Gender differences in cardiovascular disease

An epidemiologic study of endocrine factors

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Gender differences in cardiovascular disease

An epidemiologic study of endocrine factors

Geslachtsverschillen in hart- en vaatziekten

Een epidemiologisch onderzoek naar endocriene factoren

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Als alles een illusie blijkt,
blijft altijd de illusie.

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(dank aan Roteb)

Contents

Publications and manuscripts based on the studies described in this thesis

Chapter 2.1
Hak AE, Pols HAP, Hofman A, Witteman JCM. Body weight affects the association between smoking and progression of atherosclerosis in postmenopausal women: the Rotterdam Study (submitted).

Chapter 2.2
Hak AE, Stehouwer CDA, Bots ML, Polderman KH, Schalkwijk CG, Westendorp ICD, Hofman A, Witteman JCM. Associations of C-reactive protein with measures of obesity, insulin resistance, and subclinical atherosclerosis in healthy, middle-aged women. Arterioscler Thromb Vasc Biol 1999; 19:1986-91.

Chapter 2.3
Hak AE, Pols HAP, Stehouwer CDA, Meijer J, Kiliaan AJ, Hofman A, Breteler MMB, Witteman JCM. Markers of inflammation and cellular adhesion molecules in relation to insulin resistance in nondiabetic elderly: the Rotterdam Study. J Clin Endocrinol Metab 2001; 86:4398-405.

Chapter 3.1
Hak AE, Witteman JCM, Hugens W, Keyzer JJ, Pop VJ, Uitterlinden AG, Pols HAP. The increase in cholesterol with menopause is associated with the apolipoprotein E genotype: a population-based longitudinal study (submitted).

Chapter 3.2
Hak AE, Polderman KH, Westendorp ICD, Jakobs C, Hofman A, Witteman JCM, Stehouwer CDA. Increased plasma homocysteine after menopause. Atherosclerosis 2000; 149:163-8.

Chapter 3.3
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Chapter 3.4
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Chapter 3.5
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Chapter 4.1
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Chapter 4.2
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CHAPTER 1

Introduction

THE INITIAL INTEREST in coronary heart disease research in the 1950s centered primarily on men because of its emergence as a major cause of morbidity and mortality in men around middle age. In women, the incidence of coronary heart disease is low at younger age and increases after middle age, though the occurrence remains lower in women than in men at all ages. The fact that cardiovascular disease is the major cause of morbidity and mortality in women has been recognized already for many years and the last decade much effort has been put in describing and studying cardiovascular disease in women. Despite the research that has been carried out on the differences in cardiovascular disease between the sexes, the gender gap in coronary heart disease occurrence is not completely understood until now.[1]

The work presented in this thesis aims at gaining insight into gender specific issues of cardiovascular disease and the cause of the rising incidence of cardiovascular disease in women after middle age by studying putative endocrine and metabolic risk factors. Data from various population-based studies were used to study these issues.

In chapter 2, studies on classical cardiovascular disease risk factors attenuating the female advantage with regard to cardiovascular disease occurrence are presented. Chapter 3 contains studies on sex specific determinants of cardiovascular disease with a focus on sex steroids. In chapter 4, studies on alternative endocrine cardiovascular disease risk factors in postmenopausal women are described. In chapter 5, the results described in this thesis are placed in a broader context, some methodological considerations are discussed, and views on further research regarding gender specific issues of cardiovascular disease are put forward.

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CHAPTER 2

Classical risk factors for atherosclerosis

2.1

Body weight affects the association between smoking and progression of atherosclerosis in postmenopausal women

The Rotterdam Study

Abstract

Background: The association between body mass and cardiovascular disease is weaker among smokers, which is generally ascribed to the weight-lowering effect of smoking. An alternative explanation is that the atherogenic effects of smoking may be different among subjects with lower compared with those with higher body weight. We studied whether body mass modifies the association between cigarette smoking and progression of aortic atherosclerosis in postmenopausal women.

Methods: In 1680 postmenopausal women (mean age 65.5 years, SD ± 6.7 years) participating in the population-based Rotterdam Study, complete data on smoking habits, body mass index (BMI), and aortic atherosclerosis at baseline and after a mean follow-up period of 6.5 years (SD ± 0.5 years) were available. Aortic atherosclerosis was assessed by radiographic detection of calcified deposits in the abdominal aorta, which have been shown to reflect intimal atherosclerosis. Data were analyzed by logistic regression. Never-smokers were regarded as the reference category, and analyses were adjusted for age and duration of follow-up.

Results: In lower-weight women (BMI < median), smoking was associated with an odds ratio of 4.3 (95% CI, 2.5-7.2) for any progression of aortic atherosclerosis, whereas in their higher-weight counterparts (BMI ≥ median) the odds ratio was 2.1 (CI, 1.2-3.8) (*P* for interaction < 0.05). The difference could not be attributed to smoking habits and multivariate adjustment did not materially affect the results. In lower-weight women smoking ≥ 10 cigarettes per day, the odds ratio for moderate to severe progression of aortic atherosclerosis was 12.9 (CI, 5.8-28.4) as compared with a corresponding odds ratio of 3.0 (CI, 1.4-6.3) in higher-weight women (*P* for interaction < 0.01).

Conclusion: The association between cigarette smoking and progression of atherosclerosis in postmenopausal women is stronger in lower-weight than in higher-weight women. These results suggest that adipose tissue-derived estrogen may ameliorate the atherogenic effects of smoking.

Introduction

A high body weight is generally considered to be adversely associated with cardiovascular disease risk.[1-4] Among smoking women, effects of body mass on coronary heart disease[2] and cardiovascular mortality[3,4] were found to be less pronounced than among nonsmokers, and among female smokers, a positive relation between body mass and mortality was even virtually absent.[5] The dilution of the association between body weight and cardiovascular disease among smokers is often ascribed to the weight-lowering effect of smoking.[6] An alternative explanation, however, is that the atherogenic effects of smoking may be different among subjects with lower compared with those with higher body weight. Smoking is postulated to exert deleterious cardiovascular consequences through antiestrogenic effects.[7] In postmenopausal women, ovarian estrogen production has ceased and adipose tissue is the major source of endogenous estrogens through peripheral conversion of adrenal androgens.[8,9] The atherogenic effects of smoking may therefore be antagonized by adipose tissue in postmenopausal women. Accordingly, postmenopausal women with higher body weight would be relatively protected against the atherosclerotic consequences of smoking.

In postmenopausal women participating in the population-based Rotterdam Study, we examined whether body mass modifies the association between cigarette smoking and progression of aortic atherosclerosis during 6.5 years of follow-up.

Methods

The Rotterdam Study

The Rotterdam Study is a population-based cohort study designed to assess the occurrence and clarify the determinants of chronic diseases in an aging population.[10] The cohort includes 3105 men and 4878 women aged 55 and over (78% of the eligible population) living in a defined district in Rotterdam, The Netherlands. Baseline data were collected from 1990 until 1993. The third examination phase took place from 1997 until 1999. Between the first and third examination phase 1992 persons had died, and 35 were lost to follow-up. Fifty-five subjects were not invited for the third examination phase because they were living outside the area, resulting in 5901 invited subjects. Of the invited subjects, 1922 men and 2875 women (81%) participated. The study was approved by the medical ethics committee of Erasmus MC, Rotterdam, The Netherlands, and written informed consent was obtained from all participants.

Measurements

During a home interview at baseline and at follow-up, a trained research assistant gathered information on current and past health, medication, smoking habits, alcohol intake, and age of menopause (self-reported age of last menstruation). As an indicator of socio-economic status the highest attained level of education was assessed. Participants were subsequently invited to visit the research center for clinical examination. At baseline, height, weight, and waist and hip circumferences were measured while each participant was wearing indoor clothing without shoes. Body mass index (BMI, weight divided by height squared) and waist-to-hip ratio (WHR) were computed. Two blood pressure measurements were taken with a random-zero sphygmomanometer after a 5-minutes rest with the subject in sitting position, and averaged. Serum total cholesterol and high-density lipoprotein (HDL) cholesterol levels were assessed by an automated enzymatic procedure in a nonfasting blood sample. Diabetes mellitus was defined as the use of glucose-lowering medication or a random or post-load serum glucose level $\geq$ 11.1 mmol/l according to the World Health Organization (WHO) criteria.[11]

Aortic Atherosclerosis

At baseline and at follow-up, lateral radiographic films of the lumbar spine (T12-S1) were made from a fixed distance while the participant was seated. Atherosclerosis was diagnosed off-line by detecting calcified deposits in the abdominal aorta, as described previously,[12,13] by a technician and scored independently of the subjects' smoking status. Calcification was considered present when linear densities were present in an area parallel and anterior to the lumbar spine (L1-L4).

Progression of atherosclerosis was defined as the occurrence of new calcifications or enlargement of the calcified area present at baseline. Baseline and follow-up films were examined in pairs. The extent of progression was graded as mild, moderate, or severe, according to the length of the new area or enlargement of the calcified area present at baseline ($\leq$ 1 cm; >1 up to 2.5 cm; and > 2.5 cm, respectively). No subject showed a decrease in extent of aortic calcification. All films were read by 1 observer who was aware of the date of the radiographs. Before the scoring, a sample of the films was read by 2 observers simultaneously so as to reach agreement on the interpretation of the scoring protocol. Previously determined interobserver agreement on progression scoring (absent versus present) based on 758 pairs of lateral radiographic films of the lumbar spine at our department reached a percentage of agreement of atherosclerotic change of 88, and a κ statistic of 0.74.[12]

The validity of radiographic assessment of aortic atherosclerosis has been

studied by comparing results of this method with data obtained at autopsy. Radiographic assessment was shown to be highly specific, and in most cases visible calcification represented advanced intimal atherosclerosis.[14] Intimal calcification was also shown to be clearly distinguishable from medial calcification.[15] A comparison study involving computed tomography (CT) was performed at our department. In 56 unselected elderly persons, aortic calcifications were independently assessed by radiography and CT. Calcifications were detected on abdominal radiography in 32 subjects. In all but 1 person, these calcifications were shown to be located in the aorta on the corresponding CT images.[13]

Aortic calcification is known to be associated with risk factors for cardiovascular disease[12,13] and with atherosclerosis at other sites[16] and predicts cardiovascular morbidity and mortality.[17,18] When aortic calcification (as detected by radiography) was compared with coronary artery calcium (as detected by electron-beam computed tomography) in 457 participants in the Rotterdam Study, aortic calcification was present in 3.9% of participants in the lowest tertile of coronary artery calcium, in 13.7% of those in the middle tertile of coronary artery calcium, and in 31.5% of those in the highest tertile of coronary artery calcium (P for trend < 0.001, adjusted for age and sex).

Population for analysis

In the Rotterdam Study, 4865 women were postmenopausal at baseline. Of these, 4229 women visited the research center. Because of logistic reasons, a radiograph of the lumbar spine was unavailable at baseline for 870 women. For 75 women, calcification of the posterior wall of the abdominal aorta could not be evaluated because the aorta was not clearly depicted on the radiograph. Information on smoking habits or BMI was missing for 31 and 25 women, respectively, resulting in 3228 women to be included in the analysis of aortic atherosclerosis at baseline.

