Non-carriers	ER22/23EK	OR (95%CI)	*P*
Prevalent dementia	n/N	n/N		
Age and sex adjusted	170/5,595 (3.0%)	2/395 (0.5%)	0.14 (0.03; 0.59)	0.01
Age, sex, vascular factors adjusted†			0.21 (0.08; 0.58)	0.003
			HR (95%CI)	
Incident dementia				
Age and sex adjusted	313/5,425 (5.8%)	16/393 (4.1%)	0.63 (0.38; 1.04)	0.07
Age, sex, vascular factors adjusted†			0.64 (0.39; 1.07)	0.09

*Values represent odds ratios (OR (95% confidence intervals)) for the prevalence of dementia, and hazard ratios (HR (95% confidence intervals)) for the incidence of dementia, non-carriers are reference

n/N, number of cases / total number in the analysis, OR, odds ratio, 95% CI, 95% confidence interval, HR, hazard ratio, *p*, p-values for carriers of the ER22/23EK allele (non-carriers are reference).

† Vascular factors included in the model: systolic and diastolic blood pressure, diabetes mellitus, serum total and high-density lipoprotein cholesterol, body mass index and smoking

the ER22/23EK allele (Table 2). The association remained unchanged after adjustment for potential cardiovascular intermediates or *APOE* genotype and exclusion of strokes at baseline or censorship of incident strokes

Cognitive function

After exclusion of those who were demented, no relationship was identified between MMSE scores and the ER22/23EK allele in both studies. The adjusted difference between ER22/23EK-carriers and non-carriers was 0.05 point (95% Confidence Interval (CI):

Table 3. ER22/23EK allele and cognitive performance*

Compound score	Odds ratio (95% CI)	*P*
Memory		
Age and sex adjusted	1.02 (0.63; 1.67)	0.93
Age, sex, vascular factors adjusted†	1.01 (0.61; 1.65)	0.98
Speed		
Age and sex adjusted	2.24 (1.30; 3.89)	0.004
Age, sex, vascular risk factors adjusted†	2.22 (1.18; 3.84)	0.004
Overall cognitive function		
Age and sex adjusted	1.18 (0.70; 1.99)	0.54
Age, sex, vascular factors adjusted†	1.16 (0.69; 1.96)	0.58

* Values represent Odds ratios (95% confidence intervals (CI)) for carriers of the ER22/23EK allele to have better cognitive performance (non-carriers are reference).

† Vascular factors included in the model: systolic and diastolic blood pressure, diabetes mellitus, serum total and high-density lipoprotein cholesterol, body mass index and smoking

-0.13; 0.23) in the Rotterdam Study and 0.26 points (95% CI: -0.24; 0.77) within the Rotterdam Scan Study. However, ER22/23EK-carriers had higher scores on the compound scores for memory performance, psychomotor speed and global cognitive function in the Rotterdam Scan Study. The average differences were not statistically significant (age and sex adjusted differences (95% CI) in Z-score for memory performance, psychomotor speed and global cognitive function: 0.06 (-0.13; 0.25), 0.13 (-0.04; 0.30) and 0.10 (-0.04; 0.24) respectively), but carriers were more than twice as likely to score better on tests of psychomotor speed when dichotomized at the median level (p = 0.004, see Table 3).

Structural brain abnormalities on MRI

In the Rotterdam Scan Study, no significant differences were observed between carriers and non-carriers of the ER22/23EK allele with respect to hippocampal (p = 0.49) and amygdalar volumes (p = 0.50). At baseline, carriers of the ER22/23EK allele had slightly less severe white matter lesions, but these differences were not statistically significant. The adjusted differences for ER22/23EK-carriers compared to non-carriers were –0.38 points (95% CI: -0.84; 0.07 points) and –0.18 ml (95% CI: -0.81; 0.45 ml) for periventricular and subcortical white matter lesions respectively. However, when presence or absence of white matter lesions was compared, ER22/23EK-carriers were less than half as likely to have these lesions than non-carriers (47% for periventricular and 40% for subcortical white matter lesions respectively) (Table 4). Similarly, ER22/23EK carriers were less likely to have a brain infarct on MRI (age and sex adjusted OR 0.76 (95% CI: 0.43; 1.32), although this was not significant. In addition, ER22/23EK-carriers had almost 70% less progression of subcortical white matter lesions (Table 4). This association remained unchanged after adjustment for baseline subcortical white matter lesions (OR for any progression: 0.26 (95% CI: 0.09; 0.73). Progression of periventricular white matter lesions was not different between ER22/23EK-carriers and non-carriers (Table 4).

Table 4. ER22/23EK allele and white matter lesions on MRI*

	White matter lesions			
	Periventricular		Subcortical	
	Odds ratio (95% CI)	*P*	Odds ratio (95% CI)	*P*
Presence				
Age and sex adjusted	0.47 (0.28; 0.80)	0.005	0.40 (0.21; 0.79)	0.008
Age, sex, vascular factors adjusted†	0.37 (0.19; 0.72)	0.003	0.29 (0.13; 0.67)	0.003
Progression				
Age and sex adjusted	1.02 (0.47; 2.20)	0.96	0.34 (0.14; 0.84)	0.02
Age, sex, vascular factors adjusted†	1.03 (0.48; 2.23)	0.94	0.34 (0.14; 0.83)	0.02

* Values represent odds ratios (95% confidence intervals (CI)) for presence and progression of white matter lesions, non-carriers are reference.

† Vascular factors included in the model: systolic and diastolic blood pressure, diabetes mellitus, serum total and high-density lipoprotein cholesterol, body mass index and smoking

DISCUSSION

In the Rotterdam Study, a prospective population-based study in the elderly, we found that the functional ER22/23EK allele of the *GR* gene is associated with a nearly 40% risk reduction of incident dementia during a follow-up period of almost 6 years, supported by significantly less (reduction of 86%) prevalent dementia at baseline. In a second study population, the Rotterdam Scan Study, we observed that ER22/23EK-carriers performed better on psychomotor speed tests and less often had periventricular and subcortical white matter lesions or brain infarctions on MRI. Progression of subcortical white matter lesions was also significantly lower in ER22/23EK-carriers. Interestingly, several indicators of cerebrovascular pathology did not significantly differ between genotypes when analyzed continuously. However, when we divided the participants according to presence or absence of white matter lesions, or good or bad psychomotor speed performance, we found highly significant differences. This supports our hypothesis that this allele is related to healthy conditions in the elderly rather than to pathological conditions. In both studies we did not observe associations with memory function in non-demented subjects.

There are several possible explanations for the protective effects on the brain we observed in carriers of the ER22/23EK allele. First, the ER22/23EK allele has previously been shown to be associated with a relative resistance to glucocorticoids with respect to the negative feedback in normal individuals.[4] The regulation of glucocorticoid production is modulated by a negative feedback mechanism of glucocorticoids at the level of the hypothalamus and pituitary, which is mediated by the GR. High levels of cortisol have been associated with cognitive decline both in non-demented elderly and in patients with dementia, and high cortisol levels after dexamethasone, indicative of impaired negative feedback function, have been related to cognitive decline in non-demented elderly.[8,9,11] Also, atrophy of the hippocampus is facilitated by cortisol.[31] Cortisol levels have been shown to be increased in both vascular dementia and AD.[8,9] Thus, the lower risk on dementia and white matter lesions in ER22/23EK-carriers might be related to a decreased direct effect of cortisol on the brain, which is possibly mediated by a relative insensitive GR. However, in the present study we did not observe any differences in hippocampal or amygdalar volumes. Atrophy of the hippocampus and amygdala is associated with decreased memory function and is an early marker of AD.[17,28] In accordance, in the Rotterdam Study, we did not observe differences in memory function between non-demented carriers and non-carriers. Also in the Rotterdam Scan Study, we only found an association with psychomotor speed function, but not with memory function. This could be explained by the presence of the mineralocorticoid receptor (MR), of which the expression in the brain is restricted to the hippocampal and amygdalar regions.[32] Glucocorticoids can also bind with high affinity to the MR.[33] In rat brain, it has been

shown that glucocorticoids activate only MR when present in low concentrations (during basal conditions), whereas higher glucocorticoid concentrations (during stress conditions) activate both MR and GR.[33] Thus, glucocorticoid balance in the hippocampus and amygdala in basal state seems to be mainly regulated by the MR. Therefore, the beneficial effects of a subtle resistance of the GR might be less in these brain regions. However, we have to be careful with the interpretation of these studies of corticosteroid receptors in the brain, because most data are from animal studies and it is not known whether they can be extrapolated to the human brain.

An alternative, second explanation for the beneficial cerebral effects of the ER22/23EK allele, is that this *GR* variant has previously been associated with lower C-reactive protein[5] and lower total cholesterol and LDL-cholesterol levels.[4] Inflammation, reflected by higher levels of C-reactive protein, and atherosclerosis have both been associated with AD[34,35] and cerebral white matter lesions.[36,37] Since ER22/23EK-carriers had less often white matter lesions, as well as less progression of these lesions, the underlying mechanism might be a better vascular status in ER22/23EK-carriers. This is supported by our finding of better psychomotor speed scores in carriers of this allele, since psychomotor speed performance is associated with the presence of cerebral white matter lesions.[15] However, adjustment for markers of atherosclerotic disease did not change our results. On the other hand, a limitation of our study is that only cholesterol levels at high age are available. Since midlife cholesterol concentrations in particular have been shown to be related to an increased risk of dementia,[34] we cannot rule out the possibility that atherosclerosis underlies the beneficial effects of this allele on the brain.

A third mechanism, which possibly relates to the effects of the ER22/23EK allele, might be through glucose/insulin metabolism. In this context, the ER22/23EK allele has previously been associated with a better insulin sensitivity in the elderly.[4] Increased serum insulin concentrations and diabetes have been shown to be associated with decreased cognitive function and dementia.[38,39] The effects of changes in insulin homeostasis on the brain can be either direct or via the process of atherosclerosis.[39,40]

In conclusion, our findings suggest that the ER22/23EK variant of the *GR* gene is associated with a lower risk of dementia. Our data suggest that the mechanisms underlying this association might be a relative resistance to glucocorticoids and, at least in part, less white matter lesions. Given the low prevalence of the ER22/23EK variant in this elderly population and the modest inverse association with incident dementia, it is likely to be less important than other genetic risk factors (e.g. the *APOE* allele ε4) for AD. Importantly, however, our findings suggest that glucocorticoid resistance may be involved in the development of dementia. Further large prospective population-based cohort studies are needed to confirm and clarify the possible protective role of genetically determined glucocorticoid resistance in dementia.

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Part II

Retinal vessels and risk of dementia

Chapter 4

Determinants of retinal vessel diameters

Chapter 4.1

Markers of cardiovascular disease and retinal vessel diameters

ABSTRACT

Purpose: *A lower retinal arteriolar-to-venular ratio (AVR) has been suggested to reflect generalized arteriolar narrowing and to predict the risk of cardiovascular diseases. The contribution of the separate arteriolar and venular diameters to this AVR is unknown. Thus associations between retinal arteriolar and venular diameters, and the AVR on the one hand and blood pressure, atherosclerosis, inflammation markers and cholesterol levels on the other were examined in the Rotterdam Study.*

Methods: *In this cross-sectional population-based study, for one eye of each subject (≥55 years; n = 5,674) retinal arteriolar and venular diameters (in μm) of the blood columns were summed on digitized images. At baseline, blood pressures, cholesterol levels, and markers of atherosclerosis and inflammation were also measured.*

Results: *With increasing blood and pulse pressures retinal arteriolar and venular diameters, and the AVR decreased significantly and linearly. Smaller arteriolar diameters were associated with increased carotid intima-media thickness. Larger venular diameters were associated with higher carotid plaque-score, more aortic calcifications, lower ankle-arm index, higher leukocyte count, higher erythrocyte sedimentation rate, higher total serum cholesterol, lower HDL, higher waist-to-hip ratio and smoking. A lower AVR was related to increased carotid intima-media thickness, higher carotid plaque-score, higher leukocyte count, lower HDL, higher body mass index, higher waist-to-hip ratio and smoking.*

Conclusions: *Because larger venular diameters are associated with atherosclerosis, inflammation and cholesterol levels, the AVR does not depend only on generalized arteriolar narrowing due to the association between smaller arteriolar diameters and higher blood pressures. These data indicate that retinal venular diameters are variable and may play their own independent role in predicting cardiovascular disorders.*

INTRODUCTION

Structural changes in the retinal vasculature have long been recognized as an important predictor of systemic hypertensive damage and life prognosis.[1,2] Different classifications mainly depended on qualitative assessment of retinal vessels using ophthalmoscopy, which has a poor reproducibility and gauges arteriolar diameters against the venular ones.[3]

Recently, in the Atherosclerosis Risk in Communities (ARIC) Study a semi-automated system was developed to measure the vessel diameters on fundus photographs.[4-6] The authors attributed a lower arteriolar-to-venular ratio (AVR) to generalized arteriolar narrowing and suggested that this ratio might provide information in predicting incident cardiovascular diseases independently of known cardiovascular risk factors.[6-14] Compared to other retinal signs like hemorrhages, focal arteriolar narrowing or arterio-venous nicking, the AVR was the more reliable and commonly used parameter of vascular damage.[6,8-11,15]

It remains unclear, however, what exactly the separate arteriolar and venular diameters contributed to the AVR and what kind of vascular pathology this ratio precisely reflects. Limited data have been published on the association between AVR or retinal vessel diameters and blood pressure.[6,11-13] Associations concerning AVR and atherosclerosis were inconclusive.[15,16] Currently, no data are available on the specific relationship between arteriolar or venular diameters and atherosclerosis, inflammation markers or cholesterol levels. We therefore investigated in a population-based setting these cross-sectional associations.

METHODS

Population

The present study was performed as part of the Rotterdam Study, a population-based cohort study on chronic and disabling diseases in the elderly. A total of 7,983 subjects aged 55 years and older living in a district of Rotterdam agreed to participate in the study. Information on the baseline study population has appeared in previous reports.[17,18] Because the ophthalmic part became operational after the screening of participants had started, a smaller number ($n = 6{,}780$) participated in the ophthalmic examination.[19] The study was conducted according to the tenets of the Declaration of Helsinki, and the medical ethics committee of the Erasmus Medical Center approved the study protocol. A written informed consent was obtained from all participants. Baseline interviews and examinations were performed from 1990 to mid-1993.

Retinal vessel measurements

After the eye examination at baseline simultaneous stereoscopic fundus color transparencies centered on the optic disc (pharmacological mydriasis, 20° field, Topcon Optical Company, Tokyo, Japan) were digitized with a high-resolution scanner (Nikon LS-4000, Nikon Corporation, Japan).[18] Per subject the image with the best quality (left or right eye) was analyzed with a semi-automated system (Retinal Analysis, Optimate, WI; Department of Ophthalmology & Visual Science, University of Wisconsin-Madison) by four trained graders masked for the endpoints.[4-6] We used the improved Parr-Hubbard formula to compute the summary vessel measures. Because each eye has a different magnification due to its optical system (lens, cornea) we additionally adjusted this summary vessel measure for the refraction and corneal curvature using Littmann's formula to obtain absolute measures.[20,21] On one eye of each subject one sum value was calculated for the arteriolar blood column diameter and one for the venular (in μm), the AVR being the ratio of these. In a random sub-sample of 100 subjects we found no differences between the right and left eyes for the arteriolar and venular diameters.

Quality control sessions with a random sub-sample of 40 transparencies gave the following Pearson's correlation coefficients for inter-grader agreement 0.67-0.80 (arteriolar diameter), 0.91-0.94 (venular diameter) and 0.75-0.84 (AVR). For intra-grader agreement these were 0.69-0.88, 0.90-0.95 and 0.72-0.90, respectively.

Blood pressure, atherosclerosis, inflammation and cholesterol

Blood pressures, ankle-arm index, intima-media thickness, carotid artery plaques and aortic atherosclerosis were measured as described previously.[22-24]

Briefly, intima-media thickness of the common carotid artery was assessed by ultrasonography, using a 7.5-MHz linear-array transducer (ATL Ultra-Mark IV). For the current analyses, the average of the mean anterior and posterior intima-media thickness of both the left and the right common carotid artery was used. Atherosclerotic plaques, assessed by ultrasound at the bifurcation, common, and internal carotid artery on both sides, were defined as focal thickening of the vessel wall (of at least 1.5 times the average intima-media thickness) relative to adjacent segments with or without calcified components. The carotid plaque-score (range: 0-6) reflects the number of these locations with plaques. Aortic atherosclerosis defined as calcified deposits in the abdominal aorta on lateral radiographic films of the lumbar spine, was considered present when linear densities were seen in an area parallel and anterior to the lumbar spine (L1-L4). The extent of calcification was scored according to the length of the involved area (0, 0.5 to <1, 1 to <2.5, 2.5 to <5, 5 to <10, and ≥10 cm). All readers and technicians were masked for all clinical information.

Leukocyte count was assessed in citrate plasma using a Coulter Counter T540® (Coulter electronics, Luton, England). Blood was drawn directly into VACUTAINER® tubes

and erythrocyte sedimentation rate was read after 60 minutes. Non-fasting serum total cholesterol and high-density lipoprotein (HDL) concentrations were determined by an automated enzymatic procedure.[25] Information on smoking (categorized as current, former or never) and medication use was obtained during the home interview. Alcohol consumption was assessed as part of a dietary interview.[26]

Study sample

Of the 6,780 participants in the ophthalmic part, 6,436 persons underwent optic disc photography. From these, 762 subjects were excluded because they had ungradable fundus transparencies on both eyes, resulting in a cohort of 5,674 subjects. Measurements on blood pressure were missing in 91 participants, ankle-arm index in 547, intima-media thickness in 918, carotid plaque-score in 970, aortic calcifications in 1,510, leukocyte count in 394, erythrocyte sedimentation rate in 1,543, total cholesterol in 44, HDL cholesterol in 53, body mass index in 72, waist-to-hip ratio in 350, smoking in 65 and alcohol consumption in 884. Restricted availability of technicians was the main reason for missing data, which was independent of any subject characteristics.

Statistical analysis

Analysis of covariance (ANCOVA) was used to compare subjects with gradable and ungradable transparencies and to analyze whether associations were linear. If so, multiple linear regression models were used to assess these cross-sectional relationships. We analyzed blood pressures both per standard deviation (SD) and per 10-mmHg increase. The former was taken to compare the effect size between systolic and diastolic blood pressures, the latter to compare our results to those of other studies.

All analyses were repeated after stratifying on subjects with diseases known to affect venular width such as diabetes mellitus and hypertension. We used SPSS Windows version 11.0 (SPSS Inc., Chicago, Illinois).

RESULTS

Table 1 shows the baseline characteristics of subjects with gradable and ungradable transparencies. The means were: arteriolar diameter 146.9 μm (range: 92.2-235.7; SD: 14.4), venular diameter 222.0 μm (range: 135.1-313.6; SD: 20.9) and AVR 0.66 (range: 0.48-1.02; SD: 0.06).

Blood pressures and retinal vessel diameters

With increasing blood and pulse pressures the arteriolar diameters decreased linearly (Table 2). The figure shows that the relationship with the arteriolar diameters was stron-

Table 1. Baseline Characteristics presented as unadjusted means (SD) or percentages

	Gradable*	Ungradable	Adjusted differences† (95% CI)
Number (n)	5,674	762	
Age (years)	68.0 (8.2)	75.7 (10.1)	7.8 (7.1; 8.4)
Sex (% female)	59.0	63.0	0.1 (-3.8; 4.0)
Institutionalized (%)	3.6	22.0	9.9 (8.3; 11.6)
Diabetes Mellitus (%)	10.0	14.0	0.9 (-1.4; 3.3)
Systolic blood pressure (mmHg)	138.5 (22.1)	145.2 (24.0)	1.5 (-0.2; 3.3)
Diastolic blood pressure (mmHg)	73.7 (11.4)	74.2 (12.8)	1.4 (0.5; 2.3)
Pulse pressure (mmHg)	64.8 (17.7)	71.0 (19.7)	0.1 (-1.2; 1.4)
Ankle-arm index	1.07 (0.22)	0.97 (0.28)	-0.03 (-0.05; -0.02)
Carotid Intima-media thickness (mm)	0.79 (0.15)	0.86 (0.19)	0.02 (0.01; 0.03)
Aorta calcification ≥ 5 cm (%)	11.1	17.6	2.1 (-1.2; 5.4)
Carotid plaques ≥ 4 (%)	16.1	24.0	1.5 (-1.7; 4.7)
Leukocyte count (10^9/L)	6.7 (1.9)	6.8 (3.0)	1.1 (-0.1; 0.2)
Erythrocyte sedimentation rate	12.9 (10.7)	16.2 (14.4)	1.3 (0.2; 2.3)
Total serum cholesterol (mmol/L)	6.63 (1.21)	6.53 (1.30)	0.01 (-0.1; 0.1)
Serum HDL cholesterol (mmol/L)	1.35 (0.36)	1.34 (0.36)	0.00 (-0.02; 0.03)
Body mass index (kg/m^2)	26.3 (3.68)	26.0 (3.69)	-0.44 (-0.73; -0.14)
Waist-to-hip ratio	0.90 (0.09)	0.91 (0.09)	0.00 (-0.01; 0.01)
Smoking (%)			
Current	23.6	18.7	0.4 (-2.9; 3.7)
Former	42.9	36.8	-3.7 (-7.4; 1.9)
Alcohol consumption (%)			
≤ 10 gram/day‡	45.0	41.9	-3.3 (-8.1; 1.5)
> 10 - ≤ 20 gram/day	15.4	13.0	-1.3 (-4.8; 2.2)
> 20 gram/day	19.5	19.3	1.0 (-2.7; 4.7)

* Subjects with a gradable fundus transparency on at least one eye

† Age and sex adjusted if applicable

‡ 10 grams is on average equal to 1 drink

gest in the youngest age category (per SD increase in systolic blood pressure: -3.8 µm (95% CI: -4.7; -2.8)) and became non-significant above age 80 years. With increasing blood and pulse pressures the venular diameters showed a small decrease, and stratified on age there seemed to be no clear trend.

With increasing blood and pulse pressures the AVR decreased linearly (Table 2). The relationship with AVR was strongest in the age category 55-60 years (Fig.); per SD increase in systolic blood pressure AVR decreased by 0.016 (95% CI: 0.012; 0.019). Diastolic blood pressure showed the same trends; hence only systolic blood pressure is presented in the figure.

Table 2. Age and sex adjusted difference (95% CI) in retinal vessel diameters with increasing blood pressure levels both per SD and per 10-mmHg and with increasing severity of sub-clinical atherosclerosis

	N	Arteriolar diameter (µm)	Venular diameter (µm)	Arteriolar-to-venular ratio
Blood pressure				
Systolic Blood Pressure:				
Per SD increase	5,583	-2.4 (-2.8; -2.0)	-1.0 (-1.6; -0.5)	-0.008 (-0.009; -0.006)
Per 10-mmHg increase		-1.1 (-1.3; -0.9)	-0.5 (-0.7; -0.2)	-0.0035 (-0.004; -0.003)
Diastolic Blood Pressure:				
Per SD increase	5,583	-2.4 (-2.8; -2.1)	-0.8 (-1.3; -0.2)	-0.009 (-0.010; -0.007)
Per 10-mmHg increase		-2.1 (-2.5; -1.8)	-0.7 (-1.1; -0.2)	-0.008 (-0.009; -0.006)
Pulse Pressure:				
Per SD increase	5,583	-1.5 (-1.9; -1.1)	-0.8 (-1.4; -0.3)	-0.004 (-0.006; -0.003)
Per 10-mmHg increase		-0.8 (-1.1; -0.6)	-0.5 (-0.8; -0.1)	-0.002 (-0.003; -0.001)
Markers of sub-clinical atherosclerosis				
Carotid Intima-media thickness:				
Per SD increase	4,756	-0.9 (-1.4; -0.5)	-0.2 (-0.8; 0.5)	-0.004 (-0.005; -0.002)
Carotid plaque-score:				
Per plaque increase	4,704	-0.2 (-0.5; 0.1)	0.4 (0.1; 0.8)	-0.002 (-0.003; -0.001)
Ankle-arm index:				
Per SD decrease	5,127	0.4 (0.0; 0.8)	1.3 (0.7; 1.9)	-0.002 (-0.0004; -0.004)
Aorta calcification:				
Per category increase	4,164	0.03 (-0.3; 0.4)	0.4 (-0.1; 0.8)	-0.001 (-0.002; 0.001)

Atherosclerosis and retinal vessel diameters

The arteriolar diameters did not show an association with the different markers of atherosclerosis except intima-media thickness. After additional adjustment for blood pressure, this association became weaker (per SD increase: -0.6 µm (95% CI: -1.0; -0.1)).

The venular diameters were linearly related to several markers of atherosclerosis. Larger venular diameters were associated with a lower ankle-arm index, higher carotid plaque-score, more aortic calcifications (borderline) and a non-significantly increased intima-media thickness. Additional adjustment for blood pressure did not alter these associations. Stratification on age did not show a trend between atherosclerosis and retinal venular diameters.

A lower AVR was significantly related to a lower ankle-arm index, increased intima-media thickness and higher carotid plaque-score, but not to aortic calcifications (Table 2). After additional adjustment for blood pressure, AVR was still associated with intima-media thickness (per SD increase: -0.002; 95% CI: -0.004; -0.001) and carotid plaque-score (per plaque increase: -0.002; 95% CI: -0.003; -0.001).

Inflammation markers, cholesterol levels and retinal vessel diameters

Higher leukocyte count, higher erythrocyte sedimentation rate, lower serum HDL levels, higher total serum cholesterol, higher waist-to-hip ratio and smoking were related to

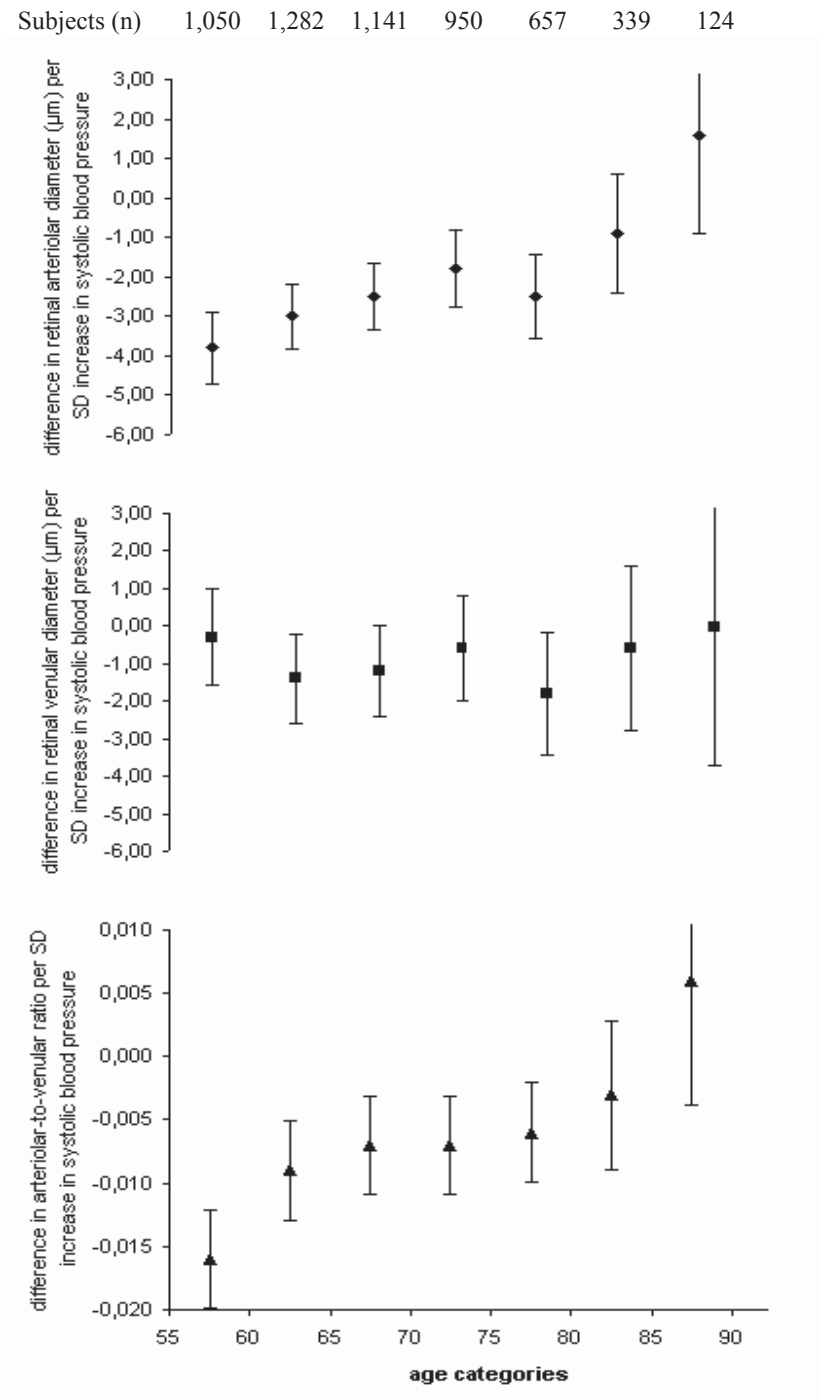

Figure. The relationship between blood pressure and retinal vessel measurements per five-years age categories (age category 90-95 years has been excluded due to low numbers n=40))

Table 3. Age and sex adjusted difference (95% CI) in retinal vessel diameters with increasing levels of inflammation, cholesterol, smoking and alcohol consumption

	N	Arteriolar diameter (µm)	Venular diameter (µm)	Arteriolar-to-venular ratio
Leukocyte count: per SD increase	5,280	1.1 (0.8; 1.5)	2.9 (2.4; 3.5)	-0.003 (-0.005; -0.002)
Erythrocyte sedimentation rate: per SD increase	4,131	0.7 (0.3; 1.2)	0.8 (0.1; 1.5)	0.001 (-0.001; 0.003)
Total serum cholesterol: per SD increase	5,630	0.2 (-0.2; 0.6)	0.4 (-0.1; 1.0)	-0.0002 (-0.002; 0.001)
Serum HDL cholesterol: per SD increase	5,621	-0.5 (-0.9; -0.1)	-1.2 (-1.8; -0.7)	0.001 (0.000; 0.003)
Body mass index: per SD increase	5,602	-0.7 (-1.1; -0.3)	0.1 (-0.4; 0.7)	-0.004 (-0.005; -0.002)
Waist-to-hip ratio: per SD increase	5,324	-0.2 (-0.6; 0.3)	1.1 (0.4; 1.7)	-0.004 (-0.006; -0.002)
Smoking:				
Current vs. non-smokers (n=1,324 vs. n=1,877)		5.0 (3.9; 6.0)	10.0 (8.3; 11.4)	-0.007 (-0.011; -0.003)
Former vs. non-smokers (n=2,408 vs. n=1,877)		1.3 (0.4; 2.3)	2.5 (1.2; 3.9)	-0.002 (-0.006; 0.002)
Alcohol consumption:				
≤ 10 gram/day vs. non-drinkers (n=2,158 vs. n=965)		0.6 (-0.5; 1.7)	0.6 (-0.9; 2.2)	0.001 (-0.004; 0.005)
> 10 - ≤ 20 gram/day vs. non-drinkers (n=737 vs. n=965)		-1.0 (-2.4; 0.4)	-0.9 (-2.9; 1.1)	-0.002 (-0.008; 0.003)
> 20 gram/day vs. non-drinkers (n=930 vs. n=965)		-1.1 (-2.4; 0.3)	1.5 (-0.4; 3.4)	-0.010 (-0.015; -0.004)

larger venular diameters and to a lesser extent to larger arteriolar diameters thus resulting in a lower AVR (Table 3).

These analyses were repeated using models in which the cardiovascular variables were categorized. Because all relationships were linear in these analyses, only the linear models are presented. Stratification on subjects with and without hypertension did not significantly alter any of the above-mentioned results. The results were again the same after stratification on diabetes mellitus. However, in subjects with diabetes mellitus the point estimates for these relationships were less stable with wider confidence intervals, because only 10% of the population had diabetes mellitus at baseline.

DISCUSSION

These results show that higher blood and pulse pressures were related to lower arteriolar diameters. More atherosclerosis as measured by the ankle-arm index, aortic calcifications and carotid plaque-score, higher leukocyte count, higher erythrocyte sedimentation rate,

higher total cholesterol, lower HDL levels, higher waist-to-hip ratio or smoking were related to larger venular diameters, the overall effect being a lower AVR.

For proper interpretation some methodological issues warrant consideration. The excluded subjects were on average older and more often institutionalized. This group probably contained more subjects with physical or mental disabilities or lens opacities that contributed to the poor quality of the transparencies. However, there were only small differences in cardiovascular risk factors between the two groups, suggesting a limited role for selection bias.

The cross-sectional setting prevented inferring causality or chronological order of events. Photographs were not taken synchronized on the cardiac cycle, so there might be variation in vessel diameter due to pulsatility. A variation of 2-17% in vessel diameter has been described.[27] However, because photography was independent of any subject characteristics, this will have caused random misclassification.

Strengths of this study include its population-based design, data collection within a short time-span and the detailed measurement of vessel diameters on 20° stereoscopic fundus transparencies (leading to higher magnification) obtained after pharmacological mydriasis. Both ARIC and Cardiovascular Health Study (CHS) used 45° photographs without pharmacological mydriasis, whereas the Blue Mountains Eye Study used 30° photographs through dilated pupils.[6,15,13] Furthermore, ours seems the first study that used the improved Parr-Hubbard formulas and Littmann's correction to approximate absolute intra-luminal diameters.[20,21]

Blood pressure and retinal vessel diameters

When analyzed separately, the impact of blood pressure was more prominent in younger subjects and more in arterioles than venules, as expected. The decrease in arteriolar diameters not only reflects vasoconstriction but also intimal thickening, medial hyperplasia, hyalinization and sclerosis.[28,29] We found smaller decrease in arteriolar diameter (1.1 μm and 2.1 μm per 10-mmHg increase in systolic and diastolic blood pressure) than reported (1.9 μm and 4.3 μm).[13] Apart from the abovementioned differences in grading and measuring techniques, this smaller decrease could be explained by the older age distribution of our (55-99 years) versus the ARIC (48-76 years) and the Blue Mountains cohorts (49+ years, only 8% above age 80 years).[6,13] In the older age groups the vessels become progressively rigid and lose their ability to adequately react to blood pressure changes.[30,31]

The association between venular diameter and blood pressure was weak, and stratified on age there was no trend. In our oldest age category the estimate might be unstable due to low numbers.

Atherosclerosis and retinal vessel diameters

There was no relationship between the arteriolar diameters and markers of atherosclerosis, except for intima-media thickness. Apart from being a marker of atherosclerosis, intima-media thickness may indicate a response of the artery wall to changes in shear and tensile stress due to hypertension.[32] This is also supported by our data, because after additional adjustment for blood pressure this association became weaker.

We did find a relationship between atherosclerosis and larger venular diameters. A clear patho-physiological explanation for this association is lacking. It is known that in diabetic retinopathy the retinal venules become wider.[33] Other examples of venular dilatation are patients with central retinal vein occlusion, carotid artery narrowing leading to so-called venous stasis retinopathy or deep venous thrombosis in the legs who also have more often atherosclerosis.[34,35] It has been suggested that hyperlipidemia, platelet activation and blood coagulation are involved in the development of both atherosclerosis and venous thrombosis.[35] Whether atherosclerosis induces venular changes, both share common risk factors or occur independently of each other, still needs to be elucidated.

In the CHS study atherosclerosis as measured by ankle-arm index, carotid plaques and intima-media thickness was, contrary to our findings, not related to AVR.[15] Selection bias might have occurred, because they performed carotid ultra-sonography five years prior to fundus photography.[15] In the ARIC study, however, a lower AVR was related to more carotid plaques,[16] and in our study the relationship with AVR persisted for intima-media thickness and carotid plaque-score after adjusting for blood pressures. The relationship between markers of atherosclerosis and AVR is mainly driven by larger venular diameters.

Inflammation markers, cholesterol levels and retinal vessel diameters

Higher leukocyte count, higher erythrocyte sedimentation rate, lower HDL levels, higher waist-to-hip ratio and smoking were associated with larger arteriolar and even more strongly with larger venular diameters. Because inflammation also plays an important part in atherosclerosis, vessel widening in these conditions may be due to similar mechanisms. We hypothesize that disruption of the endothelial surface layer (ESL) may underlie the apparent vessel widening. The endothelial surface of all vessels is coated with a matrix of proteoglycans and glycoproteins. This layer (glycocalyx) is directly bound to the plasma-membrane.[36,37] An immobile plasma layer consisting of soluble plasma proteins including glycosaminoglycans is attached to this glycocalyx. Together these layers are known as the ESL with a thickness ranging from 0.5 μm to over 1.0 μm.[36] The presence of the ESL may influence several physiological functions of the vessels including flow resistance, barrier function, leukocyte adhesion, coagulation and angiogenesis.[36] Damage to this ESL seems to be the earliest detectable injury to the vascular wall in atherosclerosis.[37] Free radicals as produced by oxidized low-density lipoproteins or activated leukocytes, can disrupt

this surface.[37] It could be that the increase in intra-luminal diameter we observed with increasing severity of atherosclerosis or inflammation partly reflects a diminishing ESL.

That higher leukocyte count, lower HDL levels, higher body mass index, higher waist-to-hip ratio and smoking were related to a lower AVR supports the suggestion that the AVR is also a marker of inflammation and endothelial dysfunction.[16]

Conclusion

Our data confirmed that elevated blood pressures were associated with smaller arteriolar diameters, but revealed that larger venular diameters were related to atherosclerosis, inflammation and cholesterol levels. Hence, the idea that the AVR overall reflects generalized arteriolar narrowing needs to be re-evaluated by taking into account the separate arteriolar and venular diameters. These data indicate that the venular diameters do not remain constant in different pathological conditions and may play their own independent role in predicting cardiovascular disease. In future research more attention needs to be paid to the role of venules in vascular disease.

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Chapter 4.2

Inflammation markers and retinal vessel diameters

ABSTRACT

Retinal vessels may provide a way to study the cerebral microcirculation. In particular larger retinal venular diameters have been associated with cerebrovascular disease. An inflammatory response may underlie this association. In a population-based cohort study among 5,279 participants aged 55 years or older with graded retinal vessel diameters we observed that higher serum levels of C-reactive protein, fibrinogen and higher Liproprotein-associated phospholipase A2 activity were strongly associated with larger venular diameters. Weaker associations were found with arteriolar diameters. Our findings support the hypothesis that larger retinal venular diameters reflect systemic inflammation and suggest that inflammation is involved in cerebrovascular disease.

INTRODUCTION

Retinal vessels may reflect the condition of intracerebral vessels and their investigation may help understand the etiology of cerebrovascular disorders.[1] A lower ratio of arteriolar-to-venular diameter (AVR), suggested to reflect generalized arteriolar narrowing due to chronic hypertension,[2] has been related to cerebral small vessel disease and an increased risk of stroke.[3,4] However, a lower AVR may not only reflect arteriolar narrowing but also venular widening. Within the Rotterdam Study we found larger venular diameters rather than smaller arteriolar ones to be associated with progression of cerebral small vessel disease[5] and an increased risk of stroke.[6] Mechanisms underlying venular widening are to date poorly understood yet might provide new insights in the etiology of cerebrovascular disease.

In the Rotterdam Study we found that larger venular diameters were associated with sub-clinical atherosclerosis, cholesterol levels, and non-specific inflammatory markers, namely white blood cell count and erythrocyte sedimentation rate.[7] Two other population-based cohorts, the Beaver Dam Eye Study and the Multi-Ethnic Study of Atherosclerosis reported associations of larger venular diameters with other inflammatory markers including interleukin 6, fibrinogen and C-reactive protein (CRP),[8,9] supporting our suggestion that venular widening may reflect systemic inflammation.

The inflammatory markers fibrinogen, CRP and Lipoprotein-associated phospholipase A2 (Lp-PLA2) are all associated with an increased risk of cardiovascular disease and stroke independent of conventional vascular risk factors.[10-12] Lp-PLA2, also known as platelet-activating factor acetylhydrolase, is considered a new biomarker of cardiovascular disease.[12] To further clarify the effect of inflammation on retinal vessel diameters, by proxy measures of the cerebral microcirculation, we examined the cross-sectional data from the Rotterdam Study to determine whether CRP, fibrinogen and Lp-PLA2 were associated with retinal vascular caliber.

METHODS

The Rotterdam Study is a large population-based prospective cohort study among 7,983 elderly aged 55 years or older in a suburb of Rotterdam, the Netherlands, and ongoing since 1990.[13] Since eye examinations became operational a few months after the baseline examinations had started, a smaller number (n = 6,780, response rate 78%) participated in the ophthalmic part of the study.[14] Fundus color transparancies were available in 6,436 participants and of these, 762 were excluded because they had ungradable fundus transparencies on both eyes, resulting in a cohort of 5,674 persons. Fundus transparencies were taken with a telecentric Topcon-TRC-SS2 camera and centered on the optic

disk (20° field, magnification 2.58x, Topcon Optical Company, Tokyo, Japan) after pharmacological mydriasis,[15] and digitized with a high-resolution scanner (Nikon LS-4000, Nikon Corporation, Japan). For each participant the qualitatively best digitized image of either eye was analyzed with the Retinal Vessel Measurement System (Retinal Analysis, Optimate, WI; Department of Ophthalmology & Visual Science, University of Wisconsin-Madison, USA).[16] The rationale and procedures to measure and summarize retinal vessel diameters have been described and Littmann's formula was used to correct for magnification changes due to refraction anomalies.[7,16] Four trained graders performed the assessments, masked to the clinical characteristics of the participants. Pearson's correlation coefficients for intergrader agreement were 0.67-0.80 for arteriolar diameters and 0.91-0.94 for venular diameters. For intragrader agreement these figures were 0.69-0.88 (arteriolar diameters) and 0.90-0.95 (venular diameters).

Inflammatory markers were measured in blood samples collected at the baseline examination. CRP was measured in serum samples kept frozen at –20°C using Rate Near Infrared Particle Immunoassay (Immage® Immunochemistry System, Beckman Coulter, USA). This system measures concentrations from 0.2 to 1440 mg/l, with a within-run precision < 5.0%, a total precision < 7.5% and a reliability coefficient of 0.995. CRP levels were available for 5,339 participants. Since CRP level distribution was highly skewed data were log-transformed. Because very high CRP levels likely reflect an acute inflammation at the time of blood sampling, individuals of whom the logarithm of the CRP level was more than 3 standard deviations (SDs) above the mean were excluded from the study sample ($n = 60$), resulting in an overall study sample for the current analyses of 5,279 persons. Fibrinogen and Lp-PLA2 activity were measured at baseline in two random subsamples of the sample with CRP measurements in platelet poor plasma samples stored at -80°C. Fibrinogen was available for 2,155 and Lp-PLA2 activity for 1,375 of the persons with gradable fundus transparencies. Fibrinogen levels were derived from the clotting curve of the prothrombin time assay using Thromborel S as a reagent on an automated coagulation laboratory (ACL 300, Instrumentation Laboratory). The coefficient of variation was 5%. Lp-PLA2 activity was measured with a high-throughput radiometric activity assay[11] and expressed as nanomoles of platelet-activating factor hydrolysed per minute per 1 ml of plasma samples. On the basis of split samples, the coefficient of variation was 8.4%.

Vascular risk factors may confound an association of inflammatory markers with retinal vessel diameters. Blood pressure, smoking, diabetes mellitus, body mass index, atherosclerotic plaques, total and HDL cholesterol have all been associated with retinal vessel diameters and were assessed according to methods that have been described.[7]

Analysis of covariance (ANCOVA) was used to compare participants with gradable and ungradable fundus transparencies and to analyze whether associations between inflammatory markers and vessel diameters were linear, adjusting for age and sex. If the

observed associations were linear, multiple linear regression models were used in which the inflammatory markers were analyzed per SD increase. To see whether associations were independent of known vascular risk factors, all analyses were additionally adjusted for the abovementioned covariates, with imputed means for missing values ($n = 994$). In addition, because retinal arteriolar and venular diameters are related,[17] we adjusted the analyses on arteriolar diameters for venular ones and vice versa.

RESULTS

Baseline characteristics of participants in the overall study sample and those in the subsamples with fibrinogen or Lp-PLA2 are shown in Table 1. In the overall study sample arteriolar diameters were on average 146.8 μm (range 92.1-235.7; SD, 14.5) and venular diameters were on average 221.8 μm (range 135.1-313.6; SD, 22.0). Mean diameters in the subsamples did not differ from those in the overall study sample (data not shown).

The associations of inflammatory markers with arteriolar and venular diameters are shown in Figure 1. Higher levels of fibrinogen or higher Lp-PLA2 activity were associated with larger arteriolar diameters. CRP levels were not related to arteriolar diameters. Higher levels of CRP, fibrinogen or higher Lp-PLA2 activity were all associated with larger venular diameters. Analyses per SD increase in the different inflammatory markers showed that associations were stronger for venular diameters than for arteriolar diameters (Table 2). Additional adjustment for cardiovascular risk factors or retinal venular

Table 1. Characteristics of the study sample with C-reactive protein and of the subsets with fibrinogen and lipoprotein-associated phospholipase A2 activity (Lp-PLA2) assessments*

Characteristic	Sample with C-reactive protein measurement	Subset with fibrinogen measurement	Subset with Lp-Pla2 measurement
Number (n)	5,279	2,155	1,375
Age (years)	67.9 (8.2)	69.4 (8.2)	67.6 (8.1)
Sex (% female)	58.7	61.9	60.7
Institutionalised (%)	3.5	4.4	2.9
Systolic blood pressure (mmHg)	138.4 (22.0)	137.5 (21.2)	137.5 (21.7)
Diastolic blood pressure (mmHg)	73.7 (11.4)	72.4 (11.0)	73.4 (10.9)
Smoking (% current)	23.6	24.2	23.7
Diabetes (%)	9.6	11.5	8.5
Body mass index (kg/m^2)	26.3 (4.0)	26.5 (3.8)	26.2 (3.6)
Carotid plaques ≥ 4 (%)	15.9	13.7	15.3
Total cholesterol (mmol/L)	6.6 (1.2)	6.7 (1.2)	6.7 (1.2)
HDL cholesterol (mmol/L)	1.4 (0.4)	1.4 (0.4)	1.4 (0.4)

* Values are means (SD), or percentages

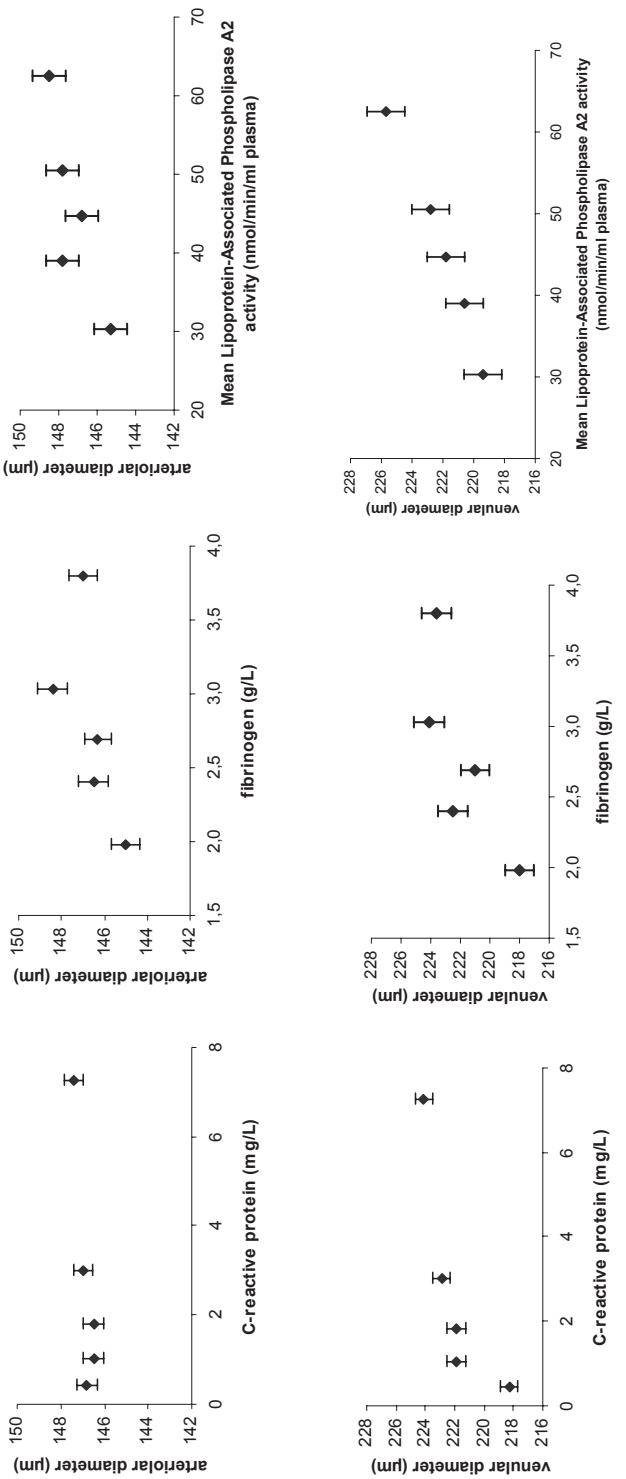

Figure 1. Retinal arteriolar and venular diameters according to quintiles of the inflammatory markers C-reactive protein, fibrinogen and Lipoprotein-Phospholipase A2 (Lp-PLA2)
Each diamond represents mean age and sex adjusted vessel diameters (standard errors), plotted at the median of each quintile of the corresponding inflammatory marker

Table 2. Association of inflammatory markers with retinal vessel diameters

	Arteriolar diameters*	Venular diameters*
C-reactive protein, per SD (1.04 mg/L) increase (*n*=5,279)		
Adjusted for age and sex	0.36 (-0.06; 0.77)	2.09 (1.51; 2.68)
Adjusted for age, sex, and cardiovascular risk factors†	0.36 (-0.07; 0.78)	1.61 (1.00; 2.22)
Adjusted for age, sex, cardiovascular risk factors and retinal vascular caliber‡	-0.32 (-0.71; 0.06)	1.43 (0.88; 1.98)
Fibrinogen, per SD (0.67 g/L) increase (*n*=2,155)		
Adjusted for age and sex	0.79 (0.16; 1.42)	1.90 (1.01; 2.79)
Adjusted for age, sex, and cardiovascular risk factors†	0.53 (-0.10; 1.17)	1.16 (0.22; 2.10)
Adjusted for age, sex, cardiovascular risk factors and retinal vascular caliber‡	-0.16 (-0.70; 0.38)	1.02 (0.23; 1.84)
Lp-PLA2, per SD (11.5 nmol/min/mL plasma) increase (*n*=1,375)		
Adjusted for age and sex	1.15 (0.40; 1.97)	2.50 (1.42; 3.58)
Adjusted for age, sex, and cardiovascular risk factors†	1.08 (0.19; 1.96)	2.33 (1.06; 3.61)
Adjusted for age, sex, cardiovascular risk factors and retinal vascular caliber‡	0.18 (-0.63; 0.99)	1.50 (0.36; 2.64)

* Values are adjusted mean differences (95% Confidence Intervals) per SD increase in inflammatory marker

† Systolic and diastolic blood pressure, smoking, diabetes mellitus, body mass index, carotid artery plaques, total and high-density lipoprotein cholesterol.