Of the 2875 women participating at follow-up, 2864 were postmenopausal at baseline. Of these, 2276 women visited the research center both at baseline and at follow-up. Because of logistic reasons, a radiograph of the lumbar spine was unavailable at baseline for 415 women and at follow-up for 312 women. Radiographs of the lumbar spine both at baseline and at follow-up were available for 1792 women. Progression of aortic atherosclerosis could not be evaluated for 55 women because the aorta was not clearly depicted on the radiograph at baseline or at follow-up. Information on smoking habits at baseline and at follow-up and information on BMI at baseline was missing for 14, 13, and 5 women, respectively. Twenty-five women started or re-started smoking during follow-up, resulting in 1680 women to be included in the analysis of progression of aortic atherosclerosis.

Statistical analysis

For current and former smokers, the number of packyears smoked was calculated by multiplying the total number of years of smoking with the number of cigarettes smoked daily divided by 20. We used general linear regression analysis to compare age-adjusted smoking habits between lower-weight (BMI < median) and higher-weight (BMI ≥ median) smoking women. Subgroups of body weight were based on the median BMI for purpose of power. We used logistic regression analysis to compute odds ratios for the association between smoking habit and (progression of) aortic atherosclerosis stratified by the median BMI. Never-smokers were regarded as the reference category. The association between smoking habit and graded progression of aortic atherosclerosis was analyzed by using polytomous logistic regression analysis. We adjusted for age by entering age as a continuous variable in the regression model and, if appropriate, number of years of follow-up. In subsequent models, we additionally adjusted for WHR, systolic blood pressure, cholesterol level, HDL cholesterol level, presence of diabetes mellitus (yes-no), alcohol intake (in 4 categories: nondrinking; less than 1 glass; 1 to 2 glasses; and more than 2 glasses per day), years since menopause, ever use of hormone replacement therapy (yes-no), and education level (in 4 categories: primary education; lower general education/lower vocational education; intermediate vocational education; and higher education/university). For lacking data on categorical confounders missing value indicators were used.[19] To test for effect modification, we added a cross-product term representing the interaction between BMI (below, equal to or above) and smoking habit in the described models.

We considered 2-sided probability values < 0.05 to be statistically significant. SPSS 10.0 for Windows (SPSS Inc., Chicago, Illinois) was used for all analyses.

Results

Baseline characteristics of the study population are shown in Table 1. In the 3228 women available for analysis at baseline, age ranged from 55.0 to 94.5 years, with a mean of 68.1 years. In the 1680 subjects available for analysis during follow-up, age ranged from 55.0 to 88.8 years, with a mean age of 65.6 years at baseline. The median BMI was 26.3 kg/m^2 in all subjects at baseline and 26.0 kg/m^2 in subjects available for analysis during follow-up. The mean body weight of the women available for analysis of progression of atherosclerosis was 70.2 kg (Standard Deviation [SD] ± 10.8 kg) at baseline and 70.5 kg (SD ± 11.9 kg) at follow-up. Seventy-six percent of these women (n=1281) kept stable weight (± 5 kg) during follow-up. At baseline, 625 women (19%) were current

Table 1. Baseline characteristics of the study population

	Subjects for analysis at baseline (n=3228)*	Subjects for analysis during follow-up (n=1680)†
Characteristic	Mean ± SD	Mean ± SD
Age, y	68.1 ± 8.1	65.6 ± 6.7
Height, m	1.62 ± 0.06	1.63 ± 0.06
Weight, kg	70.0 ± 11.1	70.2 ± 10.8
Body mass index (BMI), kg/m^2	26.8 ± 4.0	26.5 ± 3.9
Waist-to-hip ratio (WHR), cm/cm	0.87 ± 0.09	0.86 ± 0.09
Systolic blood pressure, mmHg	139 ± 22	135 ± 21
Diastolic blood pressure, mmHg	74 ± 11	73 ± 11
Total cholesterol, mmol/L	6.9 ± 1.2	6.9 ± 1.2
HDL cholesterol, mmol/L	1.5 ± 0.4	1.5 ± 0.4
Time since menopause, y	19.1 ± 9.5	16.7 ± 8.3
	Percentage	Percentage
Smoking status		
Current smokers	19	18
Continuing smokers‡	-	13
Past smokers§	29	35
Quitted smokers‖	-	5
Never smokers	52	52
Diabetes mellitus	10	6
Alcohol drinkers	74	78
Ever use of hormone replacement therapy	13	15
Education (higher education/university)	5	5
Aortic atherosclerosis	64	58

* For some women, data were missing on WHR (n=206), blood pressure (n=43), total cholesterol level (n=26), HDL cholesterol level (n=30), time since menopause (n=84), diabetes mellitus (n=89), alcohol drinking (n=401), ever use of hormone replacement therapy (n=60), and education (n=11).
† For some women, data were missing on WHR (n=95), blood pressure (n=12), total cholesterol level (n=10), HDL cholesterol level (n=12), time since menopause (n=27), diabetes mellitus (n=47), alcohol drinking (n=137), ever use of hormone replacement therapy (n=27), and education (n=3).
‡ Subjects who continued smoking during follow-up (using follow-up information).
§ Subjects who stopped smoking before the baseline examination.
‖ Subjects who stopped smoking during follow-up (using follow-up information).

cigarette smokers and 1670 subjects (52%) had never smoked. At the follow-up examination, 217 women (13%) turned out to have continued smoking during follow-up and 873 women (52%) had never smoked. Aortic atherosclerosis was present at baseline in 2066 women (64%). Of the women available for analysis during follow-up, aortic atherosclerosis was present at baseline in 58% (n=969) and in 69% of the women (n=1153) progression of aortic atherosclerosis was detected during follow-up.

Smoking, BMI, and aortic atherosclerosis

Number of cigarettes smoked per day, age at starting of smoking, and pack-

Table 2A. Baseline smoking habits in current smokers according to body mass index (BMI)

	Current smokers (n=625)	
	BMI < 26 kg/m^2 (n=367)	BMI ≥ 26 kg/m^2 (n=258)
No. of cigarettes per day	14.2 ± 0.4	13.3 ± 0.5
Age of starting, y	22.2 ± 0.5	23.0 ± 0.6
Packyears of smoking, y	29.3 ± 1.0	26.9 ± 1.1
Inhaling, %	77	62*

Values are age-adjusted means ± SE or percentages.
* $P < 0.01$ relative to BMI < 26 kg/m^2.

Table 2B. Baseline smoking habits in continuing smokers during follow-up according to body mass index (BMI)

	Continuing smokers (n=217)	
	BMI < 26 kg/m^2 (n=138)	BMI ≥ 26 kg/m^2 (n=79)
No. of cigarettes per day	14.6 ± 0.6	14.4 ± 0.8
Age of starting, y	21.9 ± 0.7	21.9 ± 0.9
Packyears of smoking, y	28.8 ± 1.4	28.9 ± 1.8
Inhaling, %	76	68

Values are age-adjusted means ± SE or percentages.

years of smoking did not differ between lower-weight (BMI < median) and higher-weight (BMI ≥ median) smoking women, but a higher proportion of lower-weight women reported inhalation of cigarette smoke (Table 2A). In lower-weight women, smoking was associated with an odds ratio of 4.4 (95% Confidence Interval [CI], 3.2-6.1) for aortic atherosclerosis, whereas in their higher-weight counterparts an odds ratio of 2.1 (CI, 1.5-2.9) was found relative to never-smokers (Table 3A). The formal test for interaction reached a *P* value of 0.004. Multivariate adjustment did not materially affect the results (*P* value for interaction unchanged).

Smoking, BMI, and progression of aortic atherosclerosis

Among continuing smokers, smoking habits at baseline did not differ between lower-weight and higher-weight women (Table 2B). During 6.5 years (SD ± 0.5 years) of follow-up, odds ratios for progression of aortic atherosclerosis in

Table 3A. Aortic atherosclerosis at baseline according to smoking status and body mass index (BMI)

		Never-smokers (n=1670)	Current smokers (n=625)
BMI < 26 kg/m^2	Atherosclerosis, % (n)	57% (447)	75% (276)
	Odds ratio (95% CI)*	1 (reference)	4.4 (3.2 ; 6.1)
	Odds ratio (95% CI)†	1 (reference)	3.6 (2.5 ; 5.2)
BMI ≥ 26 kg/m^2	Atherosclerosis, % (n)	65% (577)	70% (180)
	Odds ratio (95% CI)*	1 (reference)	2.1 (1.5 ; 2.9)
	Odds ratio (95% CI)†	1 (reference)	1.8 (1.3 ; 2.7)

* Adjusted for age.
† Adjusted for age, waist-to-hip ratio, systolic blood pressure, cholesterol level, HDL cholesterol level, diabetes mellitus (yes-no), alcohol intake (4 categories), years since menopause, ever use of hormone replacement therapy (yes-no), and education (4 categories).

Table 3B. Progression of aortic atherosclerosis during follow-up according to smoking status and body mass index (BMI)

		Never-smokers (n=873)	Continuing smokers (n=217)
BMI < 26 kg/m^2	Progression, % (n)	64% (269)	84% (116)
	Odds ratio (95% CI)*	1 (reference)	4.3 (2.5 ; 7.2)
	Odds ratio (95% CI)†	1 (reference)	3.7 (2.1 ; 6.8)
BMI ≥ 26 kg/m^2	Progression, % (n)	68% (311)	76% (60)
	Odds ratio (95% CI)*	1 (reference)	2.1 (1.2 ; 3.8)
	Odds ratio (95% CI)†	1 (reference)	2.0 (1.1 ; 3.7)

* Adjusted for age and duration of follow-up.
† Adjusted for age, duration of follow-up, waist-to-hip ratio, systolic blood pressure, cholesterol level, HDL cholesterol level, diabetes mellitus (yes-no), alcohol intake (4 categories), years since menopause, ever use of hormone replacement therapy (yes-no), and education (4 categories).

lower-weight and higher-weight continuing smoking women relative to never-smokers were 4.3 (CI, 2.5-7.2) and 2.1 (CI, 1.2-3.8), respectively, adjusted for age and duration of follow-up. The test for interaction reached a P value of 0.046 (Table 3B). Multivariate adjustment did not materially affect the results (P value for interaction=0.074). Restriction of the analysis to women with stable weight (± 5 kg) during follow-up (n=1281) did not substantially change the results. The odds ratios for progression of aortic atherosclerosis in lower-weight and higher-weight continuing smoking women relative to never-smokers with stable weight during follow-up were 4.2 (CI, 2.3-7.4) and 2.0 (CI, 1.0-4.2), respectively, adjusted for age and duration of follow-up (P value for interaction=0.065).