‡ Analyses on retinal arteriolar diameters are adjusted for retinal venular diameters, analyses on retinal venular diameters are adjusted for retinal arteriolar diameters

caliber attenuated the associations of fibrinogen and Lp-PLA2 with arteriolar diameters, which became non-significant. All other associations attenuated but remained significant (Table 2).

DISCUSSION

Our results show that higher serum levels of the inflammatory markers CRP and fibrinogen, and higher Lp-PLA2 activity are all associated with larger retinal venular diameters, independent of vascular risk factors. Only higher Lp-PLA2 activity was also associated with larger retinal arteriolar diameters, but this attenuated after adjustment for retinal venular caliber.

Strengths of this study are the population-based design, the detailed assessment of vessel diameters on 20° stereoscopic transparencies leading to higher magnification than on 35° fields, and the adjustment for refractive errors of the eye which cause magnification differences on the fundus transparencies. We previously reported that differences between those with and without gradable fundus transparencies were small once age differences were taken into account,[7] suggesting a limited role for selection bias. Furthermore, photography and assessment of vessel diameters were unrelated to clinical characteristics of the participants. Therefore, measurement error most likely caused non-differential misclassificiation which, if anything, leads to an underestimation of effects.

Our findings are in line with previous reports relating in particular larger retinal venular diameters to several inflammatory markers.[7-9] Both CRP and fibrinogen are acute-phase proteins and high levels serve as nonspecific markers of inflammatory processes. Fibrinogen also has hemostatic properties, it affects platelet aggregation and endothelial function and may predispose to thrombosis and enhance atherogenesis.[10] Lp-PLA2 hydrolyzes oxidized phospholipids to generate lysophosphatidylcholine and oxidized fatty acids, which exert pro-inflammatory properties but may also directly promote atherogenesis.[18] The role of inflammation in the initiation and progression of atherosclerosis is well established,[19] and vessel widening in these conditions may be due to similar mechanisms. For instance, damage to the endothelial surface layer covering the vascular endothelium seems to be one of the earliest events in the initiation of atherosclerosis. Free radicals originating from oxidized low-density lipoproteins or activated leukocytes are able to degrade this layer,[20] which may have contributed to the observed increase in intraluminal vessel diameter with higher levels of inflammatory markers.[7]

As atherosclerosis mainly develops in the arteries, an association of inflammatory markers with retinal arteriolar diameters might be expected. Higher Lp-PLA2 activity, which may promote atherogenesis, was indeed associated with larger arteriolar diameters. The absence of significant associations with CRP and fibrinogen, more general inflammatory markers, may be due to the strong association of blood pressure with smaller arteriolar diameters. These smaller diameters reflect vasoconstriction due to intimal thickening, medial hyperplasia and eventually sclerosis of the vessel wall.[7] Due to this increased arterial stiffness, arterioles are probably less capable of dilatation than venules.

The associations of all inflammatory markers with retinal vessel diameters attenuated after additional adjustment for established cardiovascular risk factors. This suggests that these factors are confounders in the causal pathway. In addition, there may be residual confounding from risk factors not measured or adjusted for in this study.

Taken together, our findings strengthen the hypothesis that larger retinal venular diameters reflect systemic inflammation. Because the retinal and cerebral microvasculature share similar embryological, anatomical and physiological properties, our findings can be reasonably assumed to have implication on cerebrovascular disease processes and indirectly reinforce the role of inflammation in the pathogenesis of cerebrovascular disease.

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Chapter 4.3

Complement factor H polymorphism and retinal vessel diameters

ABSTRACT

Purpose: *Retinal venular dilatation is associated with systemic inflammation. We hypothesized that larger retinal venular diameters are related to the His allele of the Tyr402His polymorphism in the complement factor H (CFH) gene, a major inhibitor of the complement pathway, and examined possible effect modification by smoking and inflammatory markers.*

Methods: *This cross-sectional study was performed within the Rotterdam Study, a population-based study among elderly aged 55 years and older. We genotyped the Tyr402His polymorphism of the CFH gene in 5,066 participants and graded retinal arteriolar and venular diameters on digitized fundus transparencies.*

Results: *Genotype frequencies were 41% for TyrTyr, 45% for TyrHis and 14% for HisHis carriers. The His^{402} allele was associated with smaller rather than larger venular diameters (age and sex adjusted means and standard errors (μm) were 222.5 ± 0.45 for TyrTyr, 221.9 ± 0.43 for TyrHis and 220.6 ± 0.78 for HisHis carriers (P-trend 0.03)). This association was apparent only in never smokers, and was not modified by the inflammatory markers erythrocyte sedimentation rate, leukocyte count, C-reactive protein or fibrinogen. Adjustment for cardiovascular risk factors did not change results. No associations were found with arteriolar diameters.*

Conclusions: *Our findings do not support the hypothesis that the His^{402} allele is related to larger retinal venular diameters. The association with smaller retinal venular diameters most likely is a chance finding, it was present only among never smokers and not modified by inflammatory mediators of complement. This suggests that the Tyr402His variant is not related to retinal venular diameters.*

INTRODUCTION

Retinal vessels can be visualized non-invasively and are used to assess systemic vascular damage.[1] In particular, retinal vessel diameters may reflect the condition of intracerebral vessels and their investigation may help to understand the etiology of cerebrovascular disease.[2] Larger retinal venular diameters are associated with atherosclerosis, cholesterol levels and markers of inflammation,[3-5] and with an increased risk of stroke and progression of cerebral small vessel disease.[6,7] This suggests that inflammation may be involved in the etiology of cerebrovascular disease. In addition, recent findings from a twin study and the family and genome wide association study performed in the Beaver Dam Eye Study (BDES) suggest that retinal arteriolar and venular diameters are at least in part genetically determined.[8-10] Moreover, the association between genetic factors and retinal vessel diameters in the BDES has been reported to be independent of hypertension,[10] suggesting that genetically determined processes other than hypertension may play a role in retinal vessel caliber.

Inflammation initiates and promotes atherosclerosis.[11] Complement plays a role in the promotion of inflammation as complement and complement regulatory factors are deposited in atherosclerotic plaques.[12] Complement factor H (CFH) is an essential plasma protein in the regulation of the complement pathway and may be important in the inhibition of complement early in the process of atherosclerosis.[13] Recently, the His allele of the Tyr402His polymorphism (rs1061170) in the *CFH* gene (chromosome 1q32) has been found to increase the susceptibility of aging macula disorder.[14-19] Because atherosclerosis has been implicated in the etiology of aging macula disorder, this association may at least in part reflect atherosclerosis.[20] The observation that the His^{402} allele of the Tyr402His polymorphism was also associated with an increased risk of myocardial infarction,[21] provides further support for this hypothesis.

A role for the Tyr402His variant in the *CFH* gene in atherosclerosis is biologically plausible, given its location within a region of positively charged amino acids that are implicated in the binding of heparin and C-reactive protein.[22] Normally, the ability of CFH to downregulate the effects of complement is facilitated by binding to these molecules. The substitution of a positively charged histidine for a non-charged hydrophobic tyrosine at position 402 alters CFH binding properties to heparin and C-reactive protein.[23] As a result, heterozygous and in particular homozygous carriers have malfunctioning CFH, ultimately leading to increased complement-related damage to the vascular endothelium,[18] especially in the presence of acute and chronic inflammatory mediators of the complement pathway.[19,21]

We further analyzed the influence of inflammation on the retinal vasculature by examining associations between the Tyr402His polymorphism and retinal arteriolar or venular diameters, including effect modification by smoking and inflammatory media-

tors. We hypothesized that carriers of the His[402] allele had larger retinal vessel diameters, in particular venular ones.

METHODS

Study population

The Rotterdam Study is a large population-based, prospective cohort study among 7,983 elderly aged 55 years or older residing in a suburb of Rotterdam, the Netherlands, assessing incidence and determinants of chronic diseases in the elderly.[24] In 1990 to 1993, trained research assistants visited all participants at home and obtained information on sociodemographic characteristics, medical history, current health status, medication use and determinants for these diseases. In addition, the participants were invited to the research center for a clinical examination by the research physicians. Since eye examinations became operational a few months after the baseline examinations had started, a smaller number (n = 6,780; response rate 78%) participated in the ophthalmic part of the study.[25] Fundus transparancies were available in 6,436 participants and of these, 762 participants were excluded because they had ungradable fundus transparencies on both eyes. DNA was available from 5,291 of the remaining 5,674 participants. Genotyping failed in 225, leaving 5,066 persons as the study sample for this cross-sectional study. The study was conducted according to the tenets of the Declaration of Helsinki, and the Medical Ethics Committee of the Erasmus University approved the study protocol. Written informed consent was obtained from all participants.

Retinal vessel measurements

Fundus transparencies were taken centered on the optic disk (20° field, Topcon Optical Company, Tokyo, Japan) after pharmacological mydriasis,[26] and digitized with a high-resolution scanner (Nikon LS-4000, Nikon Corporation, Japan). For each participant the digitized image with the best quality of either eye was analyzed with the Retinal Vessel Measurement System (Retinal Analysis, Optimate, WI; Department of Ophthalmology & Visual Science, University of Wisconsin-Madison, USA).[1,3] Within a half to one disk diameter from the optic disk all retinal arteriolar and venular diameters were measured to calculate summary values of the central retinal arteriolar and venular diameters. Four trained graders performed the assessments, masked to the clinical characteristics of the participants. Pearson's correlation coefficients for intergrader agreement were 0.67-0.80 for arteriolar diameters and 0.91-0.94 for venular diameters. For intragrader agreement these figures were 0.69-0.88 (arteriolar diameters) and 0.90-0.95 (venular diameters).

Genotyping

Participants were genotyped for the Tyr402His (1277T > C) (rs 1061170) polymorphism of the *CFH* gene. Genotypes were determined in 2 ng genomic DNA with the Taqman allelic discrimination assay (Applied Biosystems, Foster City, California). Primer and probe sequences were optimised by using the SNP assay-by-design service of Applied Biosystems. Reactions were performed with the Taqman Prism 7900HT 384 wells format as described by Fang et al.[27]

Covariates

Smoking status (categorized as current, former and never smoking) and medication use were assessed at the baseline interview. At the research center, blood was drawn directly into VACUTAINER® tubes and erythrocyte sedimentation rate was read after 60 minutes. Leukocyte count was assessed in citrate plasma using a Coulter Counter T540® (Coulter electronics, Luton, England). High-sensitivity C-reactive protein was measured in serum samples kept frozen at -20°C, using a rate near-infrared particle immunoassay method (Immage high sensitive CRP®, Beckman Coulter, USA). Fibrinogen was measured in platelet poor plasma kept frozen in liquid nitrogen and stored at -80°C. Fibrinogen levels were derived from the clotting curve of the prothrombin time assay using Thromborel S as a reagent on an automated coagulation laboratory (ACL 300, Instrumentation Laboratory).

As blood pressure we took the average of two measurements in sitting position with a random zero sphygmomanometer at the brachial artery. An ultrasound carotid artery atherosclerotic plaque score (range: 0-6) reflected the number of locations with plaques at the bifurcation, common, and internal carotid artery on both sides.[28] Non-fasting serum total and high-density lipoprotein (HDL) cholesterol concentrations were determined by an automated enzymatic procedure.[29] Height and weight were measured and the body mass index was calculated by dividing body weight (kg) by height squared (m^2). Diabetes mellitus was considered present if participants reported use of antidiabetic medication or when the random or post-load serum glucose level was greater than 11.1 mmol/l.

Data analysis

Hardy-Weinberg equilibrium of the Tyr402His polymorphism was tested using a chi-square test. Analysis of covariance was used to compute age and sex adjusted mean vessel diameters within genotype. To examine effect modification by complement activators, we subsequently performed separate analyses stratified on smoking status, and the inflammatory markers erythrocyte sedimentation rate, leukocyte count, serum C-reactive protein and fibrinogen. For smoking, participants were categorized as never, former and current smokers. For the inflammation markers, the median value was used to divide participants into two subgroups, one below and one above the median value of the variable. These

analyses were also adjusted for blood pressure, carotid artery plaque score, serum total and HDL cholesterol, BMI and diabetes mellitus. We next performed additional analyses stratifying on hypertensive status, diabetes, carotid artery plaque score and BMI. For hypertension and diabetes, participants were grouped into those with hypertension and diabetes, and those without. For the carotid artery plaque score and BMI the median value was used to divide participants into two subgroups, one below and one above the median value of the variable. All analyses were performed using SPSS 11.0 for Windows (SPSS Inc., Cary, North Carolina).

RESULTS

Genotype distributions were in Hardy-Weinberg equilibrium. The overall frequency of the His allele was 36% and genotype frequencies were 41% for TyrTyr, 45% for TyrHis and 14% for HisHis carriers. Baseline characteristics of all participants with gradable fundus transparencies and genotyping for the Tyr402His polymorphism are shown in Table 1.

Table 1. Baseline characteristics of the study population*

	Total (n=5,066)
Age (years)	67.9 (8.1)
Sex (% female)	58.2
Systolic blood pressure (mmHg)	138.4 (21.9)
Diastolic blood pressure (mmHg)	73.6 (11.2)
Body mass index (kg/m^2)	26.3 (0.4)
Total cholesterol (mmol/L)	6.6 (1.2)
HDL cholesterol (mmol/L)	1.3 (0.4)
Diabetes mellitus (%)	9.5
Smokers (%)	
Never	33.2
Current	23.6
Former	43.2
Erythrocyte sedimentation rate (mm/hour)	12.9 (10.7)
Leukocyte count (10^9/L)	6.7 (1.9)
Serum Fibrinogen (g/L)	2.8 (0.7)
Serum C-reactive protein (mg/L)†	1.79 (0.87-1.79)

* Values are means (standard deviations) or percentages.

† Median (interquartile range) are presented because of the skewed distribution

Table 2. The Tyr402His variant in the *CFH* gene and the association with mean summarized retinal arteriolar and venular diameters, stratified according to inflammatory mediators, hypertensive status, diabetes, carotid artery plaque score and body-mass index*

	Estimated central retinal arteriolar diameter				Estimated central retinal venular diameter			
	TyrTyr	TyrHis	HisHis	*P*-trend	TyrTyr	TyrHis	HisHis	*P*-trend
Number (%)	2,088 (41)	2,279 (45)	699 (14)		2,088 (41)	2,279 (45)	699 (14)	
All	146.8 (0.31)	147.0 (0.30)	146.8 (0.54)	0.94	222.5 (0.44)	222.0 (0.43)	220.5 (0.77)	0.03
Smoking								
- never	145.1 (0.54)	145.7 (0.51)	145.0 (0.93)	0.80	218.2 (0.77)	217.1 (0.72)	213.8 (1.33)	0.01
- former	146.5 (0.48)	146.2 (0.47)	146.8 (0.85)	0.99	222.1 (0.67)	221.8 (0.65)	221.3 (1.18)	0.53
- current	150.1 (0.66)	150.8 (0.61)	149.7 (1.09)	0.97	229.8 (0.95)	229.5 (0.88)	228.7 (1.57)	0.57
Sedimentation rate								
≤ 10 mm/hr	148.3 (0.51)	147.4 (0.49)	148.9 (0.89)	0.98	225.3 (0.72)	222.3 (0.69)	224.3 (1.26)	0.08
> 10 mm/hr	147.4 (0.57)	148.3 (0.52)	146.5 (0.93)	0.79	222.2 (0.78)	222.6 (0.72)	218.5 (1.28)	0.06
Leukocyte count								
≤ 10 (10^9/ L)	146.7 (0.46)	146.2 (0.44)	146.2 (0.82)	0.43	220.6 (0.64)	219.3 (0.61)	218.0 (1.14)	0.03
> 10 (10^9/ L)	147.6 (0.46)	148.0 (0.43)	147.6 (0.76)	0.79	224.8 (0.65)	224.3 (0.62)	222.6 (1.09)	0.11
C-reactive protein								
≤ 1.78 mg/L	147.0 (0.46)	146.9 (0.44)	147.0 (0.80)	0.98	221.5 (0.66)	220.7 (0.63)	219.1 (1.13)	0.08
> 1.78 mg/L	147.0 (0.46)	147.0 (0.44)	145.9 (0.80)	0.31	223.5 (0.65)	223.0 (0.62)	221.4 (1.13)	0.14
Fibrinogen								
≤ 2.6 g/L	146.0 (0.65)	146.7 (0.62)	147.3 (1.14)	0.27	223.1 (0.95)	220.3 (0.91)	221.5 (1.66)	0.13
> 2.6 g/L	146.9 (0.67)	146.4 (0.62)	148.6 (1.15)	0.43	222.6 (0.99)	221.8 (0.92)	220.0 (1.70)	0.19
Hypertension								
No	148.3 (0.42)	148.9 (0.40)	147.6 (0.40)	0.68	223.6 (0.60)	223.4 (0.56)	220.8 (1.01)	0.05
Yes	143.8 (0.65)	143.6 (0.63)	143.9 (1.11)	0.99	220.4 (0.93)	220.2 (0.91)	219.0 (1.59)	0.53
Diabetes mellitus								
No	146.7 (0.33)	147.0 (0.32)	146.7 (0.57)	0.90	222.6 (0.47)	221.9 (0.45)	220.6 (0.81)	0.04
Yes	147.8 (0.94)	147.9 (0.86)	147.6 (1.61)	0.95	221.8 (1.50)	223.2 (1.37)	219.4 (2.55)	0.70
Carotid plaque score								
≤ 1	148.0 (0.46)	148.0 (0.42)	147.8 (0.79)	0.82	223.4 (0.64)	222.3 (0.58)	220.9 (1.08)	0.04
> 1	146.3 (0.54)	146.4 (0.53)	145.7 (0.94)	0.68	223.1 (0.77)	221.9 (0.76)	219.6 (1.35)	0.03
Body mass index								
≤ 26.0 kg/m^2	147.4 (0.46)	147.6 (0.43)	147.5 (0.78)	0.87	222.7 (0.65)	222.0 (0.61)	220.5 (1.11)	0.10
> 26.0 kg/m^2	146.3 (0.43)	146.4 (0.42)	145.9 (0.75)	0.78	222.3 (0.61)	222.1 (0.60)	220.4 (1.07)	0.19

* Values are age and sex adjusted means (standard errors)

Table 2 shows the association of the Tyr402His polymorphism with retinal vessel diameters, both in all participants and after stratification on complement activators or cardiovascular risk factors. The His^{402} allele was not associated with arteriolar diameters, whereas it was associated with smaller venular diameters in an allele-dose dependent way. Stratification on the participants' smoking status showed that this association was present

among never smokers, but absent in current or former smokers, although this interaction was statistically not significant (*P*-value for interaction = 0.15). No effect modification was observed when we stratified on serum inflammatory markers (*P*-values for interaction all >0.65). Additional adjustment for established cardiovascular risk factors did not change the results. When stratifying on cardiovascular risk factors, the association of the His^{402} allele with larger venular diameters was stronger among those without hypertension or diabetes, but the interaction terms were statistically not significant (*P*-values for interaction 0.62 and 0.93 respectively). Also, no effect modification was observed when stratifying on the carotid artery plaque score or BMI (*P*-values for interaction 0.52 and 0.32 respectively).

DISCUSSION

Carriers of the His^{402} allele had smaller retinal venular diameters in an allele-dose dependent way. This association was present only in never smokers and was not modified by inflammatory mediators of complement, or adjustment or stratification for established cardiovascular. Retinal arteriolar diameters were not related to the Tyr402His variant. Thus, we could not support our hypothesis that larger retinal venular diameters are associated with the His^{402} allele of the Tyr402His polymorphism.

Strengths of this study are the population-based design, the detailed assessment of vessel diameters on 20° stereoscopic transparencies leading to larger magnified images, and the adjustment for refractive errors of the eye. This enabled us to estimate the intra-luminal arteriolar and venular diameters more in detail, where others reported uncorrected vessel diameters in pictures with smaller magnification.[1,30,31] Another advantage is the use of a genetic marker. This approach deals with reverse causation as genotype is determined before disease onset.[32] Limitations related to the semi-automated system assessing the retinal vessel diameters have been described.[1] For instance, photographs were taken independent of the cardiac cycle and effects of pulsatility on vessel width cannot be ruled out. Most likely these limitations led to an underestimation of our effects due to random misclassification because photography and assessment of vessel diameters was unrelated to clinical characteristics of the participants. Allele frequencies were in correspondence with previous reports from the Rotterdam Study.[19,21] We therefore consider selection bias due to selective non-participation of HisHis carriers unlikely.

CFH is an important regulator of the complement pathway. Activation of this pathway initatiates a proteolytic cascade that releases pro-inflammatory anaphylatoxins and causes formation of a membrane-attack complex ultimately leading to cell lysis. CFH is a potent inhibitor of the complement pathway by binding and inactivating complement component C3b. This prevents the production of C3 convertase in the alternative cascade and production of C5 convertase in the common pathway. As a result, CFH

interferes with progression of the entire cascade.[23] A malfunctiong CFH may lead to less inactivation of the complement component C3b and as a result of the entire complement cascade, presumably by altered binding to C-reactive protein and heparin. Heterozygous and in particular homozygous carriers of the His^{402} variant are genetically predisposed to a malfunctioning CFH. This seems of importance in particular when the complement cascade is switched on by acute and chronic inflammatory mediators including C-reactive protein, leukocyte count, white blood cell count, fibrinogen and smoking.[19,21]

Because higher levels of the above mentioned inflammation markers and smoking are all associated with larger retinal vessel caliber, and in particular larger venular diameters,[3-5] we hypothesized that carriers of the His^{402} allele would have larger venular diameters, especially in the presence of inflammatory mediators. In contrast, we found an association of the His^{402} allele with smaller venular diameters compared to carriers of the Tyr^{402} allele. This association was not modified by inflammatory mediators and was present in never rather than current smokers. Given that only 222 participants among the never smokers were carrier of the HisHis allele, we consider this result most likely to be a chance finding due to the relatively low numbers.

Alternative explanations should be discussed. First, the His^{402} allele could be related to smaller venular diameters through mechanisms other than inflammation. The Tyr402His variant is located within a cluster of positively charged amino acids that is implicated in the binding of CFH to not only C-reactive protein but also to heparin,[22] which normally increases the affinity of CFH for cell surface-bound C3b.[23] Carriers of the His^{402} allele might have a less effective binding to heparin. The association of larger retinal venular diameters with inflammation has previously been ascribed to damage to the endothelial surface layer caused by oxidized low density lipoproteins and activated leukocytes.[3] Heparin attenuates leukocyte and low density lipoprotein related damage to the venular endothelial surface.[33] If less effective binding of *CFH* to heparin leads to higher plasma levels of heparin, this could indirectly have lead to the observed decrease in venular diameter. However, the potential influence of the Tyr402His polymorphism on *CFH* function and interaction with heparin is currently unknown.[23] Also, we cannot explain why this mechanism would be of importance among never smokers only. Second, it needs to be elucidated whether the Tyr402His polymorphism is the variant that actually underlies the association with venular diameters, rather than merely reflecting another marker within the *CFH* gene in complete or partial linkage disequilibrium.

In conclusion, our findings do not support the hypothesis that larger retinal venular diameters are related to the His^{402} allele of the Tyr402His polymorphism in the *CFH* gene. The association of the His^{402} allele with smaller retinal venular diameters is most likely to be a chance finding, it was present only among never smokers and not modified by inflammatory mediators of complement. Taken together, our findings suggest that the Tyr402His variant is not related to retinal vessel diameters.

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Chapter 4.4

Arterial oxygen saturation, cerebral blood flow and retinal vessel diameters

ABSTRACT

Purpose: *Retinal vessel diameters, in particular larger venular diameters, have been associated with cerebrovascular disease. Larger retinal venular diameters may reflect cerebral ischemia. We investigated whether arterial oxygen saturation (SaO_2) and total cerebral blood flow (CBF), indicators of cerebral oxygen supply, are associated with retinal arteriolar or venular diameters.*

Design: *Cross-sectional study performed within the population-based Rotterdam Study.*

Participants: *Randomly selected participants aged 55 years or older (n = 696), who underwent both an eye examination and brain magnetic resonance imaging (MRI).*

Methods: *SaO_2 was determined by pulse oximetry on the right index finger. CBF was assessed using a phase-contrast MRI sequence that measured the flow in the basilar and both internal carotid arteries. Brain volume was measured to express CBF per 100 ml brain volume. Retinal arteriolar and venular diameters were measured on digitized fundus color transparencies on one eye of each participant. Regression models were used to investigate the association of SaO_2 and CBF with retinal vessel diameters.*

Main outcome measures: *Mean retinal arteriolar and venular diameters (in μm).*

Results: *Lower SaO_2 was associated with larger venular diameters. Persons with SaO_2 <96% (n = 113) had on average 5 μm larger venular diameters compared to those with SaO_2 ≥96% (n = 583) (age and sex adjusted mean difference 5.6 μm, 95%CI, 1.2;10.0). CBF was not related to venular diameters when analyzed separately. Additional analyses showed that the association between SaO_2 and venular widening was confined to participants within the lowest tertile of CBF. No associations were found between SaO_2 or CBF and arteriolar diameters. Additional adjustment for established cardiovascular risk factors did not change the results.*

Conclusions: *We observed an association of lower SaO_2 with larger retinal venular diameters, in particular in the presence of lower CBF. Our findings suggest that venular widening may reflect a lower oxygen supply, especially to the brain.*

INTRODUCTION

Retinal vessels share many characteristics with intracerebral vessels and their investigation may help to understand the etiology of cerebrovascular disorders. A lower ratio of arteriolar-to-venular diameters (AVR) was suggested to reflect generalized arteriolar narrowing due to hypertension and has been associated with presence of white matter lesions and subclinical brain infarcts on magnetic resonance imaging (MRI) and an increased risk of cerebral stroke.[1-3] Yet, a lower AVR may either result from arteriolar narrowing or venular widening. Recently, larger venular rather than smaller arteriolar diameters were found to be associated with cerebrovascular disease, both progression of white matter lesions and an increased risk of stroke.[4,5]

The mechanisms underlying retinal venular dilatation remain incompletely understood. Although larger venular diameters are associated with inflammation and atherosclerosis,[6-8] other mechanisms may also be involved. Retinal venular dilatation is observed in the early stages of diabetic and venous stasis retinopathy, both of which are characterized by retinal hypoxia,[9,10] and are also associated with an impaired cerebral blood flow (CBF).[10,11] Venular dilatation has therefore been hypothesized to be a marker of not only retinal but also of cerebral ischemia.[4]

The total amount of oxygen available to the brain is determined by both arterial oxygen content and CBF. The assessment of the arterial oxygen pressure is a relatively invasive procedure, and therefore difficult to apply in population-based studies. The arterial oxygen saturation (SaO_2) as assessed by pulse oximetry is a non-invasive measure that approximates the arterial oxygen pressure.[12] The total CBF can be assessed non-invasively by measuring the flow in the supplying vessels of the brain using magnetic resonance imaging (MRI).[13]

We considered SaO_2 and CBF as indicators of brain oxygenation and hypothesized that there would be an association of lower SaO_2 and lower CBF with larger retinal vessel caliber, in particular larger retinal venular diameters. We therefore examined the association of SaO_2 and CBF with retinal arteriolar and venular diameters using cross-sectional data from the population-based Rotterdam Study.

METHODS

Study population

The Rotterdam Study is a population-based prospective cohort study among 7,983 elderly and ongoing since 1990.[14,15] From 2000 to 2002 the cohort was extended with 3,011 persons (≥55 years). Home interviews and physical examinations, including eye examinations, were performed during both the baseline (2000-2002) and a follow-up

examination (2002-2004) of this extended cohort. Between August 2005 and May 2006, we randomly invited 1,073 of the 3,011 persons to undergo brain magnetic resonance imaging (MRI) as part of the Rotterdam Scan Study II. The institutional review board approved the study. Persons with MRI contraindications were excluded, leaving 975 eligible persons of whom 907 participated and gave written informed consent (response 93%). Complete MRI examinations were available in 895 persons, 810 of whom had had eye examinations performed and fundus transparencies available in 2002-2004. Of these 810, 94 persons were excluded because they had ungradable fundus transparencies on both eyes and an additional 20 persons were excluded because SaO_2 and CBF measurements were missing, resulting in a current study population of 696 participants. The mean interval between the eye examinations and the MRI scan was 1.0 year (SD 0.5 years).

Retinal vessel measurements

Fundus transparencies were taken centered on the optic disk (20° field, Topcon Optical Company, Tokyo, Japan) after pharmacological mydriasis,[16] and digitized with a high-resolution scanner (Nikon LS-4000, Nikon Corporation, Japan). For each participant the qualitatively best digitized image of either eye was analyzed with the Retinal Vessel Measurement System.[17] Retinal arteriolar and venular diameters were measured and summarized using Parr-Hubbard-Knudtson formulas and Littmann's formula to adjust for refractive errors of the eye.[6] Two trained graders performed the assessments, masked to participants' clinical characteristics. Pearson's correlation coefficients for intergrader agreement were 0.87 for arteriolar and 0.91 for venular diameters. Intragrader agreement was 0.65-0.85 for arteriolar and 0.82-0.86 for venular diameters.

Assessment of arterial oxygen saturation, cerebral blood flow, and covariates

SaO_2 was measured twice with a pulse oximeter (Oxycount, Andos, Hamburg, Germany) on the right index finger, and was recorded with increments of 0.5 percent point. The average of the two measurements was computed and used for the analysis.

We measured CBF according to previously described methods.[13,18] Briefly, a sagittal 2D phase-contrast MR angiographic scout image was performed on which a transverse plane for ungated phase-contrast imaging was chosen perpendicular to the precavernous portion of the internal carotid arteries and to the middle part of the basilar artery. Flow was calculated from the phase-contrast images using IDL-based custom software (Cinetool, GE Healthcare, USA). Circular regions of interest (ROI) were drawn manually around the basilar and both internal carotid arteries on the phase-contrast images and encompassed the entire lumen of the vessel. The mean signal intensity in each ROI reflected the flow velocity in cm/s. Flow in ml/s was calculated by multiplying the mean flow velocity by the cross-sectional area of the vessel. Flow rates for the basilar and carotid arteries were summed and multiplied by 60 to obtain CBF in ml/min. Because low CBF may

merely reflect a lower oxygen demand in persons with smaller brain volume rather than cerebral ischemia, we calculated CBF in ml/min/100 ml brain volume. Brain volume (in ml) was assessed on structural MRI sequences using automated tissue classification.[19] CBF was divided by brain volume and multiplied by 100 to yield CBF per 100 ml brain volume. Independent of each other, two experienced technicians performed all manual ROI drawing and flow measurements. Intergrader agreement was excellent (Pearson correlation coefficient ≥0.94 for all vessels).

Cardiovascular risk factors may confound an association of CBF and SaO_2 with retinal vessel diameters. Blood pressure, smoking, diabetes mellitus, body mass index, blood pressure, atherosclerotic plaques, total and HDL cholesterol and leukocyte count have all been previously associated with retinal vessel diameters and were assessed according to methods that have been described.[6] For the current report we used cardiovascular risk factors assessed in 2002-2004.

Data analysis

Analysis of covariance (ANCOVA) was used to compare participants with gradable and ungradable fundus transparencies and to investigate whether associations between SaO_2 or CBF with vessel diameters were linear. SaO_2 was analysed according to the discrete values in the population. CBF, which is a continuous variable, was analysed in quintiles. Because initial analyses with SaO_2 suggested a threshold at 96% SaO_2, we performed additional analyses with SaO_2 as a dichotomous variable (persons with SaO_2 ≥96% were reference). Initial analyses with CBF in quintiles showed a linear relationship with retinal vessel diameters. We subsequently used multiple linear regression models to analyse the associations with retinal vessel diameters per standard deviation (SD) increase in CBF. Low SaO_2 has been reported to aggravate brain damage in concomitance with low CBF.[20] We therefore also investigated the association of SaO_2 with retinal vessel diameters stratified on CBF. Because of relatively low numbers in the group containing persons with SaO_2 <96% ($n = 113$), CBF was stratified in tertiles. All analyses were adjusted for age and sex and additionally for the abovementioned cardiovascular risk factors.

RESULTS

Baseline characteristics of persons with gradable and ungradable fundus transparencies are shown in Table 1. Those excluded were significantly older. After adjustment for age and sex, no differences were observed in other risk factors.

Figure 1 shows the association of SaO_2 and CBF with retinal vessel diameters. Lower SaO_2 was associated with larger venular diameters, but not with arteriolar diameters. Persons with SaO_2 <96% ($n = 113$) had on average 5 μm larger venular diameters compared

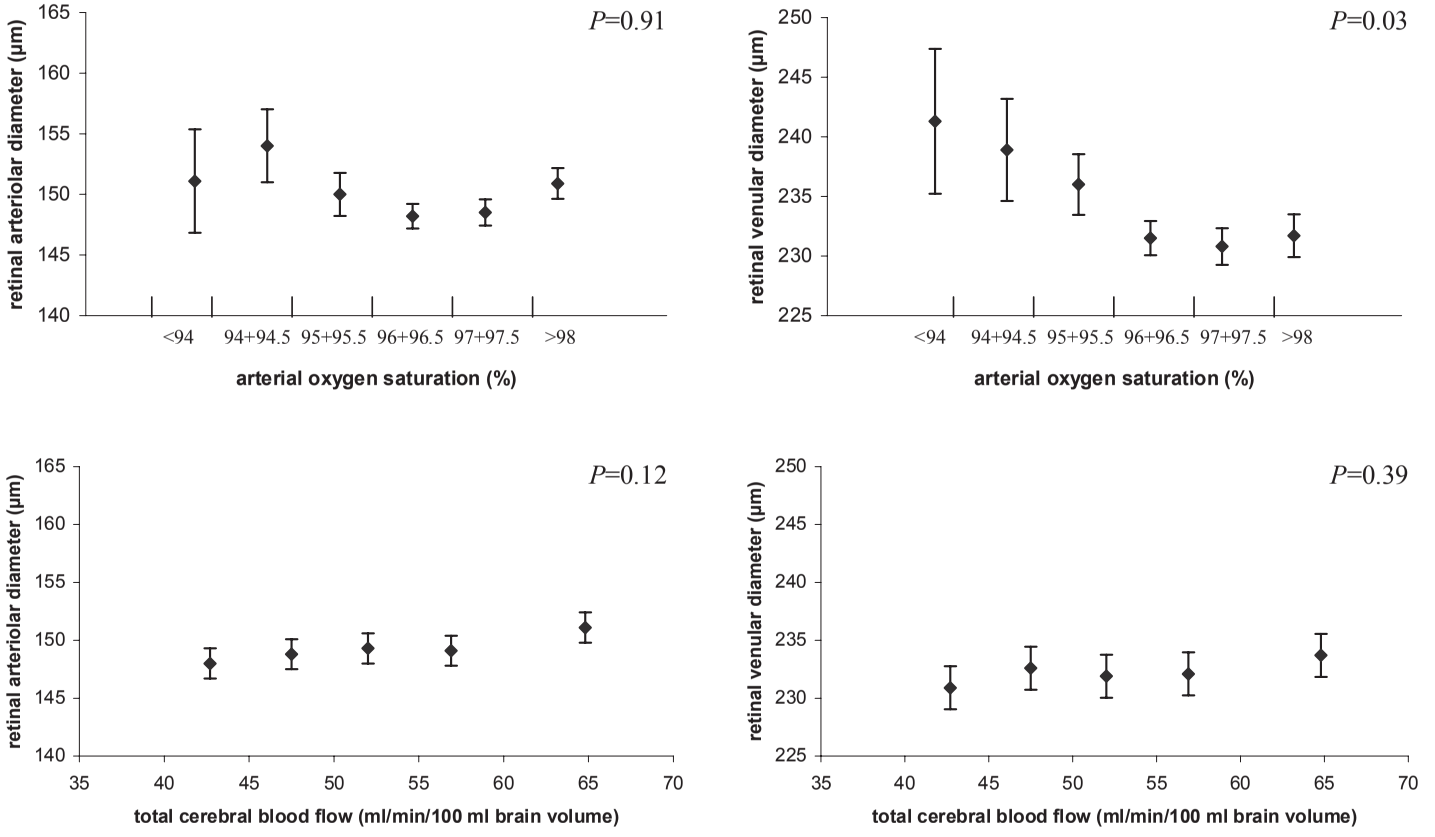

Figure 1. Arterial oxygen saturation, total cerebral blood flow, and retinal vessel diameters. Age and sex adjusted mean retinal venular diameters (SE) are plotted according to the discrete values of arterial oxygen saturation in the population (<94, 94+94.5, 95+95.5, 96+96.5, 97+97.5 and >98%) and at the median of each quintile of total cerebral blood flow (values per quintile: 42.7, 47.5, 52.0, 56.9 and 64.8 ml/min/100 ml brain volume). P-values are P-trends.

Table 1. Baseline characteristics presented as unadjusted means (SD) or percentages

	Gradable transparencies	Non-gradable transparencies	Adjusted differences* (95% CI)†
Number (n)	716	94	
Age (years)	67.1 (5.0)	69.7 (7.0)	2.9 (1.8; 4.1)‡
Sex (% female)	57	61	2.7 (-13.2; 7.7)
Systolic blood pressure (mmHg)	143.1 (17.9)	145.1 (20.1)	0.4 (-3.4; 4.2)
Diastolic blood pressure (mmHg)	80.9 (10.3)	80.2 (9.8)	0.4 (-1.7; 2.5)
Body mass index (kg/m^2)	27.5 (4.0)	27.4 (2.9)	0.1 (-7.6; 0.9)
Serum total cholesterol (mmol/L)	5.7 (1.0)	5.6 (1.0)	-0.1 (-0.3; 0.1)
Serum HDL cholesterol (mmol/L)	1.4 (0.4)	1.4 (0.4)	0.0 (-0.1; 0.1)
Carotid artery plaque score	2.9 (2.7)	3.0 (2.8)	0.3 (-0.3; 0.9)
Leukocyte count (10^9/L)	6.7 (1.8)	6.6 (1.5)	-0.1 (-0.5; 0.3)
Smoking (% current)	13	12	1.8 (-8.7; 12.3)
Diabetes mellitus (%)	8	13	4.5 (-10.5; 1.4)

* Age and sex adjusted, if applicable

† CI=confidence interval

‡ Significant ($p<0.05$) compared to those with gradable fundus transparencies

to those with SaO_2 ≥96% ($n = 583$) (age and sex adjusted mean difference 5.6 µm, 95%CI, 1.2;10.0). CBF was neither related to arteriolar nor to venular diameters (age and sex adjusted mean difference per SD increase in CBF: 0.96 µm (95% confidence interval (CI) -0.19; 2.10) for arteriolar and 0.66 µm (95%CI, -0.97; 2.29) for venular diameters). Figure 2 shows that the association of low SaO_2 with larger venular diameters was confined to persons within the lowest tertile of CBF (age and sex adjusted mean difference in venular diameter for persons with SaO_2 <96% ($n = 50$) compared to persons with SaO_2 ≥96% ($n = 189$): 11.3 µm, (95%CI, 4.7; 17.9)). Although a similar association was found with arteriolar diameters, this did not reach statistical significance. Adjustment for vascular risk factors did not change the results.

DISCUSSION

Our results show an association of lower SaO_2 with larger venular diameters, especially in the presence of low CBF, which was independent of cardiovascular risk factors. No significant associations were found for arteriolar diameters.

Strengths of this study are the population-based design, the assessment of vessel diameters on 20° stereoscopic transparencies in mydriasis and the adjustment for refractive errors of the eye to approximate absolute intra-luminal diameters. Some possible limitations need to be considered. Participants with gradable fundus transparencies were significantly younger than those with ungradable transparencies, which may have

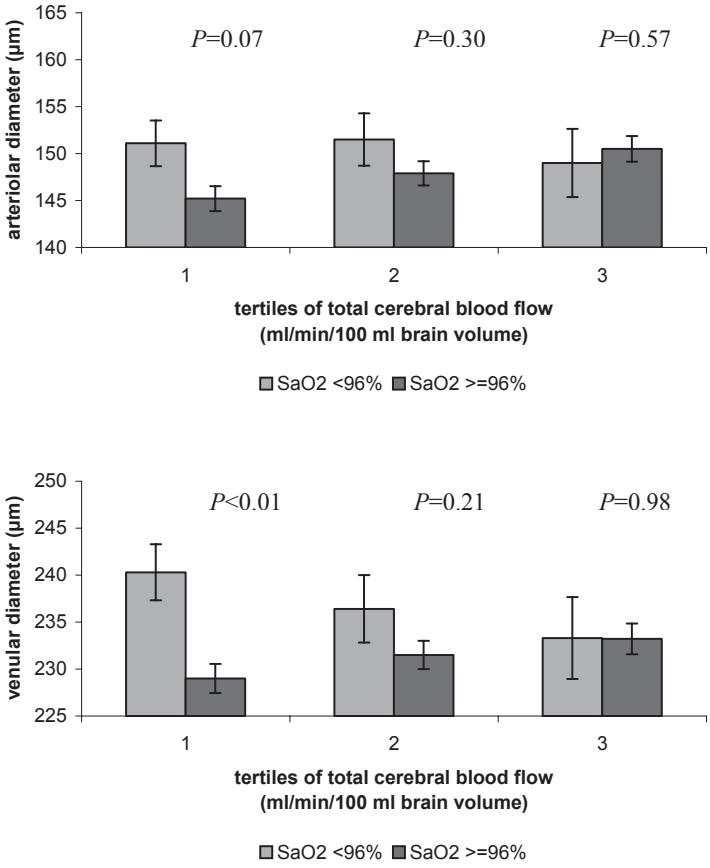

Figure 2. The association between retinal vessel diameters and arterial oxygen saturation, stratified according to tertiles of total cerebral blood flow. Values are age and sex adjusted mean retinal vessel diameters (SE). *P*=P-value for the age and sex adjusted mean difference in vessel diameters for persons with SaO_2 < 96% compared to those with SaO_2 ≥ 96%, within tertile of total cerebral blood flow.

caused selection bias. After adjustment for age and sex we observed no differences in cardiovascular risk factors between persons with gradable or ungradable transparencies, suggesting a limited role for this bias. Further, the accuracy and precision of pulse oximetry is questionable below 70-80% SaO_2.[12,21] In our study population this has not caused bias, because all participants had SaO_2 values of 89.5% or higher. Finally, both CBF and retinal vessel diameters were measured independent of the cardiac cycle. Effects of pulsatility on cerebral and retinal vessel diameters can therefore not be ruled out. Yet, Spilt et al. evaluated the reproducibility of CBF measurements using phase-contrast MRI with and without cardiac triggering and found no differences between both methods.[18] Although retinal vessel diameter variability due to the pulse cycle has been reported,[22] photography was independent of participants' clinical characteristics and will have caused random misclassification.

The total amount of oxygen that is available to the brain is dependent on both cerebral arterial oxygen content and CBF. Although CBF as assessed on MRI provides a direct measure of the total arterial CBF, SaO_2 is an indirect measure of the arterial oxygen pressure in the cerebral arteries. SaO_2 is related to the arterial oxygen pressure by the oxygen-haemoglobin dissociation curve, which is determined by the blood CO_2 concentration, temperature and 2,3-diphosphoglycerate concentration.[23] Although we did not measure these variables, pulse oximetry was performed under similar conditions for all participants. In addition, these factors mainly affect the relation between SaO_2 and oxygen pressure in capillary blood,[23] whereas pulse oximetry determines SaO_2 in arterial blood. We therefore assume that SaO_2 is an adequate reflection of arterial oxygen pressure.

The observation that larger venular diameters are associated with lower SaO_2 especially in the presence of lower CBF, supports the hypothesis that larger venular diameters may reflect a lower oxygen supply, in particular of the brain. Our findings are in agreement with observations from experimental studies suggesting that low SaO_2 exacerbates ischaemic brain damage only in addition to reduced CBF.[20] The absence of brain damage due to low SaO_2 alone has been ascribed to cerebral autoregulation increasing CBF in conditions of reduced arterial oxygen content.[24] Furthermore, it has been suggested that a lower SaO_2 is more harmful in concomitance with chronic or intermittent hypoperfusion, which is ascribed to impaired cerebral autoregulation, rather than in conditions inducing acute hypoperfusion.[25]

Because both CBF and SaO_2 were measured in the arterial rather than the venular system, an association with retinal arteriolar diameters might be expected. Yet, smaller retinal arteriolar diameters are strongly associated with higher blood pressures.[6,17] The weaker association of arteriolar diameters with CBF and SaO_2 may reflect a diminished capability of retinal arterioles to dilate due to hypertension and subsequent thickening of the intima-media and eventually sclerosis of the vessel wall at higher age.[6] This may have precluded a degree of widening in the retinal arterioles equal to that observed in retinal venules.

Furthermore, how a lower SaO_2 and CBF are related to in particular retinal venular widening, remains to be further investigated. The cross-sectional design of our study precludes any conclusion on the temporal relationship between SaO_2 and CBF on the one hand, and retinal venular caliber on the other hand. In addition, findings from in vitro experiments suggest that the microvasculature is highly dynamic, leading to structural remodeling of the vascular wall and rapid changes in vascular caliber in response to alterations in blood pressure, blood flow, or vasoactive agents.[26] How these factors are related to microvascular remodeling, and in what direction this would affect vascular caliber, remains poorly understood. Furthermore, microvascular remodeling has been mainly studied in coronary arterioles, and hardly in the cerebral or retinal circulation. To what extent it applies to venules is also unclear.

In conclusion, we observed an association of lower SaO_2 with larger retinal venular diameters especially in the presence of lower CBF. Our findings suggest that larger venular diameters may reflect a lower oxygen supply, in particular of the brain. Further studies are needed to clarify the underlying mechanism and the clinical relevance of our finding.

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Chapter 5

Retinal vessel diameters, cerebrovascular disease and dementia

Chapter 5.1

Retinal vessel diameters and cerebral small vessel disease

ABSTRACT

The direct visualization of retinal vessels provides a unique opportunity to study cerebral small vessel disease, because these vessels share many features. It was reported that persons with smaller retinal arteriolar-to-venular ratio tended to have more white matter lesions on MRI. It is unclear if this is due to arteriolar narrowing or venular dilatation. We investigated if smaller arteriolar or larger venular diameters were related to severity and progression of cerebral small vessel disease.

We studied 490 persons (60-90 years) without dementia from a population-based cohort study. At baseline (1990-1993), retinal arteriolar and venular diameters were measured on digitized images of one eye of each participant. In 1995-1996, participants underwent cerebral MRI scanning. We rated severity of periventricular white matter lesions on a 9-point scale, approximated a total subcortical white matter lesion volume (range: 0-29.5 ml), and rated the presence of lacunar infarcts. On average 3.3 years later, 279 persons had a second MRI. We rated changes in periventricular and subcortical white matter lesions with a semi-quantitative scale, and classified progression as no, minor and marked. An incident infarct was a new infarct on the follow-up MRI.

Neither venular, nor arteriolar diameters were related to the severity of cerebral small vessel disease. Larger venular diameters were, however, associated with marked progression of cerebral small vessel disease. Age and sex adjusted odds ratios (OR) per standard deviation increase were 1.71 (95% confidence interval [CI]: 1.11; 2.61) for periventricular, 1.72 (95% CI: 1.09; 2.71) for subcortical white matter lesion progression, and 1.59 (95% CI: 1.06; 2.39) for incident lacunar infarcts. These associations were independent of other cardiovascular risk factors, only the OR for incident lacunar infarcts attenuated (1.24; 95% CI: 0.72; 2.12). No association was observed between arteriolar diameters and progression of cerebral small vessel disease.

In conclusion, retinal venular dilatation was related to progression of cerebral small vessel disease. The mechanisms underlying venular dilatation deserve more attention, as they may provide new clues into the pathophysiology of cerebral small vessel disease.

INTRODUCTION

Cerebral magnetic resonance imaging (MRI) in elderly people frequently reveals white matter lesions and lacunar infarcts. Prevalence of white matter lesions ranges from 5% to 90%,[1] whereas 20% of elderly have at least one brain infarct on MRI.[2] These lesions are related to incident stroke and may contribute to the development of dementia.[3-6] They reflect ischemic small vessel disease, though the exact patho-physiological mechanism is unknown.[7] Several studies point towards increasing age, hypertension and markers of atherosclerosis as main risk factors.[8-11] The pathological status of cerebral small vessels is difficult to assess in vivo. Most non-invasive markers of vascular pathology are related to major blood vessels outside the brain and may not represent local cerebral abnormalities. The retinal vessels provide unique opportunities to study cerebral small vessel disease, because they share similar anatomy, physiology, and embryology.[12]

Recently, a semi-automated system was developed to measure retinal vessel diameters.[13,14] Because these studies did not have enough data to correct for magnification differences due to refractive errors of eyes, an arteriolar-to-venular ratio (AVR) was introduced to bypass this problem. Subsequently, a smaller AVR was suggested to reflect generalized arteriolar narrowing and was associated with cardiovascular diseases.[13,14] In the Atherosclerosis Risk in Communities Study, persons with a smaller AVR tended to have more white matter lesions.[15] However, it can be questioned whether the AVR reflects solely retinal arteriolar narrowing, because venular width does not remain constant in various pathological conditions. We reported that smaller arteriolar diameters were related to higher blood pressures, whereas larger venular diameters were related to markers of atherosclerosis and inflammation.[16]

We investigated whether arteriolar or venular diameters in the retina were related to the severity and progression of cerebral small vessel disease.

METHODS

Study population

The present study was performed as part of the Rotterdam Study, a population-based cohort study on chronic diseases in the elderly.[17] All inhabitants of a district of the city of Rotterdam aged 55 years or over were invited in random order to the study, and 7,983 actually participated (overall response 78%). The study was conducted according to the Declaration of Helsinki, and the Medical Ethics Committee of the Erasmus Medical Center approved the study protocol. A written informed consent was obtained from all participants. Because the ophthalmic part became only operational after the study had started, a total of 6,780 participants underwent baseline ophthalmic examination (1990-

1993).[16] From 1995 to 1996, we randomly selected participants (aged 60 to 90 years) of the Rotterdam Study cohort in strata of sex and 5-year age categories for participation in the Rotterdam Scan Study, a study on age-related brain changes on MRI.[18] Complete information, including a cerebral MRI scan, was obtained in 490 individuals, who also had had the ophthalmic examination at baseline. Of these, 435 participants were still alive and without contraindications in 1999-2000 and 279 of them underwent a second MRI scan.

Retinal vessel measurements

The ophthalmic examination at baseline included taking simultaneous stereoscopic fundus transparencies centered on the optic disc of both eyes with a telecentric fundus camera (pharmacological mydriasis, 20° field, Topcon Optical Company, Tokyo, Japan).[16] These transparencies were digitized with a high-resolution scanner (Nikon LS-4000, Nikon Corporation, Japan) and for each participant one eye with the best image quality was analyzed with a semi-automated system (Retinal Analysis, Optimate, WI; Department of Ophthalmology & Visual Science, University of Wisconsin-Madison). Per eye one summary value was calculated for the diameters of the blood column in the retinal arterioles and one for the venules (in μm) after correction for differences in magnification due to refractive status of the eye. The AVR was defined as the ratio of arteriolar to venular diameters.[19,20]

In a random sub-sample of 100 participants we found no statistically significant differences between right and left eyes for the arteriolar and venular diameters. Four trained graders performed all measurements masked for participant characteristics. Both inter- and intragrader studies ($n = 40$) showed good to excellent agreement (r = 0.67-0.95).[16]

MRI scanning

In 1995-1996, axial T1-, T2- and proton density (PD)-weighted cerebral MR scans were made on a 1.5-Tesla MRI scanner (MR VISION, Siemens).[21] In 1999-2000, participants underwent a second MRI on the same MR VISION scanner with the same sequences.