When smoking and progression of aortic atherosclerosis were divided into categories, lower-weight continuing cigarette smoking women who smoked ≥ 10

Table 4. Graded progression of aortic atherosclerosis during follow-up according to smoking status and body mass index (BMI), stratified by number of cigarettes smoked

		Never-smokers	Continuing smokers*		
		(n=873)	1-9 cig/day (n=55)	10-19 cig/day (n=91)	≥20 cig/day (n=68)
Mild progression					
BMI < 26 kg/m^2	Progression, % (n)	34% (140)	25% (8)	32% (20)	45% (19)
	Odds ratio (95% CI)†	1 (reference)	0.8 (0.3 ; 2.1)	5.4 (2.0 ; 15.0)	6.9 (2.2 ; 21.3)
BMI ≥ 26 kg/m^2	Progression, % (n)	33% (151)	26% (6)	31% (9)	27% (7)
	Odds ratio (95% CI)†	1 (reference)	1.3 (0.4 ; 4.6)	1.6 (0.6 ; 4.3)	1.3 (0.4 ; 4.2)
Moderate progression					
BMI < 26 kg/m^2	Progression, % (n)	24% (102)	31% (10)	52% (32)	36% (15)
	Odds ratio (95% CI)†	1 (reference)	2.1 (0.8 ; 5.4)	14.8 (5.4 ; 40.9)	11.1 (3.4 ; 36.4)
BMI ≥ 26 kg/m^2	Progression, % (n)	29% (133)	35% (8)	38% (11)	46% (12)
	Odds ratio (95% CI)†	1 (reference)	2.2 (0.7 ; 7.3)	2.8 (0.9 ; 8.0)	4.1 (1.4 ; 12.0)
Severe progression					
BMI < 26 kg/m^2	Progression, % (n)	7% (27)	6% (2)	8% (5)	10% (4)
	Odds ratio (95% CI)†	1 (reference)	1.8 (0.3 ; 9.1)	8.9 (2.1 ; 36.9)	11.7 (2.3 ; 58.7)
BMI ≥ 26 kg/m^2	Progression, % (n)	6% (27)	17% (4)	3% (1)	4% (1)
	Odds ratio (95% CI)†	1 (reference)	4.5 (1.1 ; 18.9)	1.2 (0.1 ; 11.2)	1.3 (0.1 ; 11.5)

Percentages and number of subjects with mild, moderate, and severe progression of aortic atherosclerosis add up to any progression of aortic atherosclerosis (polytomous logistic regression model).

* Due to missing data on number of cigarettes smoked per day 3 subjects could not be categorized accordingly.

† Adjusted for age and duration of follow-up.

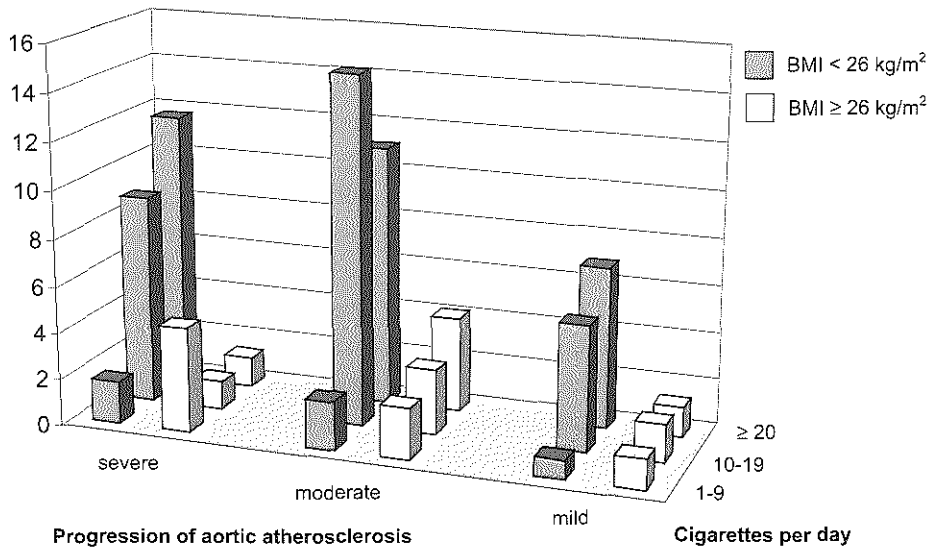

Figure. Odds ratios for graded progression of aortic atherosclerosis during follow-up according to number of cigarettes smoked and body mass index (BMI) among continuing smokers relative to never-smokers, adjusted for age and duration of follow-up (see Table 4 for data)

cigarettes per day tended to reach higher odds ratios for progression of aortic atherosclerosis than their higher-weight counterparts relative to never-smokers (Table 4 and Figure). Aggregated results showed that in lower-weight women smoking ≥ 10 cigarettes per day, an odds ratio for moderate to severe progression of aortic atherosclerosis of 12.9 (CI, 5.8-28.4) was computed, whereas in higher-weight women smoking an equal amount of cigarettes the odds ratio was 3.0 (CI, 1.4-6.3) (*P* value for interaction=0.003).

In 478 past smokers, information on time since smoking cessation was available. In past smokers quitted less than 5 years ago (n=45), the odds ratio for progression of aortic atherosclerosis among lower-weight women was 3.2 (CI, 0.9-11.7), whereas in higher-weight women an odds ratio of 1.7 (CI, 0.7-4.1) was computed, adjusted for age and duration of follow-up. Corresponding odds ratios for past smokers who quitted 5 to 10 years ago (n=49) were 2.1 (CI, 0.9-5.0) and 1.1 (CI, 0.4-3.0), respectively. In past smokers quitted more than 10 years ago (n=384), no association between smoking and progression of aortic atherosclerosis was found.

When we based subgroups on the median body weight instead of the median BMI results did not materially change.

Discussion

Our results indicate that the association between cigarette smoking and progression of atherosclerosis in postmenopausal women is stronger in lower-weight than in higher-weight women. These results suggest that adipose tissue-derived estrogen may ameliorate the atherogenic effects of smoking.

When interpreting our results, some methodological issues should be taken into account. The first concerns the design of the study. Our population-based follow-up study provided insight into the effect of body mass on the association between smoking and progression of atherosclerosis that was not influenced by the disease status. Information on progression of aortic atherosclerosis was available only for women who visited the research center both at baseline and at follow-up. Selection bias would be present when the association between smoking and progression of aortic atherosclerosis is modified by body mass differently among women with or without the availability of follow-up information, which seems unlikely. We measured aortic atherosclerosis radiographically by detecting calcified deposits in the abdominal aorta. A high level of body fat may hamper the radiographic detection of calcification and because of that the detection of progression of atherosclerosis may be underestimated in obese subjects. However, this would not have affected the results differently across smoking categories and therefore the observed interaction remains valid.

Second, we need to consider potential confounding factors. In our population, a higher proportion of lower-weight smoking women reported inhalation of cigarette smoke. Although differences were small, the possibility of residual confounding by smoking habits has to be considered, particularly since severe smokers may be inclined to underreport their smoking habits. However, the association between smoking rate and body weight is curvilinear, indicating that heavy smokers have generally higher body weights than moderate smokers.[20] Therefore, it is unlikely that confounding by amount of smoking induced the more pronounced association between smoking and progression of atherosclerosis in lower-weight women. Furthermore, we have to consider the possibility that weight loss or increase in weight rather than weight itself modifies the association between smoking and progression of aortic atherosclerosis. Restricting our analysis to subjects who kept stable weight (± 5 kg) during follow-up, however, did not affect the results.

A general issue we have to keep in mind is the fact that aortic atherosclerosis and progression of aortic atherosclerosis were observed in a high percentage of subjects (64% and 69%, respectively). Therefore, the odds ratios as derived from logistic regression analysis are overestimates of the corresponding relative risks. However, this does not influence the comparison of estimates across

strata of body weight. A next issue concerns the generalizability of our results. We consider aortic atherosclerosis to be a measure of generalized atherosclerosis. It is possible, however, that the aorta is more vulnerable to the effects of smoking and potential modifying effects of body mass than other arteries. Yet, the fact that aortic atherosclerosis is associated with an up to 9-times increased risk of ischemic stroke[21] is indicative of its importance in relation to cardiovascular disease.

In our study, effects of smoking on progression of aortic atherosclerosis disappeared after 5 years of cessation in higher-weight women, whereas in lower-weight women effects were detectable up to 10 years after smoking cessation. There is evidence of increases in body weight in women after they stop smoking,[22,23] therefore body weight, and consequently BMI, as assessed at baseline may be higher than body weight during the preceding smoking period. However, this misclassification of body weight would only have led us to underestimate the observed effect modification.

It has been shown that lean postmenopausal women are at increased cardiovascular disease mortality risk, possibly due to lower levels of adipose tissue-derived estrogen.[24] In this study,[24] no data were available on smoking. Our data suggest that differences would be more pronounced if associations were observed among smokers. Estrogen mediated effects of body mass among female postmenopausal smokers are also supported by findings in other estrogen related diseases. In postmenopausal smoking women, bone loss was much less pronounced in obese than in slender women,[25] and in postmenopausal women, the risk-enhancing effect of body weight for endometrial cancer was absent among smokers.[26]

Antiestrogenic effects of smoking in women are supported by the observations that relative to nonsmoking women, women who smoke have an earlier menopause,[27,28] a decreased risk of cancer of the endometrium,[26,29] a greater likelihood of osteoporosis[30] and osteoporotic hip fractures,[31] and attain lower levels of estrogen after exogenous estrogen therapy.[32,33] The mechanism underlying anti-estrogenic effects of smoking are not clear.[7] It is unlikely that smoking-related changes in estrogen levels can explain these effects, since smoking is not related to estrogen levels.[32,34] Smoking, however, appears to alter the metabolism of estrogens. It has been shown that compared with female nonsmokers, women who smoked had a higher rate of 2-hydroxylation of estradiol, leading to decreased formation of active estrogen metabolites.[35] These findings could indicate that nonsmokers have more circulating active estrogen than smokers. Decreased estrogenic activity in postmenopausal smokers may also result from increased estrogen-protein binding.[34] Furthermore, the pro-androgenic effects of smoking in postmenopausal women[34] may be counterregulated by body mass,

possibly through its capacity for peripheral aromatization to estrogen.

In summary, we observed that the association between cigarette smoking and progression of atherosclerosis in postmenopausal women is stronger in lower-weight than in higher-weight women. Our results are in agreement with data showing that lower-weight older women are at increased cardiovascular disease mortality risk[24] and indicate that adipose tissue-derived estrogen may ameliorate the atherogenic effects of smoking. They warrant attention for the potential effect of endogenous estrogen in postmenopausal women.

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2.2

Associations of C-reactive protein with measures of obesity, insulin resistance, and subclinical atherosclerosis in healthy, middle-aged women

Abstract

Obesity, the insulin resistance syndrome, and atherosclerosis are closely linked and may all be determinants of an increased acute-phase response. In this study, we examined the relationship of C-reactive protein (CRP) with measures of obesity, variables of the insulin resistance syndrome, and intima-media thickness of the common carotid arteries in 186 healthy, middle-aged women selected from the general population. Associations were assessed by regression analysis. CRP was strongly associated with body mass index (BMI) and waist circumference. CRP was also associated with other variables of the insulin resistance syndrome, including blood pressure, insulin, high-density lipoprotein cholesterol, triglycerides, apolipoprotein A1 (inversely), plasminogen activator inhibitor-1 antigen, and tissue-type plasminogen activator antigen. Associations between CRP and the variables of the insulin resistance syndrome disappeared after controlling for BMI but remained significant for plasminogen activator inhibitor-1 antigen. The association of CRP with common carotid artery intima-media thickness was weak and limited to ever-smokers. BMI explained 29.7% of the variance of CRP, whereas common carotid artery intima-media thickness explained only 3.7%. The results of this population-based study indicate that adiposity is strongly associated with CRP in healthy, middle-aged women. In this population, BMI accounted for the relationship between CRP and other variables of the insulin resistance syndrome. Further studies should determine whether losing weight ameliorates the inflammatory state.