White matter lesions

White matter lesions were considered present when visible as hyper-intense on proton density and T2-weighted images, without prominent hypo-intensity on T1-weighted scans. We considered white matter lesions to be in the periventricular region if they were directly adjacent to the ventricle; otherwise we called them subcortical. We scored periventricular white matter lesions semi-quantitatively in three regions (lesions adjacent to the frontal horns, the lateral walls, and the occipital horns of the lateral ventricle) resulting in a total score ranging from 0-9. For subcortical white matter lesions we ap-

proximated a total volume based on number and size of lesions (volume range: 0-29.5 ml). Both intra-and inter-rater studies ($n = 100$) showed a good to excellent agreement ($\kappa = 0.79\text{-}0.90$, $r = 0.88\text{-}0.95$).[21]

For measuring change of white matter lesion severity over time, we used a specifically developed and validated white matter lesions change scale.[22] Two raters independently assessed progression of white matter lesions severity on digital T2- and PD-weighted images by direct scan comparison. Raters were masked to all clinical information. To systematically evaluate differences in white matter lesion severity in all different brain regions they separately scored differences in the three periventricular regions of both hemispheres (periventricular score range –6 to +6) and in the subcortical white matter of the four lobes of both hemispheres (subcortical score range: -8 to +8). The rating showed good interobserver (interclass correlation coefficient 0.75-0.93) and intraobserver agreement (intraclass correlation coefficient 0.70-0.93). If raters disagreed one point or less on the scale, the mean of the ratings was used; if more, a consensus meeting was held. Progression was defined as an increase of one point or more between baseline and follow-up. Because this scale of white matter lesion progression was qualitative rather than quantitative, we categorized progression into no (score < 1), minor (score 1-2.5), and marked progression (score 3 or higher).

Lacunar infarcts

We defined brain infarcts as areas of focal hyperintensity on T2-weighted images sized ≥3 mm. Areas of hyperintensity in the white matter also had to have corresponding prominent hypointensity on T1-weighted images, in order to distinguish them from white matter lesions. Lacunar infarcts were defined as infarcts 3 to 20 mm in size and located in the subcortical white matter or basal ganglia. Non-lacunar infarcts were excluded from the analyses of lacunar infarcts. A new infarct on the follow-up MRI was classified as incident lacunar infarct.[23]

Cardiovascular risk factors

Baseline blood pressure was measured in sitting position at the right brachial artery with a random-zero sphygmomanometer. In the analyses we used the average of two measurements taken at one occasion. Body mass index (BMI) was computed as weight divided by height squared. Non-fasting serum total and HDL cholesterol levels were determined by an automated enzymatic procedure.[24] Diabetes mellitus was considered present if participants reported use of antidiabetic medication or when random or post-load serum glucose level was greater than 11.1 mmol/l. The presence of atherosclerotic plaques was assessed by ultrasound at the bifurcation, common, and internal carotid artery on both sides, resulting in a carotid artery plaque score ranging from 0 to 6.[25] Serum levels of high-sensitivity C-reactive protein (CRP) were determined by the Rate Near Infrared

Particle Immunoassay method (Immage® high-sensitive CRP, Beckman Coulter, USA). Information on smoking (categorized as current, former or never) was obtained during the home interview.

Statistical analyses

We used age and sex adjusted analysis of covariance to analyze whether baseline risk factors differed between people with and without a second MRI assessment. With linear regression analyses we assessed the relationship between baseline retinal vessel diameters and severity of periventricular and subcortical white matter lesions on the first MRI scan and with multinomial logistic regression analyses their relationship with white matter lesion progression. We used logistic regression analyses to study the association between retinal vessel diameters and prevalent as well as incident lacunar infarcts. All analyses were performed adjusted for age and sex, and additionally for other cardiovascular risk factors with SPSS Windows version 11.0 (SPSS Inc., Chicago, Illinois).

Table 1. Baseline characteristics

	All participants	Participants with repeated MRI assessment	Non-participants at follow-up	Adjusted differences* (95% CI)†
Number (n)	490	279	211	
Age (years)	68.4 (7.8)	67.0 (7.6)	70.3 (7.8)	3.3 (1.9; 4.7)‡
Sex (% female)	49	49	49	0.8 (-8.4; 9.9)
Diabetes Mellitus (%)	6.3	4.8	8.3	2.3 (-2.5; 7.0)
Smoking (% current)	22	23	20	0.0 (-7.5; 7.6)
Systolic blood pressure (mmHg)	136.7 (19.9)	134.2 (19.3)	139.9 (20.2)	3.5 (-0.03; 6.9)
Diastolic blood pressure (mmHg)	73.1 (10.8)	72.6 (10.6)	73.7 (11.2)	1.5 (-0.4; 3.5)
Body mass index (kg/m^2)	26.3 (3.3)	26.1 (3.2)	26.5 (3.3)	0.3 (-0.3; 0.9)
Carotid artery plaque score ≥ 4 (%)	17	15	21	2.0 (-5.0; 8.9)
C-reactive protein (mg/L)	2.85	2.51	3.31	0.57 (-0.30; 1.44)
Serum total cholesterol (mmol/L)	6.65 (1.3)	6.71 (1.3)	6.58 (1.2)	-0.10 (-0.32; 0.12)
Serum HDL cholesterol (mmol/L)	1.33 (0.4)	1.33 (0.3)	1.33 (0.5)	0.00 (-0.07; 0.07)
Periventricular WML§ (range: 0-9)	2.68	2.46	2.97	0.08 (-0.28; 0.43)
Subcortical WML§ (ml)	1.81	1.59	2.10	-0.01 (-0.60; 0.57)
All infarcts (%)	26	23	30	1.1 (-6.7; 8.8)
Lacunar infarcts¶ (%)	22	20	25	0.0 (-7.0; 7.9)
Retinal arteriolar diameter (µm)	147.8 (14.2)	147.8 (13.9)	147.9 (14.6)	0.3 (-2.4; 2.9)
Retinal venular diameter (µm)	223.8 (20.6)	223.3 (20.1)	224.5 (21.2)	3.0 (-0.7; 6.9)
Retinal arteriolar-to-venular ratio	0.66 (0.06)	0.66 (0.06)	0.66 (0.06)	-0.008 (-0.018; 0.003)

Presented as unadjusted means (standard deviation) or percentages; * Age and sex adjusted if applicable; † CI=confidence interval ; ‡ Significant ($p < 0.05$) compared to those with repeated MRI assessment; § WML=white matter lesions; ¶ Non-lacunar infarcts excluded

RESULTS

Baseline characteristics of participants with and without repeated MRI assessments are presented in Table 1. Non-participants at the follow-up MRI examination were on average older than those who did have a repeated MRI assessment. With respect to all other cardiovascular risk factors, there were no significant differences between the two groups after adjusting for age and sex.

The mean follow-up period between the first and second MRI was 3.3 years (standard deviation (SD): 0.2 years). During this period, 82 participants showed white matter progression in the periventricular region, of whom 31 had marked progression. A total of 89 participants showed progression in the subcortical region, of whom 27 had marked progression. There were 27 participants who had a new lacunar infarct on the follow-up MRI.

Table 2. Cross-sectional association between retinal vessel diameters and severity of cerebral small vessel disease (n=490)

	Periventricular WML* (grade)	Subcortical WML* (ml)	Lacunar infarct (OR, (95% CI))†
Arteriolar narrowing‡	0.002 (-0.17; 0.17)	0.01 (-0.21; 0.36)	1.14 (0.91; 1.44)
Venular dilatation§	-0.004 (-0.18; 0.17)	0.08 (-0.21; 0.37)	1.07 (0.85; 1.35)
Arteriolar-to-venular ratio¶	0.04 (-0.14; 0.22)	0.18 (-0.11; 0.47)	1.24 (0.98; 1.58)

All models adjusted for age and sex

* WML=white matter lesions

† OR=odds ratio; CI=confidence interval

‡ Per standard deviation decrease in arteriolar diameter

§ Per standard deviation increase in venular diameter

¶ Per standard deviation decrease in arteriolar-to-venular ratio

Table 3. Odds ratios* of white matter lesion progression and incident lacunar infarct per standard deviation difference in baseline retinal vessel measurements (n=279)

	Periventricular WML† progression			Subcortical WML† progression			Incident lacunar infarct
	Any (n=82)	Minor (n=51)	Marked (n=31)	Any (n=89)	Minor (n=64)	Marked (n=25)	All (n=27)
Arteriolar narrowing‡	0.93 (0.70; 1.22)	1.10 (0.78; 1.56)	0.77 (0.52; 1.14)	0.85 (0.65; 1.11)	0.92 (0.67; 1.25)	0.76 (0.49; 1.16)	0.81 (0.54; 1.22)
Venular dilatation§	1.33 (0.99; 1.78)	1.12 (0.79; 1.60)	1.71 (1.11; 2.61)	1.22 (0.93; 1.61)	1.06 (0.78; 1.45)	1.72 (1.09; 2.71)	1.59 (1.06; 2.39)
Arteriolar-to-venular ratio¶	1.22 (0.92; 1.61)	1.20 (0.85; 1.67)	1.25 (0.84; 1.92)	1.03 (0.79; 1.33)	0.95 (0.71; 1.28)	1.28 (0.83; 2.00)	1.29 (0.84; 1.98)

* Age and sex adjusted odds ratios with corresponding 95% confidence intervals

† WML=white matter lesions

‡ OR per standard deviation decrease in retinal arteriolar diameters

§ OR per SD increase in retinal venular diameters

¶ OR per SD decrease in arteriolar-to-venular ratio

Table 4. Odds ratios* of white matter lesion progression and incident lacunar infarcts per standard deviation increase in retinal venular diameters additionally adjusted for other cardiovascular risk factors (n=279)

	Periventricular WML† progression		Subcortical WML† progression		Incident lacunar Infarcts
	Minor	Marked	Minor	Marked	
Age and sex	1.12 (0.79; 1.60)	1.71 (1.11; 2.61)	1.06 (0.78; 1.45)	1.72 (1.09; 2.71)	1.59 (1.06; 2.39)
Age, sex and cholesterol‡	1.10 (0.77; 1.57)	1.80 (1.17; 2.78)	1.05 (0.76; 1.44)	1.80 (1.12; 2.89)	1.59 (1.06; 2.39)
Age, sex and body mass index	1.14 (0.80; 1.63)	1.63 (1.07; 2.52)	1.05 (0.77; 1.44)	1.68 (1.06; 2.66)	1.58 (1.05; 2.39)
Age, sex and carotid artery plaques	1.16 (0.80; 1.69)	1.66 (1.08; 2.55)	1.11 (0.80; 1.54)	1.95 (1.21; 3.17)	1.43 (0.92; 2.22)
Age, sex and C-reactive protein	1.07 (0.75; 1.53)	1.60 (1.03; 2.47)	1.03 (0.75; 1.41)	1.58 (1.00; 2.52)	1.52 (1.00; 2.30)
Age, sex and smoking	1.08 (0.74; 1.57)	1.64 (1.05; 2.57)	1.06 (0.77; 1.46)	1.69 (1.07; 2.68)	1.67 (1.10; 2.54)
Age, sex and diabetes mellitus	1.25 (0.85; 1.84)	1.79 (1.13; 2.83)	1.06 (0.75; 1.51)	1.77 (1.10; 2.85)	1.40 (0.90; 2.17)
Age, sex and blood pressure§	1.12 (0.78; 1.61)	1.73 (1.12; 2.68)	1.07 (0.78; 1.47)	1.83 (1.13; 2.97)	1.62 (1.07; 2.45)
Fully adjusted	1.25 (0.79; 1.96)	1.74 (1.02; 2.95)	1.09 (0.74; 1.60)	2.50 (1.30; 4.81)	1.24 (0.72; 2.12)

* Odds ratios with corresponding 95% confidence intervals

† WML=white matter lesions

‡ Total and high-density lipoprotein cholesterol

§ Systolic and diastolic blood pressure

Retinal vessel diameters were associated neither with the severity of white matter lesions, nor with prevalent lacunar infarcts on the first MRI scan (Table 2). Larger retinal venular diameters were associated with marked progression of both periventricular and subcortical white matter lesions and with incident lacunar infarcts (Table 3). Smaller arteriolar diameters were neither related to white matter lesion progression, nor to incident lacunar infarcts.

Finally, Table 4 presents the relation between retinal venular diameters and cerebral small vessel disease after additional adjustment for other cardiovascular risk factors. Each SD increase in venular diameters resulted in a 1.8 times increased risk of marked periventricular white matter lesion progression, and in a 2.8 times increased risk of marked subcortical white matter progression. Persons with larger venular diameters tended to have more incident lacunar infarcts, however this association attenuated after additional adjustments.

DISCUSSION

Especially larger retinal venular diameters were associated in this study with marked progression of periventricular and subcortical white matter lesions independent of other cardiovascular risk factors. Also, persons with larger venular diameters tended to have more incident lacunar infarcts.

We observed that venular dilatation was only related to marked white matter lesion progression, but not to the severity of white matter lesions on the first MRI scan. How

can this discrepancy be explained? It is known that persons who already have a relatively high white matter lesion load are the ones who show the greatest progression towards more extensive white matter lesions at follow-up.[26,27] This probably led to a broader range of white matter lesions for the longitudinal analyses and allowed us, in contrast to the cross-sectional analyses, to detect the association with venular dilatation. If we restricted the cross-sectional analyses to the people who also had follow-up scans the results remained virtually identical, suggesting that selective dropout does not underlie this discrepancy.

In our study, the first MRI was performed on average three years after baseline fundus photography. Because in a subsample we found hardly any changes in retinal vessel diameters over a period of six years, we concluded that baseline measurements are a good reflection of retinal vessel diameters at the time of the first MRI.

Previously, we showed that larger venular diameters were related to atherosclerosis (as measured by more plaques in the carotid arteries), higher levels of total cholesterol, lower levels of HDL, higher levels of inflammatory markers (such as erythrocyte sedimentation rate and leukocyte count) and smoking.[16] Because these risk factors are also related to the development of white matter lesions and lacunar infarcts, they may explain the observed associations.[8,28] After adjusting one-by-one for these factors, the associations between venular dilatation and white matter lesions still remained significant. The associations attenuated most after including CRP, pointing towards the involvement of inflammation in the pathogenesis of cerebral small vessel disease. When we adjusted for all these risk factors simultaneously the association between venular dilatation and cerebral small vessel disease hardly changed suggesting that other mechanisms are involved.

It has been hypothesized that retinal venular dilatation occurs in response to retinal hypoxia.[29] Venular dilatation has been described as one of the earliest changes not only in diabetic retinopathy, but also in the less well-known venous stasis retinopathy, both of which are characterized by retinal hypoxia.[30,31] Furthermore, treatment with photocoagulation decreases the oxygen requirement of retinal tissue eventually leading to disappearance of venular dilatation.[32,33] Based on these observations, a possible explanation might be that venular dilatation is a general marker of diffuse retinal and cerebral ischemia leading to increased susceptibility of brain tissue to the development of white matter lesions and lacunar infarcts.

Another explanation might be that retinal venular dilatation in some way reflects cerebral venular abnormalities. In one study, brains with severe white matter lesions showed wall thickening and obstruction of the periventricular veins, a condition known as periventricular venous collagenosis.[34,35] It was suggested that these alterations resulted in an increased venous pressure, venular dilatation, and venular blood-brain barrier disruption. This venous insufficiency could result in ischemic stress from impaired clearance of cellular metabolites in the deep white matter eventually leading to white matter

lesions.[35,36] It is unkown to what extent such cerebral venular changes might be reflected in the retinal venules.

With regard to arterioles, we previously suggested that arteriolar narrowing reflects vasoconstriction, intimal thickening and medial hyperplasia.[16] The lack of an association between smaller arteriolar diameters and cerebral small vessel disease could be due to the fact that, despite increased arterial stiffness, arterioles still possess the ability to dilate even in retinal or cerebral hypoxia, though less so than the venules.[16,29,31] These opposing effects in the same arterioles, narrowing and widening, might result in no apparent association.

In conclusion, we found retinal venular dilatation to be associated with progression of cerebral small vessel disease. In case future research would focus more on the mechanisms underlying venular abnormalities including dilatation this might provide new clues into the pathophysiology of cerebral small vessel disease.

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Chapter 5.2

Retinal vessel diameters and brain atrophy

ABSTRACT

Background: *In elderly persons, cardiovascular risk factors are associated with brain atrophy, especially of the white matter (WM). Retinal vessels may reflect the condition of intracerebral vessels. In particular larger retinal venular diameters have been associated with progression of white matter lesions and an increased risk of stroke. Whether retinal vessel diameters are related to brain atrophy remains unclear.*

Methods: *We studied 400 randomly selected elderly without dementia (age 60-90 years) from a population-based cohort study. Summary retinal arteriolar and venular diameters were measured on digitized baseline (1990-1993) fundus transparencies. We automatically segmented brain MR-images made in 1995-1996 to obtain volumes of grey matter (GM), WM and total brain (expressed as percentage of intra-cranial volume (%ICV)). We also assessed hippocampal and amygdalar volumes (ml).*

Results: *Smaller arteriolar diameters were associated with smaller total brain volume, but not with WM or GM volume separately. Persons with larger venular diameters had smaller WM volume (age and sex adjusted difference in WM volume -1.27%ICV (95% confidence interval, -2.52;-0.02) for participants in the highest quintile compared to all other quintiles of venular diameters). In contrast, larger venular diameters were related to larger GM as well as to larger hippocampal and amygdalar volumes.*

Conclusions: *Retinal vessel diameters are associated with brain atrophy on MRI in non-demented elderly. Larger retinal venular diameters were related to WM but not GM atrophy. This is in line with observations regarding cerebrovascular disease and consistent with the hypothesis that vascular pathology contributes specifically to WM atrophy in the elderly.*

INTRODUCTION

Brain atrophy is frequently observed on magnetic resonance imaging (MRI) in the elderly. Both cerebral small vessel disease and cardiovascular risk factors, in particular hypertension, diabetes mellitus and smoking, have been associated with global brain atrophy.[1-4] Only few studies investigated the various brain tissues separately. Those that did, reported that small vessel disease and cardiovascular risk factors were associated with atrophy of the white matter (WM), but not of the grey matter (GM).[2,3]

Retinal vessels may reflect the condition of intracerebral vessels. Retinal arteriolar and venular diameters are used to study the cerebral microcirculation and their investigation might provide new insights in the etiology of cerebrovascular disease.[5] A lower ratio of arteriolar-to-venular diameters (AVR) has been suggested to reflect generalized arteriolar narrowing due to hypertension[6] and was associated with an increased risk of stroke and subclinical brain infarcts on MRI.[7,8] However, a lower AVR may either result from arteriolar narrowing or venular widening. Larger venular diameters are related to atherosclerosis and systemic inflammation.[9,10] Furthermore, larger venular rather than smaller arteriolar diameters have recently been associated with progression of cerebral small vessel disease and an increased risk of stroke.[11,12]

Whether retinal vessel diameters are also related to brain atrophy remains unclear. Although a lower AVR has been related to global brain atrophy on MRI,[13] arteriolar and venular diameters have not been studied separately. Moreover, it remains unclear whether retinal vessel diameters are related to atrophy of the WM, GM, or both. We therefore investigated how retinal arteriolar and venular diameters were associated with global brain atrophy and with WM and GM atrophy separately, using cross-sectional data from the population-based Rotterdam Study.

METHODS

Study population

The Rotterdam Study is a population-based prospective cohort study investigating determinants and consequences of chronic diseases in persons aged 55 years and older.[14] Baseline examinations were performed between 1990 and 1993, and comprised an eye examination including taking fundus transparencies. Between 1995 and 1996, a random sample of 563 non-demented persons from the Rotterdam Study underwent MRI of the brain as part of the Rotterdam Scan Study, a study on age-related brain changes on MRI.[15] Participants gave their written informed consent according to the tenets of the Declaration of Helsinki. The study was approved by the appropriate medical ethics committees.

Retinal vessel measurements

Baseline fundus color transparencies were taken centered on the optic disk (20° field, Topcon Optical Company, Tokyo, Japan) after pharmacological mydriasis and digitized with a high-resolution scanner (Nikon LS-4000, Nikon Corporation, Japan). For each participant the qualitatively best digitized image of either eye was analyzed with the Retinal Vessel Measurement System (Retinal Analysis, Optimate, WI; Department of Ophthalmology & Visual Science, University of Wisconsin-Madison, USA).[6]

Summary measures for arteriolar and venular diameters separately were calculated with the improved Parr-Hubbard-Knudtson formulas and were corrected for magnification changes caused by refractive errors of the eye.[9] The AVR, defined as the ratio of these summated arteriolar and venular diameters, was used to compare our results with those of other studies. Four trained graders performed the assessments, masked to the clinical characteristics of the participants. Intra- and intergrader agreement was good to excellent.[9]

Baseline fundus transparencies were not available for one person and were ungradable on both eyes in 72 other persons, leaving 490 participants.

MRI assessments

In 1995 to 1996, brain MRI was performed on a 1.5-Tesla scanner (VISION MR,

Siemens AG, Erlangen, Germany). The protocol included T1, T2 and proton density (PD) weighted axial scans and an additional three-dimensional half-Fourier acquisition single-short turbo spin echo (HASTE) sequence.

We used the PD, T2 and HASTE sequences for automated brain tissue classification to obtain volumes of GM, normal WM, white matter lesions (WML) and cerebrospinal fluid (CSF).[2] Total WM volume was defined as the sum of normal WM and WML volume. All brain volume measures were expressed as percentage of intracranial volume (= GM + total WM + CSF). Total brain volume was obtained by summing GM and total WM.

In addition, right and left hippocampal and amygdalar structures were manually outlined on coronal slices that were reconstructed from the HASTE sequence perpendicular to the long axis of the hippocampus.[16] Volumes were calculated by summing the areas multiplied by slice thickness. Total hippocampal and amygdalar volumes were calculated by summing the volumes of each structure in both hemispheres. The intracranial cross-sectional area was measured on a middle sagittal MRI slice, to obtain normalised hippocampal and amygdalar volumes corrected for head size (men and women separately).[17]

Brain volume measures were unavailable in 90 of the 490 persons with gradable fundus transparencies, mostly due to claustrophobia during MRI acquisition, leaving a study sample for the current analysis of 400 participants.

Covariates

Smoking status (categorized as current, former and never smoking) and medication use were assessed at the baseline interview. At the research centre, blood pressure was measured twice with a random zero sphygmomanometer at the brachial artery with the subject in sitting position, and the measurements were averaged. Atherosclerotic plaques were assessed by ultrasound at the bifurcation, common, and internal carotid artery on both sides and defined as focal thickening of the vessel wall (of at least 1.5 times the average intima-media thickness) relative to adjacent segments with or without calcified components. The carotid artery plaque score (range: 0-6) reflects the number of these locations with plaques. Height and weight were measured and the body mass index (BMI) was calculated by dividing body weight (kg) by height squared (m^2). Non-fasting serum total and HDL cholesterol concentrations were determined by an automated enzymatic procedure.[18] Leukocyte count was assessed in citrate plasma using a Coulter Counter T540® (Coulter electronics, Luton, England). Diabetes mellitus was considered present if participants reported use of antidiabetic medication or when the random or post-load serum glucose level was greater than 11.1 mmol/l.

Statistical analysis

Analysis of covariance (ANCOVA) was used to assess linearity of the associations between quintiles of retinal vessel diameters or AVR and brain volume measures. We subsequently used multiple linear regression models to quantify the associations per SD increase. Because some analyses suggested non-linear relations, we also compared participants in the lowest quintile to those in the four higher quintiles of arteriolar diameters or AVR, and the highest compared to the four lower quintiles for venular diameters. All analyses were adjusted for age and sex, and additionally for blood pressure, carotid artery plaques, BMI, total and HDL cholesterol, leukocyte count, smoking and diabetes.

RESULTS

Characteristics of the study population are shown in Table 1. The mean summary arteriolar diameter was 147.3 (SD 14.0) μm, the mean venular 223.8 (SD 20.3) μm.

Table 2 shows the associations of retinal arteriolar and venular diameters and their AVR with brain tissue volumes on MRI. Smaller arteriolar diameters were related to a smaller total brain volume, but no association was found with either WM or GM volume separately. Larger venular diameters were associated with smaller WM volume, both total WM and normal WM volume. Yet, persons with larger venular diameters had a larger GM volume. Venular diameters were not related to WML or total brain volume. Persons with a lower AVR had smaller total brain volume as well as smaller total WM and

Table 1. Characteristics of the study population (1995-1996)*

Selected characteristics	Sample with retinal vessel and brain volume measures (n=400)
Age (years)	72.9 (7.7)
Sex (% female)	50
Systolic blood pressure (mmHg)	145.8 (20.3)
Diastolic blood pressure (mmHg)	76.4 (11.5)
Body mass index (kg/m^2)	26.3 (3.5)
Serum total cholesterol (mmol/L)	5.9 (1.1)
Serum HDL cholesterol (mmol/L)	1.3 (0.3)
Smoking (% current)	22
Diabetes mellitus (%)	6
Cerebrospinal fluid (% of intracranial volume)	22.3 (3.6)
Grey matter (% of intracranial volume)	46.5 (4.1)
Normal white matter (% of intracranial volume)	29.8 (6.3)
White matter lesions (% of intracranial volume)	1.3 (1.5)
Hippocampal volume (ml)	6.4 (0.9)
Amygdalar volume (ml)	4.6 (0.7)

* Values are means (SD) or percentages

normal WM volumes. A lower AVR was also associated with more WML, but this was not significant. The AVR was not related to GM volume.

Table 3 shows the association of retinal arteriolar and venular diameters and their AVR with hippocampal and amygdalar volumes on MRI. Persons with smaller arteriolar diameters had smaller hippocampal volume, but no association was found with amygdalar volume. In contrast, persons with larger venular diameters had both larger hippocampal and amygdalar volume. The AVR was not related to both measures. Additional adjustment for cardiovascular risk factors did not change the results.

DISCUSSION

Our study shows that retinal vessel diameters are associated with brain atrophy on MRI. Smaller arteriolar diameters were related to global brain atrophy, larger retinal venular diameters were associated especially with WM, and not with GM, atrophy. A lower AVR was related to global brain atrophy, as well as WM atrophy, but not with GM atrophy. All associations were independent of conventional vascular risk factors.

Strengths of our study are the quantitative assessment of different brain tissues on MRI, were others only used measures of global brain atrophy,[13] the large number of participants and the population-based setting. A limitation is that the fundus transparencies on which the retinal vessel diameters were measured, were taken 5 years prior

Table 2. Associations between summary retinal vessel diameters and brain tissue volume on MRI*

Retinal vessel characteristic	Brain tissue volume on MRI (n=400)				
	Total brain volume	Grey matter volume	White matter volume		
			Normal	Lesions	Total
Arteriolar diameters					
Per SD decrease†	-0.41 (-0.67; -0.16)	-0.14 (-0.54; 0.26)	-0.30 (-0.83; 0.24)	0.03 (-0.11; 0.16)	-0.28 (-0.77; 0.22)
Q1‡	77.0 (0.30)	46.5 (0.46)	29.1 (0.61)	1.31 (0.15)	30.4 (0.57)
Q2	77.3 (0.29)	46.2 (0.45)	29.6 (0.61)	1.45 (0.15)	31.0 (0.56)
Q3	77.8 (0.30)	47.0 (0.46)	29.6 (0.61)	1.27 (0.15)	30.8 (0.57)
Q4	78.3 (0.29)	46.1 (0.45)	30.8 (0.62)	1.30 (0.15)	32.1 (0.56)
Q5	78.1 (0.30)	46.8 (0.45)	30.2 (0.61)	1.25 (0.15)	31.4 (0.57)
Q1 vv Q2-5§	-0.85 (-1.50; -0.19)	0.02 (-0.98; 1.02)	-0.89 (-2.24; 0.45)	-0.01 (-0.34; 0.32)	-0.90 (-2.15; 0.35)
Venular diameters					
Per SD increase†	0.19 (-0.10; 0.49)	0.52 (0.08; 0.96)	-0.35 (-0.95; 0.25)	0.02 (-0.13; 0.17)	-0.33 (-0.88; 0.23)
Q1‡	77.8 (0.29)	46.2 (0.44)	30.2 (0.60)	1.27 (0.15)	31.4 (0.56)
Q2	77.0 (0.31)	45.8 (0.46)	29.9 (0.62)	1.39 (0.15)	31.3 (0.58)
Q3	77.8 (0.30)	45.9 (0.46)	30.9 (0.60)	1.35 (0.15)	32.2 (0.56)
Q4	78.0 (0.30)	47.2 (0.45)	29.6 (0.61)	1.14 (0.15)	30.7 (0.56)
Q5	77.9 (0.30)	47.6 (0.45)	28.7 (0.61)	1.44 (0.15)	30.1 (0.57)
Q5 vv Q1-4 ¶	0.22 (-0.45; 0.88)	1.31 (0.32; 2.30)	-1.43 (-2.77; -0.09)	0.16 (-0.18; 0.49)	-1.27 (-2.52; -0.02)
AVR					
Per SD decrease†	-0.33 (-0.62; -0.04)	0.37 (-0.07; 0.80)	-0.75 (-1.34 ; -0.16)	0.06 (-0.09; 0.20)	-0.70 (-1.24 ;-0.15)
Q1‡	77.3 (0.30)	46.9 (0.46)	28.7 (0.61)	1.57 (0.15)	30.3 (0.57)
Q2	77.3 (0.30)	46.8 (0.45)	29.5 (0.61)	1.16 (0.15)	30.7 (0.57)
Q3	77.6 (0.30)	46.5 (0.45)	30.1 (0.61)	1.16 (0.15)	31.2 (0.57)
Q4	78.1 (0.30)	46.5 (0.45)	30.3 (0.60)	1.35 (0.15)	31.6 (0.56)
Q5	78.1 (0.30)	46.0 (0.46)	30.6 (0.61)	1.35 (0.15)	31.9 (0.57)
Q1 vv Q2-5#	-0.44 (-1.10; 0.22)	0.45 (-0.55; 1.45)	-1.39 (-2.73; -0.04)	0.32 (-0.02; 0.65)	-1.07 (-2.33; 0.18)

***** expressed as percentage of total intracranial volume

AVR; arteriolar-to-venular ratio

† Values represent age and sex adjusted regression coefficients (95% Confidence Intervals)

‡ Values represent age and sex adjusted means (standard errors)

§ Lowest quintile of arteriolar diameter, indicating arteriolar narrowing, compared to higher four quintiles (reference)

¶ Highest quintile of venular diameter, indicating venular widening, compared to lower four quintiles (reference)

Lowest quintile of arteriolar-to-venular ratio compared to higher four quintiles (reference)

to the MRI. Selective survival may therefore have caused an underestimation of our effects.

In the Atherosclerosis Risk in Communities (ARIC) study, a lower AVR has been studied in relation to measures of global brain atrophy on MRI.[13] No association was found with sulcal widening, a measure of cortical atrophy. Although a lower AVR was related to ventricular enlargement, more a measure of *sub*cortical atrophy, this association at-

Table 3. Associations between summary retinal vessel diameters and hippocampal or amygdalar volume on MRI

Retinal vessel characteristic	Hippocampal volume (ml)	Amygdalar volume (ml)
Arteriolar diameters		
Per SD decrease†	-0.08 (-0.17; 0.00)	-0.05 (-0.12; 0.02)
Q1‡	6.26 (0.09)	4.60 (0.08)
Q2	6.31 (0.09)	4.52 (0.08)
Q3	6.56 (0.09)	4.56 (0.08)
Q4	6.30 (0.09)	4.59 (0.08)
Q5	6.52 (0.09)	4.68 (0.08)
Q1 vv Q2-5§	-0.17 (-0.37; 0.04)	0.01 (-0.16; 0.18)
Venular diameters		
Per SD increase†	0.08 (-0.01; 0.17)	0.08 (0.01; 0.16)
Q1‡	6.25 (0.09)	4.45 (0.08)
Q2	6.42 (0.09)	4.64 (0.08)
Q3	6.25 (0.09)	4.48 (0.08)
Q4	76.30 (0.09)	4.65 (0.08)
Q5	6.53 (0.09)	4.74 (0.08)
Q5 vv Q1-4 ¶	0.18 (-0.03; 0.38)	0.18 (0.01; 0.35)
AVR		
Per SD decrease†	-0.02 (-0.11; 0.07)	0.02 (-0.05; 0.10)
Q1‡	6.32 (0.09)	4.59 (0.08)
Q2	6.45 (0.09)	4.65 (0.08)
Q3	6.39 (0.09)	4.57 (0.08)
Q4	6.44 (0.09)	4.59 (0.08)
Q5	6.34 (0.09)	4.54 (0.08)
Q1 vv Q2-5#	-0.09 (-0.30; 0.11)	0.002 (-0.17; 0.17)

AVR; arteriolar-to-venular ratio

† Values represent age and sex adjusted regression coefficients (95% Confidence Intervals)

‡ Values represent age and sex adjusted means (standard errors)

§ Lowest quintile of arteriolar diameter, indicating arteriolar narrowing, compared to higher four quintiles (reference)

¶ Highest quintile of venular diameter, indicating venular widening, compared to lower four quintiles (reference)

Lowest quintile of arteriolar-to-venular ratio compared to higher four quintiles (reference)

tenuated after adjustment for WML. The authors therefore suggested that ventricular enlargement might specifically reflect loss of WM rather than GM. Our findings support this hypothesis, because a lower AVR was associated with global brain atrophy and WM, but not with GM, atrophy.

Our further analyses showed that larger venular but not smaller arteriolar diameters were associated with atrophy of both normal WM and total WM. This is in agreement with observations in cerebrovascular disease, as larger retinal venular diameters have been associated with progression of WML and an increased risk of lacunar infarctions and stroke.[11,12] It has been hypothesized that these cerebrovascular disorders reduce the

volume of not only normal WM but also of total WM in the elderly.[2] Larger venular diameters were neither associated with GM atrophy, nor with hippocampal and amygdalar atrophy. The latter two are considered putative early MRI markers of Alzheimer disease which specifically reflect neuronal loss and neurofibrillary tangles.[19] Surprisingly, larger venular diameters were associated with larger GM volumes. This association was not only present for overall GM volume but also for hippocampal and amygdalar volumes, indicating a consistent association of larger venular diameters with larger GM volume. As perfusion studies show that the blood volume in the GM is 2.5 times larger than that in the WM, we can speculate that larger venular diameters reflect a larger blood volume in the GM, but this remains to be further investigated. If anything, our findings suggest that larger venular diameters are specifically related to atrophy of WM and not to atrophy of GM.

Smaller retinal arteriolar diameters were related to global brain atrophy only, and not specifically to atrophy of the WM or GM. As smaller retinal arteriolar diameters are related to higher blood pressures,[6,9] this is consistent with earlier reports showing an association of hypertension or hypertensive retinopathy signs (such as retinal microaneurysms, hemorrhages and soft exudates) with global brain atrophy.[2,13] Taken together, the different associations we found between retinal arteriolar and venular diameters and brain atrophy on MRI, may provide further insight in the pathophysiological mechanisms underlying various brain disorders including cerebral small vessel disease and dementia.

In conclusion, our study shows that retinal vessel diameters are associated with quantitative measures of brain atrophy on MRI in non-demented elderly in the general population. Larger retinal venular diameters were related to especially WM atrophy, which is in line with observations in cerebrovascular disease and consistent with the hypothesis that vascular pathology may contribute to WM atrophy in the elderly.

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Chapter 5.3

Retinal vessel diameters and risk of dementia

ABSTRACT

Background: *Vascular pathology may be important not only in vascular dementia, but also in Alzheimer disease although the precise role remains unclear. Retinal vessel diameters, in particular larger venular diameters, have been associated with cerebrovascular disease and may help to elucidate the role of vascular pathology in Alzheimer disease.*

Methods: *We investigated whether retinal arteriolar and venular diameters are associated with risk of dementia, Alzheimer disease and vascular dementia in the prospective population-based Rotterdam Study. Digitized retinal images were available in 5,553 participants aged 55 years or over and dementia-free at baseline (1990-1993). Participants were re-examined in 1993-1994, 1997-1999 and 2002-2004 and were continuously monitored for development of dementia. Diagnoses of dementia and its major subtypes were made according to internationally accepted criteria.*

Findings: *During follow-up (mean 9.5 years), 511 participants developed dementia, Alzheimer disease was diagnosed in 396 and vascular dementia in 63 participants. Arteriolar diameters were not associated with dementia-risk. Larger venular diameters were associated with an increased risk of dementia, in particular vascular dementia (age and sex adjusted Hazard Ratio (HR), 95% CI, per SD increase in venular diameter: 1.29,1.02; 1.63), but not of Alzheimer disease (HR, 95% CI: 1.07,0.97; 1.18). Further, larger venular diameters were associated with a lower age-at-onset of Alzheimer disease, but not of vascular dementia. Adjusting for cardiovascular risk factors or excluding stroke did not change results.*

Interpretation: *Our findings suggest that vascular pathology as reflected by larger retinal venular diameters accelerates clinical expression of Alzheimer disease rather than being a cause.*

INTRODUCTION

Vascular pathology may play an important role not only in vascular dementia but also in the clinical course and progression of Alzheimer disease.[1,2] Although cerebrovascular disease including white matter lesions and lacunar infarcts frequently co-exists with Alzheimer disease, it remains unclear whether vascular factors are causally involved in Alzheimer pathology.[2] The cerebral microcirculation is difficult to assess and most non-invasive indicators of vascular pathology relate to vessel beds outside the brain. Retinal vessels may provide a way to study vascular damage to the brain more directly, because embryological, anatomical and physiological characteristics are similar to the cerebral circulation and the retina is easy to visualize in a non-invasive way.[3]

During the late 1990s, a semi-automated system became available to reliably quantify retinal arteriolar and venular diameters.[4] A lower arteriolar-to-venular ratio (AVR) was suggested to reflect generalized arteriolar narrowing due to hypertension[3] and was subsequently associated with an increased risk of cardiovascular disease and stroke.[5,6] However, a lower AVR may be due to arteriolar narrowing, venular widening or both.[7] More recent studies showed that although smaller arteriolar diameters were indeed strongly related to higher blood pressure,[7,8] in contrast larger venular diameters were associated with higher levels of inflammation markers, cholesterol, and sub-clinical atherosclerosis.[7,9] We found that larger venular diameters, rather than smaller arteriolar ones, were associated with an increased risk of stroke and progression of cerebral small vessel disease.[10,11] This suggests that retinal vessel diameters and in particular larger venular diameters, may be a marker for cerebrovascular damage, which may also yield exciting new clues regarding the pathophysiological mechanisms underlying dementia.

We therefore studied the associations between retinal arteriolar and venular diameters, and the risk of dementia and its major subtypes Alzheimer disease and vascular dementia, using data from a population-based cohort study.

METHODS

The Rotterdam Study

The Rotterdam Study is a large population-based prospective cohort study designed to study chronic diseases in the elderly.[12] All inhabitants of a district of Rotterdam aged 55 years or over were invited in random order to the study, and 7,983 actually participated (overall response 78%). A smaller number (n = 6,780) participated in the ophthalmic part of the study, since eye examinations became operational a few months after the baseline examinations had started. Fundus transparancies were available in 6,436 participants and of these, 6,432 participants were screened for dementia of whom 213 were

diagnosed to be demented at baseline. The cohort at risk of dementia thus comprised 6,219 participants who gave their written informed consent according to the tenets of the Declaration of Helsinki. The study was approved by the appropriate medical ethics committees. Follow-up examinations were conducted in 1993-1994, 1997-1999 and 2002-2004. In addition, through linkage with records of general practitioners, the total cohort was continuously monitored for morbidity and mortality. Follow up for dementia was virtually complete until January 1, 2005.

Dementia diagnosis

Participants were screened for dementia with a three-step procedure, which was similar at baseline and follow-up examinations.[13] First, participants were cognitively screened with the Mini-Mental State Examination (MMSE) and the Geriatric Mental State schedule (GMS) organic level. Second, if participants scored below 26 on the MMSE or above 0 on the GMS organic level, the Cambridge Examination of Mental Disorders in the Elderly (CAMDEX) was administered, and an informant was interviewed. Finally, participants suspected of having dementia were further examined by a neurologist, a neuropsychologist and, if possible, had magnetic resonance imaging of the brain. In addition, continuous monitoring of the cohort for incident dementia cases took place through a direct link between the study database and computerized medical records from general practitioners and through surveillance of Regional Institute for Outpatient Mental Health Care reports. The diagnosis of dementia and subtype of dementia was made in accordance with internationally accepted criteria for dementia (DSM-III-R), Alzheimer disease (NINCDS-ADRDA) and vascular dementia (NINDS-AIREN). Diagnoses were made on all available information by an expert panel including the neurologist, neuropsychologist and research physician.

Grading of retinal vessel diameters

At the baseline ophthalmic examination, fundus color transparencies were taken centered on the optic disk (20° field, Topcon Optical Company, Tokyo, Japan) after pharmacological mydriasis and digitized with a high-resolution scanner (Nikon LS-4000, Nikon Corporation, Japan). For each participant the digitized image with the best quality of either eye was analyzed with the Retinal Vessel Measurement System (Retinal Analysis, Optimate, WI; Department of Ophthalmology & Visual Science, University of Wisconsin-Madison, USA).[4]

The rationale and procedures to measure and summarize retinal vessel diameters have been described.[4,7] Summary measures for arteriolar and venular diameters were based on improved Parr-Hubbard-Knudtson formulas and were corrected for magnification changes due to refractive errors of the eye. The AVR was defined as the ratio of the summated arteriolar-to-venular diameters. Four trained graders performed the assessments,

masked to the clinical characteristics of the participants. A random sub-sample of 40 transparancies was used to monitor quality of the data at regular intervals. Pearson's correlation coefficients for intergrader agreement were 0.67-0.80 (arteriolar diameters), 0.91-0.94 (venular diameters) and 0.75-0.84 (AVR). For intragrader agreement these figures were 0.69-0.88 (arteriolar diameters), 0.90-0.95 (venular diameters) and 0.72-0.90 (AVR).

Other variables

Smoking habits (categorized as current, former and never smoking) and use of antihypertensive medication were assessed during the baseline interview. Blood pressure was measured twice with a random zero sphygmomanometer at the brachial artery with the subject in sitting position, and the measurements were averaged. Intima-media thickness of the common carotid artery was assessed by ultra-sonography, using a 7.5-MHz linear-array transducer (ATL Ultra-Mark IV). The mean anterior and posterior intima-media thickness of both the left and the right common carotid artery were averaged, and used for the analysis. Atherosclerotic plaques were assessed by ultrasound at the bifurcation, common, and internal carotid artery on both sides and defined as focal thickening of the vessel wall (of at least 1.5 times the average intima-media thickness) relative to adjacent segments with or without calcified components. The carotid artery plaque score (range: 0-6) reflects the number of these locations with plaques.[14] Non-fasting serum total and HDL cholesterol concentrations were determined by an automated enzymatic procedure. Height and weight were measured and the body mass index (BMI) was calculated by dividing body weight (kg) by height squared (m^2). Leukocyte count was assessed in citrate plasma using a Coulter Counter T540® (Coulter electronics, Luton, England). Blood was drawn directly into VACUTAINER® tubes and erythrocyte sedimentation rate was read after 60 minutes. Serum levels of high-sensitive C-reactive protein (CRP) were determined by the Rate Near Infrared Particle Immunoassay method (Immage® high-sensitive CRP, Beckman Coulter, USA). Diabetes mellitus was considered present if participants reported use of antidiabetic medication or when the random or post-load serum glucose level was greater than 11.1 mmol/l. Apolipoprotein E (*APOE*) genotype was assessed on coded DNA samples using polymerase chain reaction without knowledge of the dementia diagnosis.[15] Participants were categorized on the basis of the presence or absence of the *APOE* ε4 allele.

Statistical analysis

Analysis of covariance (ANCOVA), adjusted for age and sex, was used to compare baseline characteristics of participants with and without gradable fundus transparencies.

Associations between baseline retinal vessel diameters and incident dementia or its two major subtypes Alzheimer disease and vascular dementia were assessed with Cox

proportional hazards models. Participants were followed until diagnosis of dementia, death, or end of study, whichever came first. Hazard ratios (HR) were adjusted for age and sex. Age was additionally entered as a squared term to adjust for potential residual confounding by age. Retinal arteriolar and venular diameters and AVR were first entered in quintiles to check whether their relations with dementia were linear. Since associations were not obviously non-linear, all analyses were subsequently performed entering retinal vessel characteristics as a linear term in the model. HRs were expressed per standard deviation (SD) difference in retinal vessel diameter or AVR to allow comparison of strength of associations across the different vessel characteristics. The AVR is presented to allow comparison of our results with those of other studies. We tested the proportional hazard assumption by including the interactions of the vessel characteristics with time as covariate in the model. Interaction terms of both arteriolar and venular diameters with follow-up time were significant ($P<0.001$), indicating that the association between vessel diameters and dementia differed according to length of follow-up. We therefore performed separate analyses with cases identified until 2000 (short follow-up: median 6.3, range 0.1-9.4 years) and cases identified between 2000-2004 (long follow-up: median 10.7, range 0.1-14.4 years). To see whether retinal vessels might be related to the clinical expression of dementia, we assessed the associations of retinal vessel characteristics with age-at-onset of dementia or its major subtypes using linear regression models, adjusting for sex and follow-up duration.

All analyses were additionally adjusted for the abovementioned cardiovascular risk factors. Because the *APOE* ε4 allele is an important risk factor for Alzheimer disease,[16] and may modulate the effects of vascular disease on the brain,[17] we also performed the analyses within strata of *APOE* ε4 allele. To evaluate whether associations between retinal vessel diameters and dementia could be due to stroke, analyses were finally repeated after excluding previous stroke and censoring incident stroke cases. All analyses were performed using SPSS statistical software version 11 (SPSS Inc., Chicago, Illinois).

RESULTS

Fundus transparancies were gradable in at least one eye in 5,553 (89.3%) of the 6,219 participants who underwent the eye examination at baseline. A comparison of baseline characteristics between participants and non-participants is given in Table 1. Adjusted mean differences show that those excluded were significantly older, more often institutionalized and had higher erythrocyte sedimentation rates. There were no significant differences in other risk factors. The mean summated arteriolar diameter was 147.0 μm (SD: 14.4 μm; range 92.2-235.7 μm) the mean summated venular diameter 222.2 μm (SD: 20.8 μm; range 135.1-313.6 μm) and AVR 0.66 (SD: 0.06; range 0.48-1.02).

Table 1. Baseline characteristics of the study population (1990-1993)

	Gradable*	Ungradable	Adjusted differences† (95% CI)‡
Number (n)	5,553	666	
Age (years)	67.7 (8.0)§	74.5 (9.8)	6.7 (6.0; 7.4)
Sex (% female)	58.6	60.8	- 1.4 (-5.0; 3.0)
Institutionalized (%)	2.8	15.2	4.3 (2.5; 6.1)
Number of carotid artery plaques ≥ 4 (%)	16	22	0.6 (-2.7; 4.0)
Total cholesterol (mg/dL)	256.4 (46.7)	254.4 (49.4)	1.93 (-1.93; 5.79)
HDL cholesterol (mg/dL)	52.1 (13.9)	51.7 (13.9)	0.38 (-0.77; 1.94))
Body mass index (kg/m^2)	26.3 (3.7)	26.1 (3.7)	- 0.3 (-0.6; -0.2)
Leukocyte count (10^9/L)	6.66 (1.92)	6.76 (2.97)	0.08 (-0.09; 0.25)
Erythrocyte sedimentation rate (mm/hour)	12.9 (10.7)	16.2 (14.4)	1.09 (0.01; 2.17)
High-sensitive C-reactive protein (mg/L)	3.15 (6.08)	4.10 (11.0)	0.49 (-0.07; 1.05)
Smoking (% current)	23.7	19.6	- 2.5 (-5.9; 1.0)
Diabetes (%)	9.5	13.7	1.3 (-1.1; 3.8)
Systolic blood pressure (mmHg)	138.4 (22.0)	145.3 (23.8)	2.4 (0.6; 4.2)
Diastolic blood pressure (mmHg)	73.7 (11.3)	74.2 (12.4)	1.4 (0.4; 2.3)
Use of antihypertensive medication (%)	31.0	39.5	1.1 (-2.7; 4.8)

Values are unadjusted means (SD) or percentages.

* Participants with a gradable fundus transparency on at least one eye

† age and/or sex adjusted mean differences between participants with a gradable fundus transparency and those with ungradable fundus transparencies

‡ CI: confidence interval

§ Standard deviations between brackets

After a follow-up of 52,698 person-years (mean: 9.5 years (SD: 3.0)), 511 participants had developed dementia, of whom 396 were diagnosed with Alzheimer disease and 63 with vascular dementia. The remaining 52 cases were ascribed to other subtypes (including dementia in Parkinson's disease, multi system atrophy and Lewy body dementia). Table 2 shows the association of retinal arteriolar and venular diameters with risk of dementia. Larger arteriolar diameters were not associated with the risk of dementia, neither when analyzed in quintiles, nor when analyzed continuously. Larger venular diameters were associated with a higher risk of dementia. Participants in the highest quintile of venular diameters had an almost 40% increased risk of dementia compared with those in the lowest quintile. When analyzed continuously, the risk of dementia increased by 10% per SD increase in venular diameter (20.8 μm). A lower AVR was also associated with a higher risk of dementia, although this did not reach statistical significance (HR per SD decrease in AVR 1.08, 95% Confidence Interval (CI) 0.99; 1.17). Subtype analyses showed that arteriolar diameters were neither related to Alzheimer disease nor to vascular dementia. The increased risk of dementia associated with venular diameters was

Table 2. Risk of dementia, Alzheimer disease and vascular dementia according to retinal arteriolar and venular diameters

Retinal vessel diameters	Dementia		Alzheimer disease		Vascular dementia	
	No. of Cases	Hazard ratio (95% CI)*	No. of Cases	Hazard ratio (95% CI)*	No. of Cases	Hazard ratio (95% CI)*
Arteriolar diameter quintiles (µm)						
< 135.3	112	1.00 (ref.)	85	1.00 (ref.)	16	1.00 (ref.)
≥ 135.3 and < 143.1	101	0.98 (0.75; 1.28)	81	1.04 (0.76; 1.41)	14	0.98 (0.48; 2.01)
≥ 143.1 and < 149.7	102	1.06 (0.81; 1.39)	80	1.12 (0.82; 1.52)	11	0.79 (0.37; 1.71)
≥ 149.7 and < 158.2	107	1.07 (0.82; 1.40)	80	1.07 (0.79; 1.45)	12	0.84 (0.40; 1.77)
≥ 158.2	89	1.00 (0.75; 1.32)	70	1.06 (0.77; 1.46)	10	0.77 (0.35; 1.71)
Per SD (14.4 µm) increase, model 1†	511	1.02 (0.93; 1.11)	396	1.03 (0.93; 1.13)	63	0.93 (0.73; 1.19)
Per SD (14.4 µm) increase, model 2‡	511	1.05 (0.94; 1.17)	396	1.04 (0.92; 1.18)	63	1.03 (0.75; 1.42)
Venular diameter quintiles (µm)						
< 204.5	120	1.00 (ref.)	93	1.00 (ref.)	17	1.00 (ref.)
≥ 204.5 and < 216.3	111	1.06 (0.82; 1.37)	93	1.19 (0.89; 1.58)	6	0.32 (0.12; 0.86)
≥ 216.3 and < 226.9	79	0.85 (0.64; 1.12)	70	1.00 (0.73; 1.37)	7	0.48 (0.20; 1.16)
≥ 226.9 and < 239.1	98	1.16 (0.88; 1.51)	74	1.18 (0.87; 1.64)	11	0.83 (0.39; 1.76)
≥ 239.1	103	1.36 (1.04; 1.77)	66	1.24 (0.88; 1.67)	22	1.70 (0.90; 3.22)
Per SD (20.8 µm) increase, model 1†	511	1.10 (1.01; 1.20)	396	1.07 (0.97; 1.18)	63	1.29 (1.02; 1.63)
Per SD (20.8 µm) increase, model 2‡	511	1.12 (1.01 ; 1.23)	396	1.08 (0.97; 1.21)	63	1.38 (1.04; 1.84)

*CI indicates confidence interval.