Introduction

Recent data suggest that inflammation is involved in atherogenesis.[1,2] C-reactive protein (CRP), a major acute-phase protein, has been associated with the presence and severity of atherosclerosis[3] and has been found to predict cardiac events in subjects with[4-6] and without[7-9] prevalent cardiovascular disease. Raised concentrations of inflammatory mediators may reflect inflammation in the arterial wall associated with atherosclerosis but may also be causally involved in the disease process.[10,11] Sources of inflammation include infections[10-12] and smoking.[13] Moreover, levels of obesity have been shown to be associated with low-grade inflammation.[14,15]

Recent data also indicate that the insulin resistance syndrome is accompanied by an increased acute-phase response.[16,17] A link between the insulin resistance syndrome and the inflammatory state is further suggested by increased levels of the acute-phase proteins plasminogen activator inhibitor-1 (PAI-1) and fibrinogen in the insulin resistance syndrome[18-20] and by the finding that dyslipidemia in the insulin resistance syndrome and during the acute phase response show strong similarities.[21-23]

Obesity, the insulin resistance syndrome, and atherosclerotic disease are closely linked and may all be determinants of an increased acute-phase response. However, it is not clear whether these factors are independently associated with the inflammatory state. Previous studies on associations between CRP level as a measure of inflammation and cardiovascular risk factors were conducted in middle-aged men and elderly men and women, all of whom are at relatively high risk of atherosclerosis.[14,15] Atherosclerosis and smoking are potential sources of inflammation and possibly obscure the relation of CRP with other risk factors.

In the present study, we investigated the relationship between CRP and measures of obesity, the insulin resistance syndrome, and subclinical atherosclerosis in a population of healthy, middle-aged women with a low exposure to tobacco smoke.

Methods

Study population

We studied a population of 186 women, aged 43-55 years, selected from the general population and participating in a study on the cardiovascular effects of natural menopause. Women were selected from respondents to a mailed questionnaire about menopause, which was sent to all women aged 40 to 60 years living in the town of Zoetermeer, The Netherlands (n=12,675). Women were con-

sidered premenopausal if they had experienced 1 or more regular bleeding episodes in the past 12 months and were free from climacteric symptoms, defined as perspiration and/or hot flushes. Women were considered postmenopausal if their menses had ceased naturally for at least 12 months. Exclusion criteria were diabetes mellitus, prevalent clinical cardiovascular disease, and use of antihypertensive medication or cholesterol-lowering drugs. Women reporting use of female hormones (hormone replacement therapy or oral contraceptives) within 6 months before the clinical examination were excluded, as were subjects currently smoking 5 or more cigarettes per day. Of the eligible subjects, 93 premenopausal and 93 post-menopausal age-matched women were selected (response rate 76% of eligible and invited women). All women gave written informed consent, and the study was approved by the medical ethics committee of the Erasmus University Medical School.

Measurements

During a visit at the research center, a medical history was taken by a physician. Height, weight, and waist and hip circumferences were measured while the subjects wore indoor clothes without shoes. Body mass index (BMI, weight divided by height squared) and waist-to-hip ratio (WHR) were computed. Cigarette smoking history was obtained by a standardized questionnaire. Blood pressure was assessed with a DINAMAP automatic blood pressure recorder (Critikon, Inc.). After a 5-minutes rest in the supine position, blood pressure was measured 4 times at the right upper arm with an appropriately sized cuff, and the mean was used in the analyses.

Venous blood samples were drawn from each subject after a 12-hour fast. The samples were stored at -80°C. Total cholesterol was measured with an automated enzymatic method using the CHOD-PAP high performance reagent kit from Boehringer Mannheim. High-density lipoprotein (HDL) cholesterol was measured by the phosphotungstate method. Low-density lipoprotein (LDL) cholesterol was computed by the Friedewald formula.[24] Triglycerides were determined by using a reagent kit from Boehringer Mannheim after enzymatic hydrolysis of the triglycerides and subsequent determination of liberated glycerol by colorimetry. No correction was made for serum free glycerol. Apolipoproteins A1 and B were measured by an automated turbidimetric immunoassay using the reagent kits from Orion Diagnostics. Glucose was enzymatically determined by the hexokinase method (Instruchemie). Serum insulin was determined by metric assay (Biosource Diagnostics). This assay has no cross-reactivity with either proinsulin or C-peptide. PAI-1 antigen and tissue-type plasminogen activator (tPA) antigen levels were determined by ELISA (Innotest PAI-1, Innogenetics NV, and Imulyse, Biopool, respectively). CRP was measured

by an in-house ELISA with rabbit anti-CRP (Dako) as the catching and tagging antibody.[25] Intraassay and interassay coefficients of variation for CRP were 3.8% and 4.7%, respectively. Fasting insulin levels were used as a measure of insulin resistance.[26] In addition, insulin sensitivity was calculated according to the formula of the homeostasis model assessment method (HOMA): insulin resistance = fasting insulin x fasting glucose / 22.5.[27]

Carotid artery intima-media thickness (IMT)

Common carotid artery IMT was used as an indicator of generalized atherosclerosis.[28] Ultrasonography of the right common carotid artery was performed with a 7.5-MHz linear array transducer (ATL UltraMark IV) as described in detail previously.[29] For each individual, the common carotid artery IMT was determined as the average of near- and far-wall measurements. Carotid artery IMT measurements have been shown to be reproducible.[30] In short, mean differences (and SDs) in far-wall IMT of the common carotid arteries between paired measurements of sonographers, readers, and visists were 0.040 mm (0.07), 0.069 mm (0.04), and 0.071 mm (0.09), respectively. The intraclass correlation coefficients were 0.63, 0.88, and 0.74, respectively. These results are in agreement with the reproducibility of IMT measurements found in other studies.[31] In the present study, all measurements were conducted by 1 sonographer and 1 reader.

Statistical analysis

The clinical and biochemical features of the population are presented as mean ± SD, median (and interquartile range) for variables with a skewed distribution, or percentages. Because the distribution of CRP was highly skewed, it was natural-log-transformed for all analyses. The strength of the associations between CRP and clinical and biochemical variables was assessed by linear regression of ln CRP on each variable separately, adjusted for age. Because strong associations were found between CRP and measures of obesity, we adjusted for them in additional models. Regression analysis was further used to estimate the explained proportion of variance in CRP (R^2). The difference in CRP between premenopausal and postmenopausal women adjusted for age and measures of obesity was studied with regression analysis. We considered 2-sided probability values < 0.05 to be statistically significant. SPSS 7.5 for Windows was used for all analyses.

Results

Characteristics of the population are described in Table 1. BMI ranged from 16.8

to 41.1 kg/m^2; 42 subjects had a BMI > 27 kg/m^2. CRP varied from 0.05 to 14.38 mg/L; 2 subjects had values > 10 mg/L (10.70 and 14.38 mg/L), the cutpoint generally used to identify clinically relevant inflammation.[32] Fasting insulin levels ranged from 18 to 232 pmol/L. Common carotid artery IMT ranged from 0.43 to 0.97 mm.

CRP was significantly associated with measures of obesity: BMI, waist and hip circumferences, and WHR (Table 2). Associations with CRP were stronger for BMI and waist and hip circumferences than for WHR (r=0.54 for BMI, r=0.55 for waist circumference, r=0.53 for hip circumference, and r=0.33 for WHR, all adjusted for age). After adjustment for BMI, hip circumference and WHR were no longer associated with CRP, whereas waist circumference still was. We next visualized this relationship between BMI, WHR, and CRP by subdividing the

Table 1. Clinical and biochemical characteristics of 186 middle-aged women

Variable	All subjects
Age, y	50.9 ± 2.3
Body mass index (BMI), kg/m^2	24.9 ± 4.0
Waist circumference, cm	81.5 ± 9.5
Hip circumference, cm	105.7 ± 8.6
Waist-to-hip ratio (WHR), cm/cm	0.77 ± 0.05
Smoking status, %	
Never	53.2
Past	40.3
Current*	6.5
Systolic blood pressure, mmHg	121 ± 14
Diastolic blood pressure, mmHg	68 ± 10
Hypertension, %†	2.2
Glucose, mmol/l	5.5 ± 0.6
Insulin, picomol/L‡	44.0 (32.0 - 59.0)
HOMA, picomol x mmol/L^2‡, §	10.8 (7.7 - 15.5)
Total cholesterol, mmol/L	6.2 ± 1.0
HDL cholesterol, mmol/L	1.6 ± 0.4
LDL cholesterol, mmol/L	4.1 ± 0.9
Triglycerides, mmol/L‡, ‖	1.0 (0.8 - 1.3)
Apolipoprotein A1, mg/dL	154.5 ± 31.6
Apolipoprotein B, mg/dL	102.0 ± 26.3
PAI-1 antigen, ng/mL‡	53.0 (34.0 - 85.3)
tPA antigen, ng/mL	6.3 ± 2.4
C-reactive protein (CRP), mg/L‡	0.68 (0.33 - 1.44)
Common carotid artery IMT, mm	0.61 ± 0.09

Data are mean ± SD, median (interquartile range) for variables with skewed distributions, or percentages.
* Subjects who smoked 5 or more cigarettes per day were excluded from study participation.
† Hypertension was defined as systolic blood pressure ≥ 160 mmHg and/or diastolic blood pressure ≥ 95 mmHg.
‡ Skewed data.
§ HOMA = fasting insulin x fasting glucose / 22.5.
‖ Highest level of triglycerides was 3.80 mmol/L.

study population by the median BMI (23.9 kg/m^2) and WHR (0.77) (Figure, geometric means). BMI explained 29.7% of the variance of CRP; waist circumference 31.3%; hip circumference 28.7%; and WHR 11.4%, after adjustment for age.

The other variables included in or associated with the insulin resistance syndrome were also significantly associated with CRP: blood pressure, insulin, HDL cholesterol, triglycerides, apolipoprotein A1 (inversely), PAI-1 antigen, and tPA antigen (Table 3). No associations were found with glucose or with total and LDL cholesterol, whereas an association with apolipoprotein B was present. Separate analyses after exclusion of subjects with levels of CRP > 10 mg/L did not affect the results (data not shown).

After controlling for BMI, the associations between CRP and variables of the insulin resistance syndrome disappeared except for the association with PAI-1

Table 2. Regression coefficients* for ln C-reactive protein (CRP) as the dependent variable and measures of obesity as independent variables in 186 women

	Adjusted for age		Adjusted for age & BMI	
	β*	(95% CI)	β*	(95% CI)
Body mass index (BMI), 1 kg/m^2	0.14†	(0.11 ; 0.18)	-	-
Waist circumference, 10 cm	0.62†	(0.48 ; 0.75)	0.39‡	(0.11 ; 0.67)
Hip circumference, 10 cm	0.65†	(0.50 ; 0.80)	0.29	(-0.051 ; 0.67)
Waist-to-hip ratio (WHR), 0.05	0.34†	(0.20 ; 0.49)	0.12	(-0.021 ; 0.26)

* β indicates regression coefficient; an increase of the independent variable by 1 unit is associated with an increase of CRP by a factor of e^{β}.
Regression significant at the ‡0.01 and †0.001 levels, respectively (all 2-tailed).