† Model 1: adjusted for age and sex

‡ Model 2: adjusted for age, sex, systolic and diastolic blood pressure, antihypertensive medication, carotid artery plaque score, serum total and HDL cholesterol, serum C-reactive protein, leukocyte count, erythrocyte sedimentation rate, body mass index, smoking and diabetes mellitus.

confined to vascular dementia: for every SD increase in venular diameter, risk of vascular dementia increased significantly by 29% and of Alzheimer disease by 7%, which was not significant. For every SD decrease in AVR the HR for Alzheimer disease was 1.04 (95% CI, 0.94; 1.14) and for vascular dementia it was 1.46 (95% CI, 1.13; 1.87). Adjusting for cardiovascular risk factors did not change the association of larger venular diameters with risk of dementia (Table 2).

Subsequent analyses with different time-to-event periods showed that larger venular diameters were associated with an increased short-term risk of dementia (HR for every SD increase in venular diameter: 1.21, 95% CI 1.07; 1.36), but not with the longer-term risk (HR 1.00, 95% CI 0.88; 1.12). Table 3 shows a similar pattern for Alzheimer disease, whereas for vascular dementia it is shown that larger venular diameters are associated particularly with longer-term risk. In addition, both larger arteriolar and venular diameters were found to be associated with a lower age-at-onset for dementia and Alzheimer disease, but not vascular dementia. The association between age-at-onset and arteriolar diameters attenuated and became non-signifcant after adjusting for venular diameters.

Table 3. Risk of dementia, Alzheimer disease and vascular dementia per SD increase in arteriolar and venular diameters according to time-to-event

	Short follow-up (median 6.3, range 0.1 – 9.4 years)		Long follow-up (median 10.7, range 0.1 – 14.4 years)	
	Alzheimer disease (n=169)	Vascular dementia (n=41)	Alzheimer disease (n=227)	Vascular dementia (n=22)
	Hazard ratio (95% CI)*	Hazard ratio (95% CI)*	Hazard ratio (95% CI)*	Hazard ratio (95% CI)*
Arteriolar diameters (per SD)	1.09 (0.94; 1.26)	0.89 (0.65; 1.21)	0.98 (0.86; 1.12)	1.01 (0.67; 1.53)
Venular diameters (per SD)	1.20 (1.04; 1.39)	1.20 (0.89; 1.13)	0.97 (0.85; 1.11)	1.49 (1.01; 2.19)

*Analyses are adjusted for age and sex. CI indicates confidence interval.

Table 4. Retinal arteriolar and venular diameters and age at onset of dementia, Alzheimer disease and vascular dementia

Retinal venular diameters	Dementia	Alzheimer disease	Vascular dementia
	Age at onset mean (SE)*	Age at onset mean (SE)*	Age at onset mean (SE)*
Quintiles (µm)			
< 204.5	82.5 (0.65)	83.7 (0.74)	78.2 (1.57)
≥ 204.5 and < 216.3	81.7 (0.65)	82.4 (0.74)	80.8 (1.62)
≥ 216.3 and < 226.9	80.9 (0.64)	81.5 (0.73)	79.4 (1.53)
≥ 226.9 and < 239.1	80.4 (0.65)	79.8 (0.74)	85.1 (1.59)
≥ 239.1	79.6 (0.65)	80.4 (0.73)	78.3 (1.57)
P-trend	<0.001	<0.001	0.44
Per SD (20.8 µm) increase, model 1†	-0.85 (-1.38; -0.32)§	-1.09 (-1.72; -0.46)	0.63 (-0.67; 1.94)
Per SD (20.8 µm) increase, model 2‡	-0.78 (-1.43; -0.13)§	-1.10 (-1.86; -0.32)	0.84 (-0.93; 2.61)

* age and sex adjusted mean (standard error)

† Model 1: adjusted for follow-up duration and sex

‡ Model 2: Adjusted for follow-up duration, sex, systolic and diastolic blood pressure, antihypertensive medication, carotid artery plaque score, serum total and HDL cholesterol, serum C-reactive protein, leukocyte count, erythrocyte sedimentation rate, body mass index, smoking and diabetes mellitus.

§ values are regression coefficients (95% confidence intervals) per SD increase in retinal venular diameter

The onset of Alzheimer disease was on average 3 years earlier in cases within the highest quintile of venular diameters compared with those in the lowest quintile (Table 4). Every SD increase in venular diameters decreased the onset of Alzheimer disease with more than 1 year.

Adjusting for cardiovascular risk factors, exclusion of previous stroke and censoring of incident stroke did not change the results. For both arteriolar and venular diameters the association with dementia was similar for participants with or without at least one *APOE* ε4 allele.

DISCUSSION

Our results show that larger venular diameters in the retina are associated with a higher risk of dementia, in particular vascular dementia, and with the short-term but not the longer-term risk of Alzheimer disease. Arteriolar diameters were not associated with risk of dementia. Larger venular diameters were associated with a lower age-at-onset of Alzheimer disease, but not vascular dementia. These associations were independent of conventional vascular risk factors.

Important advantages of our study are the population-based design and the long follow-up, which with regard to the dementia diagnosis was virtually complete. Other advantages of our study are the detailed assessment of vessel diameters on 20° stereoscopic transparencies obtained after pharmacological mydriasis and the adjustment for refractive errors of the eye. This enabled us to estimate the intra-luminal arteriolar and venular diameters more in detail, where others reported uncorrected vessel diameters in pictures with smaller magnification.[4,8]

Some methodological issues should be discussed. Non-participants were on average older and more often institutionalized. As differences with included participants were small, we consider distortion of our associations by selection bias unlikely. Limitations related to the semi-automated system assessing the retinal vessel diameters have been described.[4] Because assessment of vessel diameters was unrelated to clinical characteristics of the participants, these limitations most likely led to an underestimation of our effects due to random misclassification.

Within the Atherosclerosis Risk in Communities Study, various retinal vessel characteristics have been studied in relation to cognitive function in middle-aged persons. Although retinopathy was found to be independently associated with cognitive impairment, generalized arteriolar narrowing quantified as the lowest quintile of the AVR was not related to cognitive function.[18] Yet, retinal arteriolar and venular diameters were not investigated separately. In our study, a lower AVR was associated with an increased risk of dementia, however this was due to larger venular rather than smaller arteriolar diameters. These results are in agreement with previous findings from the Rotterdam Study showing that larger venular diameters are associated with progression of cerebral small vessel disease and stroke,[10,11] both major risk factors for vascular dementia. The association of larger venular diameters with an increased risk of in particular vascular dementia offers further support for the idea that retinal venular dilatation may reflect cerebrovascular disease. Although larger venular diameters were also associated with an increased risk of Alzheimer disease this was apparent only at short follow-up and absent at longer follow-up duration. Moreover, larger venular diameters were related to a lower age-at-onset of Alzheimer disease but not vascular dementia. Taken together, this suggests that vascular pathology related to larger retinal venular diameters accelerates

the clinical expression of subclinical Alzheimer disease, rather than being a cause. This is in agreement with findings from the Nun Study showing that the expression of clinical dementia was crucially dependent on whether or not there were lacunar infarctions in the brain.[1] Moreover, cognitive deficits have been reported to occur with less Alzheimer-type pathology when cerebrovascular disease was present rather than absent,[1,19] in particular at the early stages of Alzheimer disease.[20]

Larger retinal venular diameters may be related to dementia in several ways. First, larger retinal venular diameters may reflect exposure to cardiovascalur risk factors, including atherosclerosis, inflammation markers, diabetes and smoking. Since adjusting for these factors did not change results, other mechanisms should be considered. Second, larger retinal venular diameter has been hypothesized to be a general marker of retinal ischemia and by proxy of cerebral ischemia.[11] Retinal ischemia has been related to lower cerebral blood flow and impaired cerebral vasoreactivity,[21,22] and lower cerebral blood flow velocity has been associated with risk of Alzheimer disease.[23] Third, venular dilatation may also reflect cerebral venular abnormalities. Histopathological studies reported wall thickening and obstruction of periventricular veins, referred to as periventricular venous collagenosis, in brains with severe white matter lesions.[24] These changes have been suggested to increase venous pressure and subsequently give rise to venular dilatation and venular blood-brain barrier disruption. Venous stasis could in turn lead to cerebral hypoperfusion and ischemia in the periventricular region through diminished clearance of cellular metabolites and as such contribute to the development of white matter lesions and ultimately dementia.[24,25] Because brain imaging was not performed routinely in all participants, we were not able to investigate whether white matter lesions could account for the association we found between venular diameters and risk of dementia.

Smaller arteriolar diameters are strongly related to higher blood pressures. Yet, arteriolar diameters were not related to dementia, including vascular dementia.. Due to increased arterial stiffness as a result of vasoconstriction, intimal thickening, medial hyperplasia, hyalinisation and sclerosis at higher age, widening of retinal arterioles may be less pronounced than in retinal venules in conditions reflecting ischemia.[11] This is supported by the fact that venular rather than arteriolar widening is more strongly associated with conditions reflecting ischemia to the retina or the brain, including carotid artery occlusion.[22]

The observation that larger retinal venular diameters are associated with an increased risk of vascular dementia is in line with observations in stroke and cerebral small vessel disease. Moreover, larger retinal venular diameters are associated with a lower age-at-onset and a higher short-term but not longer-term risk of Alzheimer disease. This suggests that vascular pathology as reflected by larger retinal venular diameters accelerates clinical expression of Alzheimer disease rather than being a cause.

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Chapter 6

General discussion

The aim of the research described in this thesis was to gain more insight in the role of hormones in the etiology of dementia (part I), and to quantify the role of retinal vessels as markers of vascular pathology in cerebrovascular disease and dementia (part II). In this chapter I will, for both parts separately, first review and discuss our main findings in the light of current knowledge on the etiology of dementia. Then, I will discuss methodological issues that are related to the performed studies, with particular reference to causal inference. Finally, I will focus on the implications of our findings for further research.

6.1 ENDOCRINE FACTORS AND RISK OF DEMENTIA

Recent findings from several experimental studies suggest that hormones may be involved in the etiology of Alzheimer disease, the most frequent subtype of dementia. Thyroid hormones, glucocorticoids, and sex hormones have all been shown to alter processing of the amyloid precursor protein and the deposition of β-amyloid, or the hyperphosphorylation of tau protein, which are considered key processes in Alzheimer pathology.[1-4] Large and well-designed prospective population-based studies may provide further evidence for the involvement of hormones in the development of dementia. Thyroid function, glucocorticoids and the sex hormone testosterone have hardly been studied in a prospective population-based setting.

6.1.1 Review and interpretation of main findings

Thyroid hormones

In the Rotterdam Scan Study, we observed no associations between either thyrotropin or thyroid hormones and risk of dementia during a nearly 6-year follow-up period (chapter 2.1). In line with that observation thyrotropin was neither associated with hippocampal nor with amygdalar atrophy, which are considered early preclinical markers of Alzheimer disease on MRI in non-demented elderly.[5] Higher levels of free thyroxine (fT_4) and reverse triiodothyronine (rT_3), and a lower ratio of T_3/rT_3 were all related to hippocampal and amygdalar atrophy, but genetically determined variation of these serum thyroid parameters was not (chapter 2.2). In the Honolulu-Asia Aging Study, thyrotropin was also not related to the risk of dementia during 5 years of follow-up (chapter 2.3). In this study, higher total and free T_4 levels, which may be indicative of hyperthyroidism, were associated with an increased risk of both dementia and Alzheimer disease, but only when measured within 3 years from baseline.

The latter finding is in agreement with an earlier report from the Rotterdam Study, showing an association of subclinical hyperthyroidism with a three-fold increased risk of dementia in a two-year period.[6] The absence of a relation between thyroid function and

risk of dementia at longer follow-up duration in our current studies, could suggest that alterations in thyroid function are a consequence rather than a cause of dementia. Because only thyroid hormone levels and not genetically determined variation thereof were associated with brain atrophy, this also argues against the involvement of thyroid hormones in the development of brain atrophy. Non-thyroidal illness could be the underlying mechanism. Several conditions including malnutrition, starvation, and inflammatory processes accompanying disease yield an increased frequency of non-thyroidal illness in the elderly.[7] In these situations, T_4 is converted preferentially to rT_3 instead of T_3, without overt thyroid dysfunction being present.[8] The observation that higher levels of fT_4 as well as of rT_3 and especially a lower ratio of T_3/rT_3 were all associated with brain atrophy, supports this hypothesis.

Testosterone

In the Rotterdam study, testosterone was not related to the risk of dementia during 9 years of follow-up, neither in men nor in women (chapter 3.1). Lower bioavailable testosterone levels were associated with an increased risk of dementia and Alzheimer disease in elderly men within two years from baseline only. Two other prospective studies yielded conflicting results, reporting either no association or an association of low testosterone with an increased risk of dementia among elderly men.[9,10] The absence of an association at longer follow-up duration, together with the lack of a relation between testosterone and putative early MRI markers of Alzheimer disease in our study, suggests that low testosterone levels could be a consequence rather than a cause of Alzheimer disease in elderly men.

Higher sex-hormone binding globulin (SHBG) levels have been suggested to account for the association of low testosterone with dementia.[11] SHBG binds testosterone and higher levels result in lower free testosterone levels. We indeed found higher SHBG levels to be associated with an increased risk of dementia among elderly men. SHBG levels are increased in various conditions, including subclinical hyperthyroidism and wasting, and also in smoking, which are all related to Alzheimer disease.[11] Additional adjustment for albumin, creatinin, and vascular factors attenuated the association between SHBG and dementia, indicating that higher SHBG may reflect other diseases or comorbidity related to dementia.

Genetically determined glucocorticoid sensitivity

The ER22/23EK variant of the glucocorticoid receptor gene is related to a relative glucocorticoid insensitivity.[12] In the Rotterdam Study, we observed both a lower prevalence and incidence of dementia among carriers of the ER22/23EK allele, although the latter finding did not reach statistical significance (chapter 3.2).

Glucocorticoids may exert either direct neurotoxic effects to the brain, chiefly the hippocampus,[13] or indirect effects through alterations in the vascular risk profile.[14] In particular, the ER22/23EK allele has been related to a better insulin resistance and lower total and higher HDL cholesterol levels.[12] Because the ER22/23EK variant was neither related to hippocampal volume on MRI nor to memory performance in our study, it is unlikely that the ER22/23EK variant prevents hippocampal neurotoxicity or Alzheimer pathology. In the Rotterdam Scan Study, we observed a better psychomotor speed performance and lower presence of white matter lesions and progression thereof on MRI among the ER22/23EK carriers. White matter lesions on MRI are thought to result from cerebral small vessel disease or large vessel atherosclerosis,[15,16] are related to psychomotor speed[17] and have been found to increase the risk of dementia.[18] Therefore, a healthier vascular risk profile may underlie the observed lower risk of dementia among ER22/23EK carriers.

Taken together, our studies do not provide evidence for the involvement of thyroid hormones and testosterone in the development of Alzheimer disease, which contrasts findings from experimental studies. We concluded that observations in imminent dementia are likely due to comorbidity associated with the disease process. However, other explanations need to be considered. Most importantly, possible limitations related to our study design or to the hormone assessments could account for the discrepancy with findings from experimental studies. This will be further discussed in paragraph 6.1.2.

In contrast with our negative findings on thyroid hormones and testosterone, we did find an association of genetically determined glucocorticoid sensitivity with the risk of dementia. We concluded that the ER22/23EK variant is involved in the development of dementia through vascular rather than neurodegenerative pathology. This may suggest that glucocorticoids and possibly other hormones are involved in the development of dementia through vascular pathology, rather than amyloid or tau pathology. Indeed, opposed to the suggested neuroprotective effects of estrogens by experimental studies, observational studies and randomized clinical trials found estrogens to increase the risk of dementia, in particular vascular dementia, and stroke.[19,20] These findings are in accord with the view that vascular pathology may play an important role not only in vascular dementia but also in Alzheimer disease.[21]

6.1.2 Methodological considerations

Prospective study design

The studies described in the first part of this thesis were conducted within the setting of several large prospective population-based studies. The prospective nature of these studies enabled us to measure hormone levels before persons became clinically demented,

which increased the potential for causal inference. However, the initial neuropathological changes in the dementia process may start many years and even decades before dementia becomes clinically apparent. Despite the long follow-up period for dementia in our studies, it is thus possible that hormone levels measured at baseline were already a consequence of, rather than contributing factor to, dementia. In addition, we therefore also studied hormones in relation to hippocampal and amygdalar atrophy, putative early MRI markers of Alzheimer disease on brain imaging in non-demented elderly.[5] Exclusion of persons that developed dementia during the first years of follow-up in our analyses on brain atrophy further reduced the possibility of reverse causality. We observed that thyroid hormones and testosterone were related to the risk of dementia within a few years from baseline only and not during longer follow-up. Together with the absence of associations between hormones and early MRI markers of Alzheimer disease this strongly argues against the involvement of these hormones in the etiology of dementia.

However, selective loss to follow-up might also at least partly explain the lack of associations during especially longer follow-up for dementia. Loss to follow-up for dementia was minimized by using an in person multi-step screening procedure performed both at baseline (1990-1993) and at regular intervals during follow-up (1993-1994, 1997-1999, 2002-2004), in combination with continuous monitoring of the participants' medical records. Yet, dementia is not always recognized or reported in its early stages, and may have been missed especially in participants that were not screened in person at the follow-up examinations. The number of participants that was screened in person decreased during longer follow-up, likely due to an increase in morbidity at higher age. Together, this may have led to an underestimation of especially late dementia cases, and may have attenuated associations between hormones and dementia in particular during longer follow-up.

Definition and assessment of relevant exposure

Our approach to the assessment of thyroid hormones and testosterone may have limited our possibility to find associations of these hormones with dementia. As our main approach, we investigated late life serum parameters of thyroid hormones and testosterone. Given the long preclinical period of dementia, plasma hormone concentrations assessed during late life may, however, be affected by the dementia process. Exposure earlier in life, or cumulative exposure over time may be more important in the etiology of late life dementia than late life exposure.

As an alternative approach, we investigated genetically determined variation of serum thyroid parameters and also glucocorticoid sensitivity, which offers several advantages. Genetically determined variation of hormone levels or sensitivity thereof is reflective of life time exposure, as genetic traits remain constant within individuals. Genetic association studies also deal with reverse causation, as genotype is determined before disease onset. Yet, a possible limitation is the lack of power to find an association. Effects of

individual polymorphisms on the development of dementia are likely small and could go unnoticed. Power depends on study size and, although the Rotterdam Study is quite large, the number of dementia cases is still relatively low when analyzing infrequent genetic variants. This also applies to the ER22/23EK polymorphism we studied.

In addition, it is uncertain to what extent a single measure of only thyroid hormones or testosterone characterizes the prevailing thyroid or androgenic state of the study participants. This is a potential limitation in particular because hormone levels are determined by complex feedback systems. Changes in the concentration of any hormone within an axis modulate concentrations and potentially also receptor expression patterns of all other hormones in that axis. The endocrine state of an individual is therefore much better characterized by measuring all, rather than only a few, hormones in an axis.

Finally, it is also a question whether serum hormone levels adequately reflect the endocrine state in the brain. The availability of the biologically most active thyroid hormone T_3 for instance is strictly regulated by local enzymatic conversion of serum T_4 to T_3 and depends less on serum T_3 levels.[22] In addition, hormone transporters and hormone receptors are also important regulators of hormone actions in the brain.

6.1.3 Future perspective

Future research should be directed at investigating how hormones are related to dementia, and to elucidate the discrepancy in the findings between experimental and observational studies. Several issues come to mind. To better define a persons' thyroid or androgenic state, hormone assessments should be expanded to the complete hormone axis under study. In particular with respect to estrogens and testosterone it has been suggested to study the complete (hypothalamic-pituitary-gonadal) axis, rather than solely relying on sex hormone levels.[23] However, this approach has not yet been undertaken in a prospective population-based setting.

If, based on findings from animal studies, the hypothesis is that hormones increase the risk of Alzheimer disease through an increase in neuritic plaques or neurofibrillary tangles, analyses should be adjusted at least for indicators of comorbidity or frailty and cardiovascular risk factors in particular smoking, which are related to both hormone levels and dementia. With respect to comorbidity, the assessment of T_3 and rT_3 and hormone binding proteins could be considered, regardless of the axis under study. Serum T_3 and rT_3 levels and in particular their ratio are adequate indicators of non-thyroidal illness.[8] Hormone binding proteins (SHBG, cortisol binding globulin, albumin) may be assessed not only to obtain the free hormone concentrations, but also to adjust for other factors related to dementia. SHBG for instance is related to cardiovascular disease and insulin sensitivity.[24]

To further clarify the role of testosterone in dementia, I suggest to study genetic variation of testosterone levels. The glutamine (CAG) repeat polymorphism in the androgen receptor gene for instance is related to testosterone levels,[25] and has been suggested to modulate the association between testosterone and Alzheimer disease.[26] To see whether SHBG is related to dementia as a consequence of comorbidity associated with the disease process or plays a more causal role, genetic variation of SHBG levels should be considered. Recently, promoter polymorphisms in the gene encoding SHBG have been identified that alter SHBG and androgen concentrations, and increase bone-mineral density,[27] which may also be relevant in relation to dementia.

The role of genetically determined glucocorticoid sensitivity in dementia can be further studied by investigating other functionally relevant polymorphisms, for instance Bcl1 and N363S, or haplotypes, in the glucocorticoid receptor gene.[28-30] Prospective studies on glucocorticoids and risk of dementia are lacking. This is likely due to the episodic nature and circadian rhythm of cortisol secretion. This causes considerable within person variability, limiting the use of single measurements of plasma cortisol. Although a cortisol day profile could be obtained by taking blood samples at different time intervals during a day, this technique is more labor intense and possibly too invasive to apply in a population-based setting. Free cortisol measured in saliva obtained at different time intervals or in overnight urine samples, provides less invasive alternatives. To increase efficiency I suggest to study the relation between glucocorticoids and dementia with a case-cohort approach.[31,32]

6.2 RETINAL VESSELS AND RISK OF DEMENTIA

Increasing evidence suggests that vascular pathology is important in the clinical course and progression of Alzheimer disease,[21,33] the most important subtype of dementia. Whether cerebrovascular changes are involved in the development of Alzheimer disease is less clear.[34] Retinal vessel share embryological, anatomical and physiological characteristics with cerebral vessels, and their investigation might provide new insights in the role of vascular pathology in cerebrovascular disease and dementia.[35]

6.2.1 Review and interpretation of main findings

Determinants of retinal vessel diameters

A lower ratio of summarized arteriolar-to-venular diameters (AVR) has been suggested to reflect generalized arteriolar narrowing due to hypertension,[36] and was subsequently associated with an increased risk of cardio- and cerebrovascular disorders.[37-40] Yet, a lower AVR may result from either arteriolar narrowing or venular widening. How the separate

arteriolar and venular diameters contributed to the AVR and what vascular pathology it precisely reflects remained, however, poorly understood. The most important and novel finding of our study was that retinal venular diameters are more variable than was previously assumed. In particular larger venular diameters, rather than smaller arteriolar ones, were related to various pathological conditions, rendering the existing notion that a lower AVR would only reflect generalized arteriolar narrowing due to hypertension, obsolete.

In chapter 4.1 we reported that smaller arteriolar diameters were associated with higher blood pressures. This is in line with findings from other population-based studies,[36,41,42] and indicates that arteriolar narrowing indeed reflects hypertension. In contrast, larger retinal venular diameters, rather than smaller arteriolar ones, were consistently related to atherosclerosis (as indicated by a lower ankle-brachial index, more aortic calcifications and a higher carotid artery plaque-score), higher total cholesterol and lower high density lipoprotein cholesterol levels, a higher body mass index, smoking, and higher levels of non-specific markers of inflammation, such as leukocyte count and erythrocyte sedimentation rate. Higher levels of high-sensitivity C-reactive protein and fibrinogen and higher Lipoprotein Phospholipase-A2 (Lp-PLA2) activity, more specific markers of inflammation, were also related to larger venular diameters (chapter 4.2). Together, our findings suggest that in particular larger venular diameters reflect systemic inflammation, which has recently been confirmed by findings of two other population-based studies.[43,44]

If inflammation is causally related to retinal venular dilatation, an association of genetically determined variation of inflammation with larger venular diameters might be expected. Complement plays a role in the promotion of inflammation as complement and complement regulatory factors are deposited in atherosclerotic plaques.[45] His[402] carriers of the Tyr402His polymorphism in the complement factor H (*CFH)* gene are genetically predisposed to a malfunctioning CFH, ultimately leading to increased complement-related damage to the vascular endothelium,[46] especially in the presence of acute and chronic inflammatory mediators of the complement pathway.[47,48] In our study, His[402] carriers were not found to have larger venular diameters (chapter 4.3). This could suggest that venular dilatation is not an early marker of inflammation, but merely reflects severity of atherosclerosis.