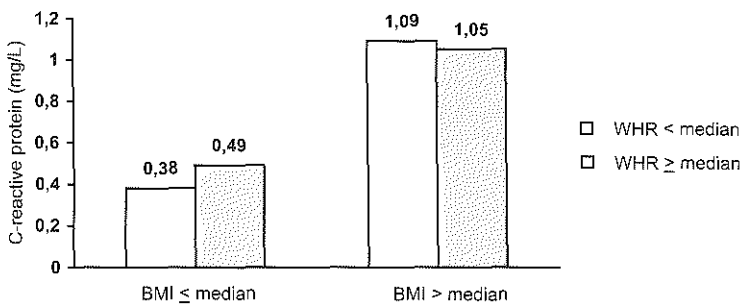

Figure. Levels of C-reactive protein (mg/L) according to body mass index (BMI) and waist-to-hip ratio (WHR) in 186 women. Values are geometric means.

Table 3. Regression coefficients* for ln C-reactive protein (CRP) as the dependent variable and clinical and biochemical characteristics as independent variables in 186 women

	Adjusted for age		Adjusted for age & BMI	
	β*	(95% CI)	β*	(95% CI)
Systolic blood pressure, 10 mmHg	0.16†	(0.057 ; 0.27)	0.025	(-0.071 ; 0.12)
Diastolic blood pressure, 10 mmHg	0.20†	(0.043 ; 0.35)	0.029	(-0.11 ; 0.17)
Glucose, 1 mmol/L	0.23	(-0.055 ; 0.51)	-0.069	(-0.32 ; 0.18)
Insulin, 10 picomol/L	0.11‡	(0.057 ; 0.16)	0.024	(-0.025 ; 0.074)
HOMA, 5 picomol x mmol/L²§	0.18‡	(0.089 ; 0.26)	0.028	(-0.058 ; 0.12)
Cholesterol, 1 mmol/L	0.11	(-0.045 ; 0.26)	0.080	(-0.050 ; 0.21)
HDL cholesterol, 0.5 mmol/L	-0.37‡	(-0.56 ; -0.17)	-0.092	(-0.28 ; 0.093)
LDL cholesterol, 1 mmol/L	0.15	(-0.020 ; 0.31)	0.090	(-0.051 ; 0.23)
Triglycerides, 1 mmol/L	0.64‡	(0.38 ; 0.90)	0.23	(-0.027 ; 0.49)
Apolipoprotein A1, 10 mg/dL	-0.063‖	(-0.11 ; -0.015)	-0.027	(-0.068 ; 0.015)
Apolipoprotein B, 10 mg/dL	0.078‖	(0.021 ; 0.14)	0.029	(-0.022 ; 0.080)
PAI-1 antigen, 1 ng/mL#	0.65‡	(0.45 ; 0.84)	0.30‖	(0.082 ; 0.51)
tPA antigen, 1 ng/mL	0.13‡	(0.067 ; 0.19)	0.052	(-0.005 ; 0.11)

BMI = body mass index.
* β indicates regression coefficient; an increase of the independent variable by 1 unit is associated with an increase of CRP by a factor of e^{β}.
§ HOMA = fasting insulin x fasting glucose / 22.5.
# PAI-1 was ln transformed to obtain a better model-fit as assessed by residual analysis; an increase of PAI-1 by 1% yields an increase of CRP by β%.
Regression significant at the †0.05, ‖0.01 and ‡0.001 levels, respectively (all 2-tailed).

Table 4. Measures of obesity and C-reactive protein (CRP) in premenopausal and postmenopausal women

	Premenopausal (n = 93)	Postmenopausal (n = 93)
	mean ± SE	mean ± SE
Age, y	50.6 ± 0.24	51.1 ± 0.24
Body Mass Index (BMI), kg/m²*	24.7 ± 0.41	25.0 ± 0.41
Waist circumference, cm*	81.3 ± 1.00	81.6 ± 0.99
Hip circumference, cm*	105.3 ± 0.90	106.1 ± 0.89
Waist-to-hip ratio (WHR), cm/cm*	0.77 ± 0.005	0.77 ± 0.005
C-reactive protein (CRP), mg/L*†	0.61 (0.49 ; 0.76)	0.71 (0.58 ; 0.88)
C-reactive protein (CRP), mg/L*†‡	0.62 (0.52 ; 0.74)	0.69 (0.58 ; 0.84)

* Adjusted for age.
† Geometric means (95% CI) are shown for CRP because its distribution is highly skewed.
‡ Adjusted for age and BMI.

antigen, although there was a substantial decline in the magnitude of this association (Table 3). Controlling for waist circumference gave the same results, whereas controlling for hip circumference decreased the described associations to a somewhat smaller extent. Controlling for WHR, on the other hand, had only a small influence on the described associations (data not shown). To evaluate whether the clustering of variables belonging to the insulin resistance syndrome might be a reflection of a general acute-phase response, associations between measures of insulin resistance (insulin and HOMA) and the other variables of the insulin resistance syndrome were adjusted for CRP. This adjustment did not modify the relation between insulin, HOMA, and the other variables (data not shown).

Measures of obesity and CRP in premenopausal and postmenopausal women separately are shown in Table 4. CRP did not differ significantly between premenopausal and postmenopausal women. Age-adjusted geometric means were 0.61 and 0.71 mg/L respectively (15% increase with menopause; 95% CI, -15% to 45%). Because menopause may be associated with changes in measures of obesity, we adjusted for these variables, which slightly influenced the results. Postmenopausal women had an age-adjusted level of cholesterol of 6.48 mmol/L versus 5.89 mmol/L in premenopausal women (10% difference; CI, 5% to 14%). PAI-1 antigen increased with menopause, but the difference lacked statistical significance. In premenopausal women, the age-adjusted geometric mean of PAI-1 antigen was 52.9 ng/mL versus 61.1 ng/mL in postmenopausal women (13% increase with menopause; CI, -8% to 33%). Because cholesterol and PAI-1-antigen are known to increase with menopause, these results indicate a correct selection of menopausal groups. The associations between CRP on the one hand and both measures of obesity and other variables of the insulin resistance syndrome on the other were found to be identical when examined in premenopausal and postmenopausal women separately (data not shown).

CRP was significantly associated with common carotid artery IMT. After stratification by smoking status, associations between CRP and common carotid artery IMT appeared to be present in ever-smokers only (Table 5). Common carotid artery IMT explained 3.7% of the variance of CRP after adjustment for age.

Discussion

Our results indicate that in healthy, middle-aged women, CRP is strongly associated with measures of obesity. CRP was associated with BMI and waist and hip circumferences but not with WHR after adjustment for BMI. CRP was also asso-

Table 5. Regression coefficients* for ln C-reactive protein (CRP) as the dependent variable and common carotid artery intima-media thickness (IMT) as the independent variable in 186 women according to smoking status

	All subjects		Ever smokers (n=87)		Never smokers (n=99)	
	β*	(95% CI)	β*	(95% CI)	β*	(95% CI)
Adjusted for age	0.021†	(0.003 ; 0.039)	0.040‡	(0.013 ; 0.067)	0.004	(-0.020 ; 0.028)
Adjusted for age & BMI	0.014	(-0.001 ; 0.030)	0.036‡	(0.014 ; 0.059)	-0.006	(-0.026 ; 0.015)

BMI = body mass index.

* β indicates regression coefficient; a 1-mm increase of common carotid artery IMT is associated with an increase of CRP by a factor of e^{β}.
Regression significant at the †0.05 and ‡0.01 levels, respectively (2-tailed).

ciated with other variables included in the insulin resistance syndrome. After controlling for BMI, however, the associations disappeared. Although in this population CRP was associated with common carotid artery IMT in ever-smokers, measures of obesity explained a much larger part of the variance of CRP than did carotid artery IMT.

One hypothesis explaining these results is that adipose tissue might be the common antecedent of both CRP and insulin resistance. The associations between CRP and variables of the insulin resistance syndrome may thus be due to the association of BMI with both insulin resistance and the acute-phase response. This idea is consistent with experimental evidence indicating that adipocytes produce tumor necrosis factor (TNF)-α.[33] TNF-α induces interleukin-6 (IL-6) synthesis,[34] a prime regulator of CRP synthesis.[35,36] Additional support for this hypothesis comes from the observation that weight reduction leads to a decrease of TNF-α mRNA expression[37] and of serum levels of TNF-α in diabetic subjects.[38] We found that CRP was strongly related to BMI and to waist and hip circumferences separately, but less to WHR. These results are compatible with previous studies, in which BMI but not WHR was related to TNF-α expression or TNF-α levels.[33,39] However, after adjustment for BMI, waist circumference was still related to CRP, whereas hip circumference was not. This suggests that abdominal fat deposition is most important in inducing inflammation.

Associations between CRP concentrations and fasting serum insulin concentrations, which persisted after adjustment for BMI, have been observed in a population of male patients with angina pectoris.[18] In addition, in healthy, middle-aged men, relationships between CRP and cardiovascular risk factors like HDL cholesterol and triglycerides persisted after adjustment for BMI.[14] One possible explanation for these discrepant results might be that the relationships between obesity, the insulin resistance syndrome, and the acute-phase response are different between men and women. Support for this hypothesis comes from the observation that sex steroids influence the metabolic activity of adipose tissue.[40] Additionally, the described studies differ from ours in that those subjects were likely to suffer from more pronounced atherosclerosis because they were male or suffering from angina pectoris. Atherosclerosis might have spuriously induced the relation between CRP and other cardiovascular risk factors. Because in our population women had a low burden of atherosclerosis, as estimated from carotid artery IMT, the potential for confounding by atherosclerosis in our study is less likely.

Associations between measures of insulin resistance and other variables included in the insulin resistance syndrome were not attenuated by adjusting for CRP levels. Therefore, our data do not suggest that the clustering of variables belonging to the insulin resistance syndrome might be a reflection of

a general acute-phase response.[16] Also, because the association between insulin resistance and measures of obesity was not affected by adjustment for CRP, our data do not support the hypothesis that adipose-tissue-derived cytokines may mediate the relation between obesity and the insulin resistance syndrome.[17,33,37,39] However, this hypothesis encompasses a causal role for TNF-α; therefore, this inference might have been more valid had we adjusted for TNF-α instead of CRP.

The selection of premenopausal and postmenopausal women is likely to be accurate, as reflected by an age-adjusted increase of cholesterol of 10 %, which is in agreement with other studies.[41,42] We did not find a clear influence of menopause on CRP levels. Both age-, and age-and-BMI-adjusted levels of CRP were slightly higher in postmenopausal (0.71 mg/L) than in premenopausal women (0.61 mg/L), but this 15% difference was not statistically significant. This result can probably be attributed to the large variation of this measure. To the best of our knowledge, no published data on the association between menopause and CRP levels are available from others studies. Estrogen replacement therapy in postmenopausal women has been shown to lower TNF-α[43] and acute-phase reactants other than CRP.[44] Experimental data suggest an inhibitory effect of estrogens on IL-6 gene expression.[45] Recent data from the Cardiovascular Health Study, however, suggest an increase of CRP with hormone replacement therapy.[46] Further studies are needed to address the association between inflammation, estrogens, and menopause.