The role of inflammation in the initiation and progression of atherosclerosis is well established[49] and venular widening in these conditions may be due to similar mechanisms. Venular widening has been observed in the early stages of diabetic and venous stasis retinopathy, in which it has been ascribed to retinal hypoxia.[50,51] Venous stasis retinopathy is frequently seen in occlusive disease of the carotid artery and is related to an impaired cerebral blood flow.[51] Venular dilatation may thus be a more general marker of ischemia. In line, we observed that persons with lower arterial oxygen saturation had

larger venular diameters, in particular in the presence of lower total cerebral blood flow (chapter 4.4). Our findings are consistent with experimental studies suggesting that hypoxia aggravates cerebral ischemic damage in the presence of cerebral hypoperfusion only.[52] The absence of brain damage due to low oxygen saturation alone has been ascribed to cerebral autoregulation increasing cerebral blood flow in conditions of reduced arterial oxygen content.[53]

Taken together, larger venular diameters reflect inflammation and atherosclerosis, and may be a more general marker of ischemia, whereas smaller arteriolar diameters reflect hypertension. Because different vascular mechanisms underlie smaller arteriolar and larger venular diameters, they should be analyzed separately when investigating the role of retinal vessels as markers of vascular pathology in cerebrovascular disease and dementia. This may provide important new clues regarding the role of vascular pathology in dementia.

Retinal vessel diameters, cerebrovascular disease and dementia

In chapter 5.1 we reported an association of larger venular diameters with progression of white matter lesions and an increased risk of lacunar infarctions. Furthermore, we observed that non-demented elderly persons with larger venular diameters had more atrophy of the white matter, but not of the grey matter (chapter 5.2). Larger venular diameters were also not related to hippocampal and amygdalar atrophy. Atrophy of the white matter especially has been related to cerebral small vessel disease and cardiovascular risk factors, and has been hypothesized to result from white matter lesions and lacunar infarctions.[54] Our observations on brain atrophy are therefore consistent with those on cerebrovascular disease. Furthermore, within the Rotterdam Study we also found larger retinal venular diameters to increase the risk of stroke.[54] Together, our observations suggest that venular dilatation is related to cerebrovascular but not to neurodegenerative pathology.

In line, persons with larger venular diameters had an increased risk of vascular dementia during a follow-up of more than 10 years (chapter 5.3). Larger venular diameters were also associated with an increased risk of Alzheimer disease, but this was apparent only at shorter follow-up (mean 6 years) and absent at longer follow-up (mean 10.5 years). Moreover, larger venular diameters were related to a lower age-at-onset of Alzheimer disease but not of vascular dementia. Together with the absence of an association between larger venular diameters and putative MRI markers of Alzheimer disease, this suggests that vascular pathology related to larger retinal venular diameters accelerates the clinical expression of subclinical Alzheimer disease, rather than being a cause.

Venular widening may be related to dementia in different ways. Larger retinal venular diameters may reflect exposure to cardiovascular risk factors, in particular inflammation

and atherosclerosis, a lower oxygen supply, and have been related to cerebrovascular disease. All have been associated with an increased risk of dementia[18,55-58] and may have contributed to the progression of Alzheimer disease either separately or together. It has been hypothesized that advanced aging together with conditions that lower the threshold for cerebral hypoperfusion, such as vascular risk factors, places vulnerable neurons in a state of metabolic compromise, leading to neuronal death and eventually dementia.[59] Symptomatic carotid artery disease and other cardiovascular risk factors are related to impaired cerebral blood flow.[60] In particular in the presence of lower oxygen saturation this may lead to relative cerebral oxygen deprivation. It has been suggested that a lower oxygen saturation is more harmful in concomitance with chronic or intermittent hypoperfusion, which is ascribed to impaired cerebral autoregulation, rather than in conditions inducing acute hypoperfusion.[61] Cerebral ischemia in turn might compromise the brain in particular in the periventricular region, because in this area the blood supply is provided through long penetrating end-arterioles with few anastomoses, leading to white matter lesions and lacunar infarctions, atrophy of the white matter and eventually dementia.

In addition, findings from an experimental study suggest that in rat brains activated leukocytes adhere in particular to the cerebral venular endothelium and contribute to the disruption of the venular blood-brain barrier.[62] Although comparative data based on studies in humans are lacking, this could suggest that inflammation and atherosclerosis may not only be involved in retinal venular widening, but may also have acted more directly on the cerebral venular system.

Whether retinal venular widening is indicative of cerebral venular abnormalities is unknown. Besides, studies on the etiology of cerebrovascular disease have almost exclusively looked at the cerebral arterioles only. However, one histopathological study reported wall thickening and obstruction of in particular the periventricular veins, referred to as periventricular venous collagenosis, in brains with severe white matter lesions.[63] These changes have been suggested to increase venous pressure, which might lead to venular dilatation, leaky venules and venular blood-brain barrier disruption.[64] Venous stasis could in turn lead to cerebral hypoperfusion and ischemia in the periventricular region through diminished clearance of cellular metabolites and as such contribute to the development of white matter lesions and dementia.[63,65] Although long overlooked, cerebrovenous pathology and in particular venous stasis might be an important mechanism underlying cerebrovascular disease, dementia and other brain disorders. For instance, venous stasis and cerebral outflow obstruction following jugular vein occlusion have recently been proposed as a mechanism underlying transient global amnesia.[66]

Smaller arteriolar diameters are strongly related to higher blood pressures.[36] Hypertension is a strong risk factor for white matter lesions and stroke, and has also been related to an increased risk of dementia.[67] Thus, an association of smaller arteriolar diameters with

cerebrovascular disease and dementia might be expected. Yet, hypertension results in vasoconstriction, intima thickening, hyalinosis and eventually sclerosis of the arteriolar vessel wall at higher age.[41] Due to increased rigidity, the retinal arterioles may have been less able to respond to blood pressure changes. Moreover, experimental studies have shown that chronic arteriolar vasoconstriction is followed by a structural reduction in the maximal arteriolar vasodilatory capacity.[68] This may have precluded a degree of widening in the retinal arterioles equal to that observed in retinal venules in various other pathological conditions, notably atherosclerosis and ischemia. Alternatively, the absence of an association with arteriolar diameters argues in favor of a more prominent role of venular abnormalities in cerebrovascular disease and dementia.

The main message from the second part of this thesis is that pathology related to retinal venular widening is involved in the etiology of cerebrovascular disease and dementia, especially vascular dementia. Larger retinal venular diameters are associated with inflammation, atherosclerosis and lower oxygen saturation and may reflect structural damage related to these vascular processes. It is likely that these vascular mechanisms at least partly explain the observed associations of larger retinal venular diameters with progression of cerebral white matter lesions, and an increased risk of dementia. To date, knowledge about the pathophysiology of the cerebral venous system and its possible role in cerebrovascular disease and dementia is limited. In particular it remains to be elucidated whether retinal venular widening is a more general marker of ischemia due to inflammation and atherosclerosis or also reflects cerebral venular pathology.

6.2.2 Methodological considerations

Methodological issues related to the assessment of retinal vessel diameters have been discussed in chapter 4.1. Here, I will address a few general methodological considerations that are related to the design of our studies.

Studies with a cross-sectional design

The cross-sectional nature of the studies that were aimed at identifying vascular mechanisms underlying retinal vessel caliber, precludes a conclusion about the temporal relationship between hypertension, inflammation or atherosclerosis on the one hand and retinal vessel caliber on the other hand. Although smaller arteriolar diameters have been reported to precede the onset of hypertension,[69,70] the relation between venular diameters and atherosclerosis has not been studied prospectively. Retinal vessel diameters may reflect structural changes that are caused by the abovementioned vascular processes. Alternatively, retinal vessel diameters reflect severity of vascular disease. Whether they are early or late markers of vascular disease, or reflect cumulative exposure to vascular risk factors remains to be elucidated.

In addition, findings from cross-sectional studies are more prone to several kinds of bias, in particular selection bias. The population-based design of our studies reduced the potential for selection bias, because persons were randomly included. Although over 10% of the fundus transparencies was ungradable, after adjusting for age we only observed small differences in cardiovascular risk factors and other baseline characteristics. Together this suggests that selection bias has played a limited role.

Studies with long follow-up

As described in section 6.1.2, epidemiological studies with a long follow-up are best suited to establish the causality of an association. The related methodological issues have been discussed. Here, I only mention selective attrition due to competing risk. Strong associations of vascular risk factors, in particular atherosclerosis, with mortality have been observed and may attenuate associations with dementia during longer follow-up.[71] In the Rotterdam Study, in particular retinal venular dilatation was found to predict all-cause mortality, especially in younger participants.[72] This may have attenuated the association between larger retinal venular diameters and Alzheimer disease during longer follow-up. However, as the association with vascular dementia became even stronger during longer follow-up this suggests a limited role of selective attrition in this particular instance.

6.2.3 Future perspective

To further our understanding of the potential role of larger retinal venular diameters as markers of vascular pathology in the etiology of cerebrovascular disease and dementia, several possibilities for further research exist. To discern the temporal relationship between vascular pathology and venular dilatation both retinal vessel diameters and their determinants should be measured at different time intervals. To deal with reverse causation, genetic markers of vascular processes that potentially underlie venular dilatation may be considered. Related to the issue of causal inference, it is also unclear at what age the vascular processes that are reflected by venular dilatation start. Studies in cohorts with younger participants may answer this question. The development of a fully automated measurement system to quantify the retinal arteriolar and venular diameters is under way. If proven reliable, this would add to the efficiency and feasibility to generate studies with a large sample size and repeated measurements of retinal vessel diameters over time.

To further clarify the mechanisms underlying retinal venular widening, other factors potentially affecting venular width should be investigated. Endothelial dysfunction is important early in the process of atherosclerosis and may also be involved in venular blood-brain barrier integrity.[62] I therefore suggest to investigate plasma markers of endothelial function, such as plasminogen-activator 1 or von Willebrand factor,[73] or genetically

determined variation thereof. Additional genetic factors that are related to endothelial function include for instance polymorphisms in the genes encoding vascular endothelial growth factor and nitric oxide synthase 3. Other markers of atherosclerosis, for instance coronary artery calcification, are also of importance. Further, factors related to a lower perfusion or a lower oxygen saturation that may be considered are left-ventricular hypertrophy, heart failure and chronic obstructive pulmonary disease. To test the hypothesis that cerebral hypoperfusion underlies the association between larger retinal venular diameters and the risk of dementia, retinal vessel diameters should be investigated in relation to risk of dementia, with additional adjustment for cerebral blood flow.

Whether retinal venular widening reflects cerebral venular abnormalities is unclear. Relating retinal vessel diameters to autopsy data, if specifically aimed at describing cerebral venular pathology, theoretically is an option, but is currently hardly available in population-based studies. Assessment of the cerebrovenous system on 2D phase contrast MR might be an alternative. Yet, the resolution of the currently used scanners is too low to measure the smaller vessel diameters.

Furthermore, segmentation techniques are currently being developed for automated brain tissue classification on MRI that will enable separation of the brain in smaller anatomical structures and regions. Their application may also further our insights in how larger retinal venular diameters are related to cerebrovascular disease and dementia.

Finally, given that venular widening was related to cerebrovascular disease and dementia independent of established cardiovascular risk factors, this also implies that retinal venular diameters may add additional information in the risk assessment of cerebrovascular disorders. However, the predictive value of retinal vessel diameters and other retinal microvascular signs needs yet to be determined.[35] Findings across different studies must first consistently show that venular widening is independently associated with an increased risk of cerebrovascular disorders and dementia. Second, the assessment of retinal venular diameters in addition to the currently applied screening procedures must substantially improve the prediction of these diseases.

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Chapter 7

Summary / Samenvatting

SUMMARY

Dementia, in particular Alzheimer disease, is a common disease in the elderly. Yet to date the underlying causes are still poorly understood. Various factors, including hormonal and vascular factors, may play a role in the etiology of dementia. The aim of the studies described in this thesis was twofold. The first aim was to study the role of hormones in the etiology of dementia. The second aim was to quantify the role of retinal vessel diameters as markers of vascular pathology. Retinal vessels share many characteristics with cerebral vessels and may therefore provide insight in the role of vascular pathology in the etiology of dementia.

The studies described in this thesis were mainly performed within the setting of the Rotterdam Study, a large prospective population-based cohort study conducted within the Ommoord area of Rotterdam among 7,983 inhabitants aged 55 years and older. At the study baseline, all participants underwent a physical examination and blood was taken for the investigation of plasma hormone levels and various vascular factors. An ophthalmologic examination included taking fundus color transparencies. Both at baseline and during several follow-up examinations we screened participants for memory problems and other signs of dementia. For this purpose we also used information of the general practitioners and the regional institution of outpatient mental health care (RIAGG). In addition, over 500 non-demented participants underwent brain imaging. The extent of brain atrophy, in particular of the hippocampus and amygdala, on MRI in non-demented elderly is a putative early marker of Alzheimer disease. We therefore also investigated the extent of brain atrophy in non-demented elderly.

In the first part of this thesis I investigated the role of hormones in the etiology of dementia. **Chapter 2** explored the relation between thyroid hormones and dementia. These hormones were not related to the risk of dementia (**chapter 2.1**), although higher plasma concentrations of thyroid hormone levels were associated with a smaller hippocampal and amygdalar volume on MRI in non-demented elderly. In **chapter 2.2** we described that variations in the genes encoding the enzyme deiodinase type 1 were associated with higher plasma concentrations of thyroid hormones. Because these genetic variants were not related to the extent of hippocampal or amygdalar atrophy, this argues against a causal role for thyroid hormones in the etiology of atrophy of these brain structures. Within the Honolulu-Asia Aging Study, a large prospective population-based cohort study among Japanese-American elderly men, higher thyroid hormone levels were associated with the risk of dementia during shorter follow-up, but not during longer follow-up (**chapter 2.3**). **Chapter 3** was aimed at studying steroid hormones in relation to dementia. We found lower plasma concentrations of testosterone to be associated with a higher short-term risk of dementia among elderly men, but not with the longer-term risk (**chapter 3.1**). No relation was found between testosterone levels and brain atrophy. Together, these find-

ings suggest that higher thyroid hormone and lower testosterone concentrations are a consequence rather than a cause of the dementia process. In **chapter 3.2** we investigated the relation of the ER22/23EK variant in the glucocorticoid receptor gene with risk of dementia. Carriers of this variant are relatively resistant to the effects of glucocorticoids. We observed that carriers of the ER22/23EK allele had a lower risk of dementia. In addition, ER22/23EK-carriers had less often white matter lesions on MRI or progression thereof. No association was found with the extent of brain atrophy. We concluded that genetically determined glucocorticoid sensitivity may be important in the etiology of dementia, possibly through a lower risk of cerebrovascular disease.

In the second part of this thesis I investigated the role of retinal vessel diameters as markers of vascular pathology in dementia. Recently, a semi-automated measurement system became available to reliably quantify vessel diameters on fundus transparencies. A lower ratio of arteriolar to venular diameters (AVR) was ascribed to arteriolar narrowing due to hypertensive damage, and was subsequently associated with a higher risk of cardiovascular diseases. It remained however unclear whether smaller arteriolar diameters, larger venular diameters, or both, contributed to a lower AVR. Furthermore, it was unclear whether only hypertensive damage or also other vascular mechanisms contribute to a lower AVR. In **chapter 4** we investigated various vascular factors in relation to arteriolar and venular diameters. We confirmed that higher blood pressures are related to generalized arteriolar narrowing. In contrast, higher cholesterol levels, the extent of atherosclerosis, a higher body weight and smoking were all associated with generalized venular widening (**chapter 4.1**). Participants with higher plasma concentrations of several inflammatory markers also had larger venular diameters (**chapter 4.2**). Complement activation is important early in the process of atherosclerosis. However, variation in the complement factor H gene, leading to less inactivation of the complement pathway throughout life, was not related to retinal arteriolar and venular diameters (**chapter 4.3**). In **chapter 4.4** we found a lower arterial oxygen saturation to be associated with larger venular diameters, in particular in the presence of a lower cerebral blood flow. We concluded that venular widening reflects inflammation and atherosclerosis, and may be a more general marker of ischemia, whereas arteriolar narrowing is a marker of hypertensive damage. **Chapter 5** was aimed at cerebrovascular disease and dementia. Again, larger venular diameters rather than smaller arteriolar ones were associated with progression of cerebral white matter lesions and a higher risk of lacunar infarcts (**chapter 5.1**). In line with this finding, non-demented elderly with larger venules also had more white matter, but not grey matter, atrophy on MRI (**chapter 5.2**). Larger venular diameters were also related to a higher risk of dementia, in particular vascular dementia (**chapter 5.3**), whereas no association was found with the risk of Alzheimer disease. However, we did find that participants who developed Alzheimer disease who had larger venular diameters at baseline, developed dementia at a younger age than participants with smaller

venules. This suggests that vascular pathology underlying venular widening is not a cause of Alzheimer disease, but instead is involved in the progression of the disease process.

In **chapter 6** I discussed our findings in the context of current knowledge on the etiology of dementia, and gave implications for future research.

SAMENVATTING

Dementie, vooral de ziekte van Alzheimer, is een veel voorkomende ziekte bij ouderen. Tot nog toe is het onduidelijk waardoor dementie veroorzaakt wordt.Verschillende factoren, waaronder hormonale en vasculaire factoren spelen mogelijk een rol bij het ontstaan van dementie. De doelstelling van de studies beschreven in dit proefschrift was tweeledig. Als eerste onderzochten wij de rol van hormonen in het ontstaan van dementie. Daarnaast bestudeerden wij diameters van vaten in het netvlies, de retina, als markers voor vaatpathologie. Retinavaten hebben veel overeenkomsten met hersenvaten en kunnen daarom inzicht verschaffen in de rol van vaatpathologie in het ontstaan van dementie.

De studies in dit proefschrift werden voornamelijk uitgevoerd binnen het Erasmus Gezondheid en Ouderen (ERGO) onderzoek. Dit is een groot prospectief bevolkingsonderzoek in de Rotterdamse wijk Ommoord onder 7.983 inwoners van 55 jaar en ouder. Aan het begin van de studie werden de deelnemers onderzocht en er werd bloed afgenomen voor het bepalen van hormoonspiegels en verschillende vasculaire factoren. Tijdens een oogheelkundig onderzoek werden foto's van het netvlies gemaakt. Zowel aan het begin van het onderzoek als tijdens diverse vervolgonderzoeken screenden we de deelnemers op geheugenproblemen en andere tekenen van dementie. Hiervoor maakten we ook gebruik van gegevens van de huisartsen en het RIAGG. Ruim 500 niet demente deelnemers kregen daarnaast een MRI scan van de hersenen. De mate van hersenatrofie, vooral van de hippocampus en amygdala, gemeten op een MRI scan voorspelt in belangrijke mate de kans op het krijgen van dementie. Daarom onderzochten we ook de mate van hersenatrofie.

In het eerste deel van dit proefschrift werd de rol van hormonen in het ontstaan van dementie beschreven. **Hoofdstuk 2** behandelde de relatie tussen schildklierhormonen en dementie. We vonden geen relatie tussen deze hormonen en de kans op het ontwikkelen van dementie (**hoofdstuk 2.1**). Wel hadden niet-demente ouderen met hogere plasma concentraties van schildklierhormonen een kleinere hippocampus en amygdala op MRI scans van de hersenen. In **hoofdstuk 2.2** beschreven we dat variaties in genen die coderen voor het deiodinase type 1 enzym leiden tot hogere plasma concentraties van schildklierhormonen. Deze genetische varianten waren echter niet gerelateerd aan atrofie van de hippocampus of amygdala. Dit pleit tegen een rol voor schildklierhormonen in het ontstaan van atrofie van deze hersenstructuren. In de Honolulu-Asia Aging Study, een groot prospectief bevolkingsonderzoek onder Japans-Amerikaanse oudere mannen, waren hogere schildklierhormoon spiegels wel gerelateerd aan een hogere kans op dementie, echter alleen op de korte en niet op de langere termijn (**hoofdstuk 2.3**). **Hoofdstuk 3** was gericht op de relatie tussen steroïd hormonen en dementie. We vonden dat lagere plasma concentraties van testosteron gerelateerd waren aan een hogere kans op dementie

bij mannen op de korte termijn, maar niet op de langere termijn (**hoofdstuk 3.1**). Er was geen verband tussen testosteron-waarden en de mate van hersenatrofie. Deze bevindingen suggereren dat hogere schildklierhormoon- en lagere testosteron concentraties in het plasma geen oorzaak van dementie zijn, maar een gevolg van het ziekteproces. In **hoofdstuk 3.2** beschreven we de ER22/23EK variant in het glucocorticoïd receptor gen in relatie tot dementie. Dragers van deze variant hebben een genetisch bepaalde relatieve resistentie voor de effecten van glucocorticoïden (stresshormonen). Wij vonden dat de ER22/23EK variant een lagere kans gaf op het ontwikkelen van dementie. Daarnaast hadden ER22/23EK-dragers minder vaak witte stofafwijkingen en een lager risico op progressie van witte stofafwijkingen. Er was geen relatie met de mate van hersenatrofie. We concludeerden dat genetisch bepaalde gevoeligheid voor stresshormonen mogelijk van belang is in het ontstaan van dementie, via een verlaging van het risico op het ontwikkelen van cerebrovasculaire ziekte.

In het tweede deel van dit proefschrift werd de rol van retinavaten als markers van vaatpathologie in dementie beschreven. Recent werd een semi-automatisch systeem ontwikkeld waarmee de vaatdiameters op netvliesfoto's betrouwbaar kunnen worden gemeten. Een kleinere verhouding (ratio) van diameters van de kleine slagaders (arteriolen) en aders (venulen) in de retina (AVR) werd toegeschreven aan vernauwing van kleine slagaders door hoge bloeddruk en werd gerelateerd aan een hogere kans op hart- en vaatziekten onafhankelijk van bekende vasculaire risicofactoren. Het was echter onduidelijk of kleinere diameters van de arteriolen, grotere diameters van de venulen, of beide, bijdragen aan een kleinere AVR. Tevens is onduidelijk of naast hypertensieve schade ook andere vasculaire mechanismen van invloed zijn op de AVR. In **hoofdstuk 4** werden verschillende vasculaire factoren beschreven in relatie tot arteriolaire en venulaire diameters. We bevestigden dat hogere bloeddrukken gerelateerd waren aan gegeneraliseerde vernauwing van arteriolen. Hogere cholesterolspiegels, de mate van slagaderverkalking (atherosclerose), een hoger lichaamsgewicht en roken waren echter allemaal gerelateerd aan gegeneraliseerde venulaire verwijding. Deelnemers met hogere plasmaconcentraties van verschillende ontstekingseiwitten hadden eveneens wijdere aderen (**hoofdstuk 4.2**). Complement activatie is van belang in de ontwikkeling van atherosclerose. Variatie in het complement factor H gen, die leidt to minder inactivatie van de complement cascade gedurende het hele leven, was echter niet van invloed op de diameters van arteriolen en venulen in de retina (**hoofdstuk 4.3**). In **hoofdstuk 4.4** beschreven we dat een lagere zuurstofspanning in het bloed gerelateerd was aan grotere venulaire diameters, vooral in combinatie met een lagere bloedtoevoer naar de hersenen. We concludeerden dat verwijding van venulen in de retina een maat is voor ontsteking en atherosclerose elders in het lichaam, en mogelijk een meer algemene maat voor ischemie, terwijl vernauwing van arteriolen een maat is voor hypertensieve schade. **Hoofdstuk 5** richtte zich op cerebrovasculaire ziekte en dementie. We beschreven dat opnieuw grotere

diameters van de aderen gerelateerd waren aan progressie van afwijkingen in de witte stof van de hersenen en een grotere kans op doorbloedingsstoornissen van de hersenen resulterend in kleine infarcten (**hoofdstuk 5.1**) In overeenstemming met deze bevinding hadden niet-demente personen met wijdere venulen meer atrofie van de witte stof, maar niet van de grijze stof, van de hersenen (**hoofdstuk 5.2**). Grotere venulaire diameters waren tevens gerelateerd aan een hogere kans op het ontwikkelen van dementie, vooral vasculaire dementie (**hoofdstuk 5.3**). Het risico op het ontwikkelen van de ziekte van Alzheimer was niet verhoogd. Wel vonden we dat personen die de ziekte van Alzheimer ontwikkelden en wijdere venulen hadden aan het begin van het onderzoek, op jongere leeftijd dement werden dan personen met nauwere venulen. Dit suggereert dat de vaatpathologie die gerelateerd is aan grotere venulaire diameters geen oorzaak is van de ziekte van Alzheimer, maar de voortgang van het ziekteproces versnelt.

In **hoofdstuk 6** besprak ik onze bevindingen in het kader van huidige inzichten met betrekking tot het ontstaan van dementie en besteedde ik aandacht aan de implicaties voor verder onderzoek.

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About the author

Frank Jan de Jong was born on March 3rd, 1976 in Dokkum, the Netherlands. He graduated in 1994 at the 'Lauwers College' in Buitenpost to study human movement sciences at the RijksUniversiteit Groningen. He obtained his masters degree in October 1998. That same year he started medical school at Erasmus University, Rotterdam. During his medical studies he participated in the Master of Science program in Clinical Epidemiology at the Netherlands Institute for Health Sciences, for which he went to Boston, USA, to attend the 21st Annual Epidemiology Summer Program. He obtained a masters degree in medicine in March 2002 and subsequently started the work described in this thesis at the Department of Epidemiology & Biostatistics, Erasmus MC, Rotterdam (Prof.dr. M.M.B. Breteler and Prof.dr. P.T.V.M. de Jong). As part of his PhD project he visited the Laboratory of Epidemiology, Demography and Biometry (head Dr. L.J. Launer) of the National Institute on Aging, Bethesda MD, USA. In 2005, he obtained his Master of Science degree in Clinical Epidemiology, and his medical degree (*cum laude*). In May 2007 he will start his neurology training at Erasmus MC, Rotterdam (head: Prof.dr. P.A.E. Sillevis Smitt). He is married to Lia Meijer. They have two children, Nynke and Thijs.