We are the first to describe an association between CRP and common carotid artery IMT in healthy, middle-aged women, which association was limited to ever-smokers (Table 4). In a study by Tracy et al[15] in a population of elderly men and women, CRP was not related to internal carotid wall thickness but was related to ankle-arm index in ever smokers only. Data from the MRFIT (Multiple Risk Factor Intervention Trial) study also show a stronger association of CRP with coronary heart disease deaths in middle-aged male smokers than in non-smokers, as defined at baseline.[8] Taken together, these and the present data suggest that CRP may mark permanent, underlying vascular damage due in part to smoking. This may explain why the associations between inflammation and atherosclerosis are more pronounced not only in current but also in former smokers. In the Physicians' Health Study, however, smoking did not modify the relation between CRP and the risk of cardiovascular events.[7]

Some issues of our study need to be addressed. First, we did not measure exposure to infectious agents such as *Helicobacter pylori* and *Chlamydia pneumoniae*, which may be weak determinants of CRP levels.[12,17] However, it appears unlikely that exposure to these agents would confound the association between BMI and CRP level. Second, in this study we measured atherosclerosis at only

1 location in the vascular system. Although we assume that common carotid artery IMT is a measure of generalized atherosclerosis,[28] assessment of the degree of atherosclerosis might have been more accurate had we used measurements at multiple locations. Finally, this study was conducted in healthy, middle-aged women without clinical cardiovascular disease, no medication use, and a low current exposure to tobacco. Smoking and atherosclerosis are potential determinants of CRP, and therefore, the choice of our population facilitates the investigation of other factors associated with CRP. However, in this population, ever smoking was also found to modify the association between CRP and atherosclerosis.

In summary, our results indicate that adipose tissue is strongly associated with CRP in healthy, middle-aged women. In this population with a low burden of atherosclerosis and current smoking, BMI accounts for the association between CRP and variables of the insulin resistance syndrome. Because inflammatory mediators may be directly involved in atherogenesis, these results suggest an important mechanism through which obesity might affect the risk of coronary heart disease. Further studies should determine whether losing weight ameliorates the inflammatory state.

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2.3

Markers of inflammation and cellular adhesion molecules in relation to insulin resistance in nondiabetic elderly

The Rotterdam Study

Abstract

Insulin resistance, which is highly prevalent in the elderly, is suggested to be accompanied by an increased acute-phase response. Until now, it is unclear whether cellular adhesion molecules are involved in the clustering of insulin resistance.

In the present study, we examined the relationship of insulin resistance (measured by postload insulin) with levels of markers of inflammation and cellular adhesion molecules in a random sample of 574 nondiabetic elderly men and women participating in the Rotterdam Study. Associations were assessed by regression analysis, with ln-insulin as the dependent variable [regression coefficient (95% CI)].

In our population, insulin was strongly and significantly ($P<0.001$) associated with the markers of inflammation C-reactive protein (CRP) [1.52 (CI, 0.96-2.08)], α-1-antichymotrypsin (ACT) [1.25 (CI, 0.82-1.69)] and interleukin-6 (IL-6) [2.60 (CI, 1.69-3.52)], adjusted for age and gender. Associations weakened, to some extent, after additional adjustment for measures of obesity, smoking, and cardiovascular disease. Insulin was associated with the soluble intercellular adhesion molecule 1 (sICAM-1) [2.22 (CI, 1.29-3.16); $P<0.001$], whereas no association with the soluble vascular cell adhesion molecule 1 (sVCAM-1) was found. The strength of the associations of insulin with CRP, ACT, IL-6, and sICAM-1, as assessed by standardized regression coefficients, was comparable with the strength of the associations of insulin with high-density lipoprotein cholesterol, body mass index, and waist-to-hip ratio.

The results of this population-based study indicate that low-grade inflammation and the cellular adhesion molecule sICAM-1 are an integral part of insulin resistance in nondiabetic elderly. These factors may contribute to the well-known relationship between insulin resistance and cardiovascular disease risk and might potentially become therapeutic targets in insulin resistant subjects.

Introduction

The insulin resistance syndrome involves clustering of several metabolic cardiovascular disease risk factors: raised insulin, dyslipidemia, obesity, increased abdominal fat, and hypertension.[1-3] Insulin resistance is highly prevalent in the elderly[4] and is associated with cardiovascular disease risk. Recent data suggest that inflammation plays a crucial role in atherogenesis[5] and that also insulin resistance may be accompanied by an increased acute-phase response, both in subjects with[6] and without diabetes mellitus.[7,8] A link between insulin resistance and the inflammatory state is further suggested by increased levels of the acute-phase proteins plasminogen activator inhibitor-1 and fibrinogen in the insulin resistance syndrome,[9,10] and by the finding that dyslipidemia in the insulin resistance syndrome and during the acute-phase response show strong similarities.[11,12]

Increased levels of circulating cellular adhesion molecules have been shown among diabetic subjects, compared with nondiabetic controls.[13,14] Cellular adhesion molecules mediate the attachment and transmigration of leukocytes across the endothelial surface in response to several inflammatory cytokines,[15] and are hypothesized to play an important role in the initiation of atherosclerosis.[16] Until now, it is unclear whether cellular adhesion molecules are involved in the clustering of insulin resistance.

To further clarify whether inflammation and endothelial activation are an integral part of the insulin resistance syndrome, we cross-sectionally examined associations of levels of markers of inflammation and cellular adhesion molecules with insulin resistance (measured by postload insulin) in a population of nondiabetic elderly men and women participating in the Rotterdam Study.

Subjects and Methods

Study population

The Rotterdam Study is a population-based cohort study aiming to assess the occurrence of chronic diseases in an aging population and to clarify their determinants.[17] The cohort includes 3105 men and 4878 women aged 55 years old and older (78% of the eligible population), living in a defined district in Rotterdam, The Netherlands. Baseline data were collected from August 1990 until July 1993. Information on current and past health, medication, lifestyle, and risk factors for chronic diseases was gathered during a home interview by a trained research assistant. The participants were subsequently invited to a research center for clinical examination. The study was approved by the medical ethics committee

of the Erasmus Medical Center Rotterdam, Rotterdam, The Netherlands.

Levels of inflammatory markers and cellular adhesion molecules were determined in plasma in a gender-stratified random sample of 720 subjects. As part of the Rotterdam Study, glucose metabolism was studied using a nonfasting oral glucose tolerance test. Within the sample of 720 subjects, postload insulin levels were available for 602 participants not prescribed glucose-lowering medication. Because postload insulin is not considered to be a valid measure of insulin resistance in diabetic subjects, subjects with newly diagnosed diabetes mellitus on basis of postload serum glucose levels ($\geq$ 11.1 mmol/L, n=28) were excluded, leaving a population of 574 nondiabetic subjects. Given gender distribution, the prevalence of cardiovascular disease risk factors in the 574 subjects was comparable with the prevalence of these risk factors in the total nondiabetic population of the Rotterdam Study.

Clinical examination and laboratory methods

Height, weight, and waist and hip circumferences were measured while the study participants wore indoor clothes without shoes. Body mass index (BMI, weight divided by height squared) and waist-to-hip ratio (WHR) were computed. Sitting systolic and diastolic blood pressure were measured with a random-zero sphygmomanometer by a trained research assistant, after a 5-minutes rest, and a standard 12-lead electrocardiogram (ECG) was obtained (ACTA ECG recorder, Esoate, Florence, Italy). The presence of peripheral arterial disease (PAD) was evaluated by measuring the systolic blood pressure of the posterior tibial artery at both the right and the left leg using an 8-MHz continuous-wave Doppler probe (Huntleigh 500 D, Huntleigh Technology, Bedfordshire, UK) and a random-zero sphygmomanometer. For each leg, a single blood pressure reading was taken with the subject in the supine position. The ratio of the systolic blood pressure at the ankle to the systolic blood pressure at the arm was calculated for each leg.

A venipuncture was performed, and nonfasting blood samples were obtained and were directly put on ice. Serum samples were processed within 30 minutes, after which they were kept frozen at -20°C. We used an automated enzymatic procedure to determine serum total cholesterol level.[18] High-density lipoprotein (HDL) cholesterol was measured similarly, after precipitation of the non-HDL fraction. All participants not prescribed glucose-lowering medication received a glucose drink of 75 g in 200 mL water after a first venipuncture. Two hours later, a second venous blood sample was obtained. Glucose levels were determined in both blood samples by the glucose hexokinase method, whereas insulin was measured by RIA (Medgenix Diagnostics, Brussels, Belgium). This assay has a cross-reactivity with proinsulin of 40%. Because subjects using glucose-low-

ering medication did not undergo the glucose tolerance test, insulin was not measured in this group. The coefficients of variation of glucose and insulin measurements were less than 2.5% and 6.0%, respectively.

Levels of inflammatory markers and cellular adhesion molecules were measured in plasma. For the collection of plasma, blood was collected in tubes containing 0.129 mol/L sodium citrate. All tubes were stored on ice before and after blood sampling. Plasma was obtained by centrifugation of 30 minutes, at 10000 rotations/minute at 10°C, and was immediately frozen in liquid nitrogen and stored at -80°C. Plasma concentrations of C-reactive protein (CRP) and α-1-antichymotrypsin (ACT) were measured by kinetic nephelometry (Behring Nephelometer BN200, Marburg, Germany) after a 5x dilution using Behring's N-dilutent. Levels of interleukin-6 (IL-6), soluble intercellular adhesion molecule 1 (sICAM-1), and soluble vascular cell adhesion molecule 1 (sVCAM-1) were determined by means of enzyme-linked immunosorbent assay (Il-6: Quantikine; sICAM-1 and sVCAM-1: Parameter, R&D Systems Europe, Oxon, United Kingdom). Interassay coefficients of variation were 4.4%, 2.8%, 8.7%, 6.9%, and 5.0% for CRP, ACT, IL-6, sICAM-1, and s-VCAM, respectively. Corresponding intraassay coefficients of variation were 2.6%, 3.7%, 5.7%, 5.0%, and 3.1%, respectively. For 16, 16, 6, 3, and 3 subjects, respectively, we could not determine levels of CRP, ACT, Il-6, sICAM-1, and sVCAM-1 because of insufficient plasma for analysis. Levels of CRP, ACT, Il-6, sICAM-1, and sVCAM-1 were therefore available for analysis in the following number of subjects: 562 (265 men, 297 women), 562 (264 men, 298 women), 570 (271 men, 299 women), 571 (271 men, 300 women), and 572 (271 men, 301 women), respectively.

Metabolic disorders

Diabetes mellitus was defined as the use of glucose-lowering medication or a random or postload serum glucose level ≥ 11.1 mmol/L according to the World Health Organization (WHO) criteria.[19] Impaired glucose tolerance was considered present when the postload serum glucose level was between 7.8 and 11.1 mmol/L in subjects without diabetes mellitus.[4] Postload insulin was used as a measure of insulin resistance. Dyslipidemia was defined as a total cholesterol level ≥ 8.0 mmol/L, and/or an HDL cholesterol level < 0.9 mmol/L,[20] and/or use of lipid lowering medication. We defined obesity as BMI ≥ 30.0 kg/m^2 in both genders, and/or waist circumference ≥ 102 cm in men, and/or waist circumference ≥ 88 cm in women according to WHO criteria.[21] Hypertension was defined as systolic blood pressure ≥ 160 mmHg and/or diastolic blood pressure ≥ 100 mmHg and/or use of antihypertensive medication, encompassing grade 2 and grade 3 hypertension according to WHO criteria.[22]

Prevalent cardiovascular disease

The presence of myocardial infarction was assessed by self-report and by analysis of the standard 12-lead ECGs, which were stored digitally and analyzed by the modular ECG analysis system.[23,24] From subjects with self-reported myocardial infarction without ECG evidence, we collected additional information from the general practitioner or cardiologist, and myocardial infarction was confirmed if the information in the medical records met standard diagnostic criteria. From subjects without self-reported myocardial infarction but with ECG evidence, in whom the absence of symptoms was confirmed by reviewing the medical records, ECGs were reviewed by an experienced cardiologist, and (silent) myocardial infarction was included when the ECG met standard diagnostic criteria for myocardial infarction. We combined both types of myocardial infarctions to one variable for the analyses.[25] Information on a history of coronary artery bypass graft (CABG) or percutaneous transluminal coronary angioplasty (PTCA) was obtained during the interview. A history of stroke was determined on the basis of interview data and medical information from the general practitioner or from hospital discharge records.[26] PAD was considered to be present if the ankle-arm systolic blood pressure index was less than 0.90 in either leg.[27]

Statistical analysis

To obtain normal distributions, insulin, CRP, and IL-6 were natural-log transformed (ln-transformation).

The associations between levels of postload insulin and markers of inflammation and cellular adhesion molecules were assessed by separate linear regression models with ln-insulin as the dependent variable and with levels of markers of inflammation and cellular adhesion molecules as independent variables. Associations were examined for the total population and for men and women separately. Models were initially adjusted for age and, if appropriate, gender. In subsequent models, we additionally adjusted for BMI, WHR, smoking (never, former, or current), and presence of cardiovascular disease.

Furthermore, multivariate-adjusted levels of markers of inflammation and cellular adhesion molecules were assessed in tertiles of levels of postload insulin. For these analyses, we constructed variables with the values 1-3 for subsequent tertiles of levels of postload insulin for the total population, and for men and women separately. These variables were entered in general linear models as continuous independent variables with levels of markers of inflammation and cellular adhesion molecules as dependent variables. Tests of significance for the coefficients of the ordered variables of insulin were considered to be tests for trend.

Subsequently, we compared the strength of the associations of postload

insulin with markers of inflammation and cellular adhesion molecules with the strength of the associations of insulin with variables classically considered to be clustered within the insulin resistance syndrome. For this endeavor, we performed separate linear regression analyses with postload ln-insulin as the dependent variable and levels of markers of inflammation, cellular adhesion molecules, and HDL cholesterol, BMI, WHR, and systolic blood pressure as independent variables, and presented standardized regression coefficients of multivariate-adjusted analyses.

In addition, we computed levels of markers of inflammation and cellular adhesion molecules in subjects according to the presence of the number of metabolic disorders known to be clustered within the insulin resistance syndrome: impaired glucose tolerance, dyslipidemia, obesity, and hypertension. For these analyses, we constructed a variable with value 0-4 according to the number of metabolic disorders present. This variable was entered in general linear models as a continuous independent variable, with levels of markers of inflammation and cellular adhesion molecules as dependent variables. Tests of significance for the coefficients of the ordered variable of the number of metabolic disorders present were considered to be tests for trend.

We considered 2-sided probability values < 0.05 to be statistically significant. SPSS 9.0 for Windows (SPSS Inc., Chicago, IL) was used for all analyses.

Results

Characteristics of the population are described in Table 1. Levels of CRP ranged from 0.01-48.74 mg/L; 35 subjects had values > 10 mg/L, the cut-point generally used to identify clinically relevant inflammation.

Correlations between the levels of the three markers of inflammation were moderate; CRP - IL-6 r=0.53, CRP - ACT r=0.40, ACT - Il-6 r=0.26, $P<0.001$, adjusted for age and gender. The levels of CRP, ACT, and IL-6 were strongly associated with levels of postload insulin (Table 2). Associations with CRP tended to be somewhat stronger in women than in men. Multivariate adjustment decreased the strength of the associations to some extent (Table 2). Adjusting the association between CRP and postload insulin for the other 2 inflammatory markers removed the association ($\beta=0.42$, $P=0.23$), whereas the effect of controlling for the other 2 inflammatory markers on the strength of the association between ACT and insulin, and IL-6 and insulin was less pronounced ($\beta=0.95$, and $\beta=1.87$, $P\leq0.001$, respectively, adjusted for age and gender). Levels of the cellular adhesion molecule sICAM-1 were associated with levels of CRP, ACT, and IL-6 (correlation coefficients r=0.28, r=0.20, and r=0.24, respectively, all

Table 1. Clinical and biochemical characteristics of the study population

Variable	All subjects n=574	Men n=272	Women n=302
Age, y	70.2 ± 8.9	69.7 ± 8.3	70.7 ± 9.3
Body mass index (BMI), kg/m^2	26.4 ± 3.4	25.9 ± 2.8	26.8 ± 3.8
Waist circumference, cm	91 ± 11	94 ± 9	87 ± 11
Waist-to-hip ratio (WHR), cm/cm	0.91 ± 0.09	0.96 ± 0.07	0.87 ± 0.08
Obesity, % (n)*	32 (181)	18 (50)	43 (131)
Smoking status, % (n)			
Never	34 (198)	9 (25)	57 (173)
Past	44 (250)	64 (174)	25 (76)
Current	22 (126)	27 (73)	18 (53)
Systolic blood pressure, mmHg	139 ± 21	138 ± 20	139 ± 22
Diastolic blood pressure, mmHg	73 ± 11	74 ± 11	73 ± 11
Hypertension, % (n)†	26 (147)	24 (65)	27 (82)
Total cholesterol, mmol/L	6.6 ± 1.2	6.3 ± 1.2	6.9 ± 1.2
HDL cholesterol, mmol/L	1.3 ± 0.4	1.2 ± 0.4	1.5 ± 0.3
Dyslipidemia, % (n)‡	24 (135)	26 (70)	21 (65)
Glucose, mmol/L	6.4 ± 1.6	6.2 ± 1.6	6.6 ± 1.6
Postload insulin, mU/L§	52.7 (31.0 – 76.0)	51.0 (29.0 – 73.0)	53.0 (35.0– 81.0)
Impaired glucose tolerance, % (n)‖	19 (107)	14 (37)	23 (70)
Cardiovascular disease, % (n)#	22 (127)	23 (63)	21 (64)
C-reactive protein (CRP), mg/L§¶	1.6 (0.8 – 3.5)	1.4 (0.7 – 3.6)	1.8 (0.8 – 3.4)
alpha-1-ACT (ACT), mg/dL¶	47.1 ± 14.0	46.1 ± 14.1	48.0 ± 13.8
Interleukin-6 (IL-6), pg/mL§¶	1.8 (1.2 – 3.0)	1.9 (1.4 – 3.1)	1.7 (1.2 – 2.8)
sICAM-1, ng/mL¶	220.9 ± 64.6	224.4 ± 72.5	217.7 ± 56.5
sVCAM-1, ng/mL¶	541.8 ± 180.8	547.6 ± 169.5	536.5 ± 190.5

Data are mean ± SD, median (interquartile range) for variables with skewed distributions, or percentages (number of subjects).
* BMI ≥ 30.0 kg/m^2 in both genders, and/or waist circumference ≥ 102 cm in men, and/or waist circumference ≥ 88 cm in women.
† Systolic blood pressure ≥ 160 mmHg and/or diastolic blood pressure ≥ 100 mmHg, and/or use of antihypertensive medication.
‡ Total cholesterol level ≥ 8.0 mmol/L, and/or HDL cholesterol level < 0.9 mmol/L, and/or use of lipid lowering medication.
§ Skewed data.
‖ Postload serum glucose between 7.8 and 11.1 mmol/L in subjects without diabetes mellitus.
Presence of PAD and/or history of myocardial infarction, PTCA, CABG or stroke.
¶ Levels of CRP, ACT, Il-6, sICAM-1, and sVCAM-1 were available in the following number of subjects: 562 (265 men, 297 women), 562 (264 men, 298 women), 570 (271 men, 299 women), 571 (271 men, 300 women), and 572 (271 men, 301 women), respectively.

$P<0.001$), whereas no association between levels of sVCAM-1 and levels of markers of inflammation were found (r=0.05, r=-0.02, r=0.08, respectively, not statistically significant), all adjusted for age and gender. Levels of sICAM-1 showed a strong association with postload insulin as well, whereas levels of sVCAM-1

Table 2. Regression coefficients for ln-insulin* as the dependent variable and markers of inflammation and cellular adhesion molecules as independent variables

	All subjects		Men		Women	
	β†	(95% CI)	β†	(95% CI)	β†	(95% CI)
C-reactive protein (CRP), mg/L‡						
model 1§	1.52	(0.96 ; 2.08)	1.10	(0.26 ; 1.94)	1.97	(1.22; 2.72)
model 2‖	0.88	(0.24 ; 1.51)	0.43	(-0.49 ; 1.35)	1.40	(0.49 ; 2.30)
alpha-1-ACT (ACT), 100 mg/dL						
model 1§	1.25	(0.82 ; 1.69)	1.26	(0.60 ; 1.92)	1.28	(0.70 ; 1.85)
model 2‖	1.09	(0.62 ; 1.60)	1.18	(0.48 ; 1.87)	1.09	(0.42 ; 1.75)
Interleukin-6 (IL-6), pg/mL‡						
model 1§	2.60	(1.69; 3.52)	2.60	(1.26; 3.94)	2.62	(1.37; 3.87)
model 2‖	1.91	(0.92; 2.90)	2.02	(0.62 ; 3.42)	1.88	(0.46 ; 3.31)
sICAM-1, 1 µg/mL						
model 1§	2.22	(1.29 ; 3.16)	2.10	(0.85 ; 3.36)	2.44	(1.01 ; 3.87)
model 2‖	1.94	(0.96 ; 2.92)	1.57	(0.24 ; 2.90)	2.26	(0.77 ; 3.76)
sVCAM-1, 10 µg/mL						
model 1§	1.78	(-1.72; 5.27)	2.45	(-3.23; 8.12)	1.30	(-3.11; 5.71)
model 2‖	0.75	(-3.07; 4.56)	0.64	(-5.26; 6.55)	0.91	(-4.13; 5.95)

* Postload insulin.

† β indicates regression coefficient; an increase of the independent variable by 1 unit is associated with an increase of insulin by a factor e^{β}.

‡ CRP and Il-6 were ln-transformed to obtain a better model fit as assessed by residual analysis; an increase of CRP and Il-6 by 1% yield an increase of postload insulin by 0.1 β%.

§ Model 1. Adjusted for age, and, if appropriate, gender. Number of subjects in models with CRP, ACT, Il-6, sICAM-1, and sVCAM-1 were 562 (265 men, 297 women), 562 (264 men, 298 women), 570 (271 men, 299 women), 571 (271 men, 300 women), and 572 (271 men, 301 women), respectively.

‖ Model 2. Adjusted for age, BMI, WHR, smoking (never, former, or current), presence of cardiovascular disease, and, if appropriate, gender. Number of subjects in models with CRP, ACT, Il-6, sICAM-1, and sVCAM-1 were 486 (230 men, 256 women), 486 (229 men, 257 women), 494 (235 men, 259 women), 495 (235 men, 260 women), and 495 (235 men, 260 women), respectively.

did not (Table 2). Adjustment of the associations between levels of sICAM-1 and postload insulin for levels of CRP, ACT, or IL-6 did not materially affect the results (data not shown). Levels of sICAM-1 were associated with other parameters of the insulin resistance syndrome, namely WHR (r=0.09, P=0.045), and HDL cholesterol (r=-0.15, P=0.001), adjusted for age and gender, whereas levels of sVCAM-1 were not (r=0.06 and r=-0.05, respectively, not statistically significant). Exclusion of subjects with impaired glucose tolerance slightly weakened

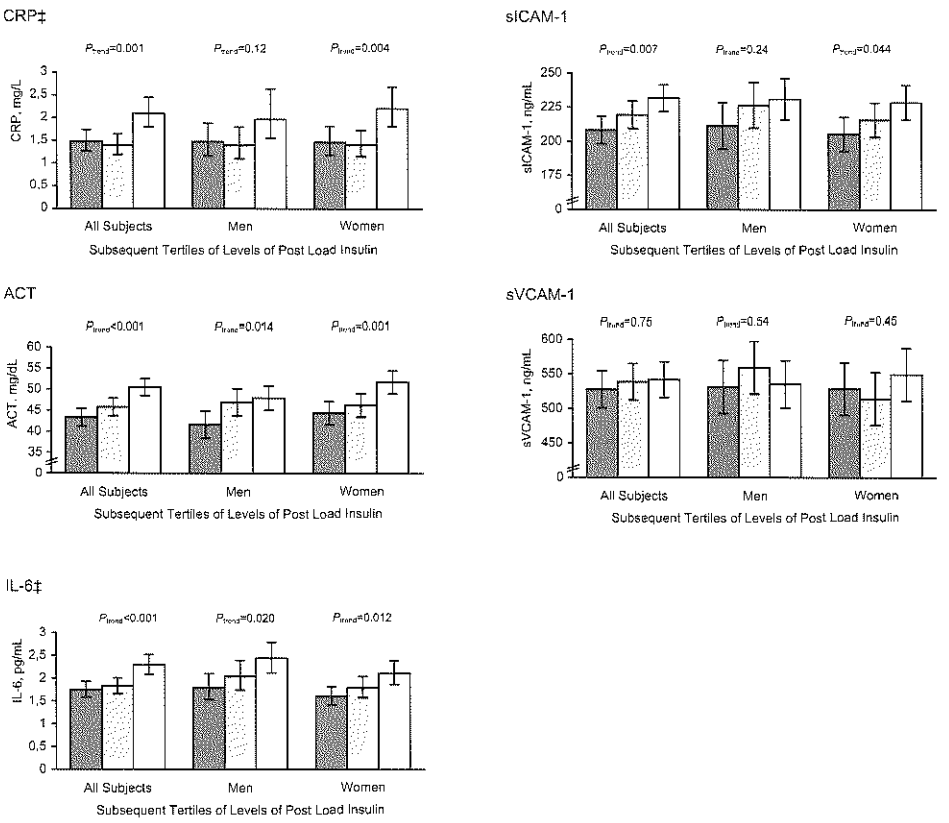

Figure 1. Multivariate-adjusted* mean levels (95% CI) of markers of inflammation and cellular adhesion molecules according to tertiles of levels of postload insulin† in the total population, and in men and women separately

* Adjusted for age, BMI, WHR, smoking (never, former, or current), presence of cardiovascular disease, and, if appropriate, gender. Multivariate-adjusted levels of CRP, ACT, Il-6, sICAM-1, and sVCAM-1 were available in the following number of subjects: 486 (230 men, 256 women), 486 (229 men, 257 women), 494 (235 men, 259 women), 495 (235 men, 260 women), and 495 (235 men, 260 women), respectively.
† Tertiles of levels of insulin were computed for the total population, and for men and women separately.
‡ Geometric mean values (95% CI) because of skewed data.

Table 3. Multivariate-adjusted* standardized regression coefficients for ln-insulin† as the dependent variable and markers of inflammation and cellular adhesion molecules and factors classically associated with insulin resistance as independent variables

	All subjects		Men		Women	
	β‡	*P*-value	β‡	*P*-value	β‡	*P*-value
C-reactive protein (CRP), mg/L§ ||	0.12	0.007	0.062	0.36	0.20	0.003
alpha-1-ACT (ACT), mg/dL§	0.20	<0.001	0.21	0.001	0.20	0.001
Interleukin-6 (Il-6), pg/mL§ ||	0.18	<0.001	0.19	0.005	0.17	0.010
sICAM-1, ng/mL§	0.17	<0.001	0.14	0.021	0.18	0.003
sVCAM-1, ng/mL§	0.017	0.70	0.014	0.83	0.022	0.72
HDL cholesterol, mmol/L§	-0.13	0.003	-0.090	0.14	-0.17	0.004
Body mass index (BMI), kg/m^2§	0.14	0.002	0.23	0.002	0.10	0.09
Waist-to-hip ratio (WHR), cm/cm§	0.25	<0.001	0.16	0.019	0.24	<0.001
Systolic blood pressure, mmHg§	0.031	0.49	0.060	0.35	-0.008	0.90

* Adjusted for age, BMI (apart from model with BMI as independent variable), WHR (apart from model with WHR as independent variable), smoking (never, former, or current), presence of cardiovascular disease, and, if appropriate, gender.
† Postload insulin.
‡ β indicates standardized regression coefficient.
§ Number of subjects in models with CRP, ACT, Il-6, sICAM-1, sVCAM-1, and HDL cholesterol to systolic blood pressure were 486 (230 men, 256 women), 486 (229 men, 257 women), 494 (235 men, 259 women), 495 (235 men, 260 women), 495 (235 men, 260 women), and 770 (358 men, 412 women), respectively.
|| CRP and Il-6 were ln-transformed to obtain a better model fit as assessed by residual analysis.

the strength of the described associations (data not shown). Exclusion of subjects with levels of CRP > 10 mg/L did not affect the results (data not shown). Associations between postload insulin and levels of markers of inflammation and sICAM-1 were still present after exclusion of subjects with prevalent cardiovascular disease (data not shown).

Multivariate-adjusted levels of CRP, ACT, IL-6, and sICAM-1 increased in subsequent tertiles of levels of postload insulin (Figure 1). All tests for trend were statistically significant, except for the trend analyses regarding the association between tertiles of levels of postload insulin and CRP and sICAM-1 in men.

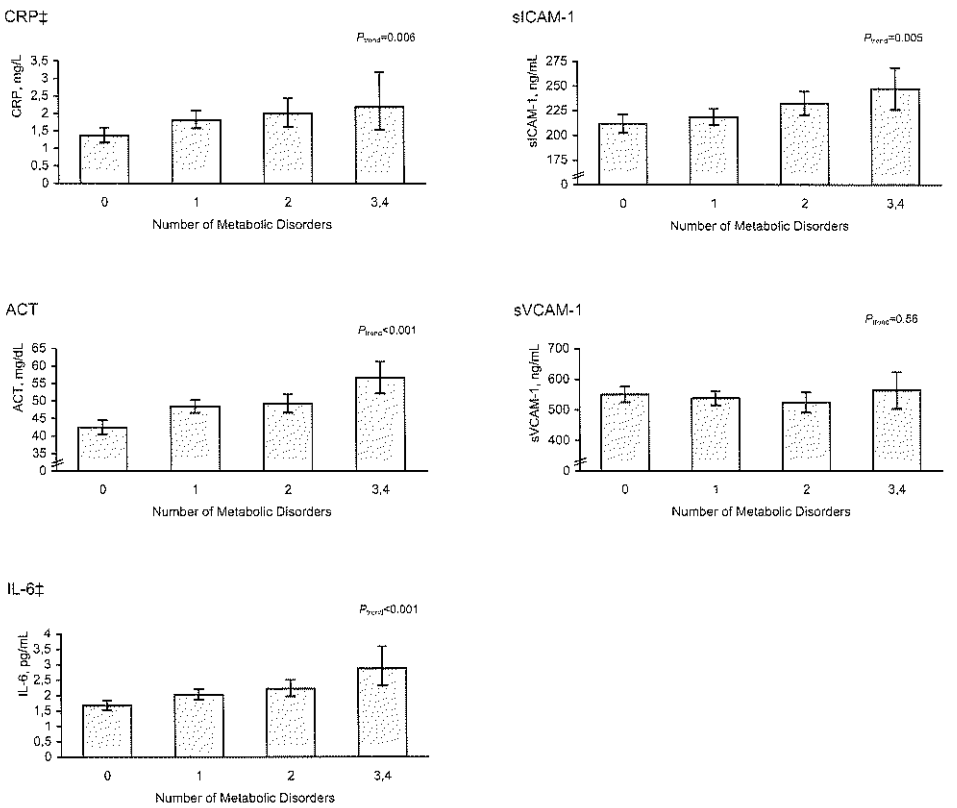

Figure 2. Multivariate-adjusted* mean levels (95% CI) of markers of inflammation and cellular adhesion molecules according to the number of metabolic disorders present† in the total population

* Adjusted for age, gender, smoking (never, former, or current), and presence of cardiovascular disease. Multivariate-adjusted levels of CRP, ACT, Il-6, sICAM-1, and sVCAM-1 were available in 559, 559, 559, 568, and 569 subjects, respectively.

† Metabolic disorders encompassed impaired glucose tolerance, dyslipidemia, obesity, and hypertension.

‡ Geometric mean values (95% CI) because of skewed data.

Additional adjustment of the levels of sICAM-1 in tertiles of levels of postload insulin for markers of inflammation did not materially affect the results (data not shown). Again, we found no association between levels of sVCAM-1 and levels of postload insulin.

The strengths of the multivariate-adjusted associations between levels of postload insulin and CRP, ACT, IL-6, and sICAM-1, as expressed by standardized regression coefficients, were comparable with the strengths of the associations between levels of insulin and HDL cholesterol, BMI, and WHR (Table 3). Adjustment of the association between levels of postload insulin and sICAM-1 for levels of markers of inflammation again did not materially affect the results (data not shown). In our population, no association between systolic blood pressure and postload insulin was found.

The number of subjects categorized in categories 0-4 indicating the number of metabolic disorders present was 192, 232, 111, 31, and 5, respectively. Because of missing data, 3 subjects could not be categorized accordingly. Because of the low number of subjects with 3 or 4 metabolic disorders, we combined these categories into 1 category for analysis. Multivariate-adjusted levels of CRP, ACT, IL-6, and sICAM-1 increased with the increasing number of metabolic disorders present (Figure 2). All tests for trend reached statistical significance. Additional adjustment of levels of sICAM-1 according to the number of metabolic disorders for markers of inflammation did not materially affect the results (data not shown). No association was found between levels of sVCAM-1 and the number of metabolic disorders present. After stratification by gender, results were comparable with those presented for the total population, apart from CRP in men (P_{trend}=0.34) and sICAM-1 in women (P_{trend}=0.17) (data not shown).

Discussion

Our results indicate that, in an elderly population, markers of inflammation are strongly and independently associated with insulin resistance, as measured by postload insulin. In addition, the cellular adhesion molecule sICAM-1 is associated with insulin, whereas sVCAM-1 is not.

Some methodological issues should be taken into account when interpreting our results. Nonfasting postload insulin was used as a measure of insulin resistance. Previous results from the Rotterdam Study indicate that these levels are similar to fasting postload levels[28] and it is shown that postload insulin provides a good measure of insulin resistance in subjects without diabetes mellitus.[29] If anything, the validity of our results does not depend on the precision of the measurement of insulin resistance used. The immunoassay used to measure

insulin is known to cross-react with proinsulin. Although proinsulin is increased in impaired glucose tolerance, it constitutes only a minor part of the total insulin measured[30] and is therefore probably not responsible for the observed association with levels of markers of inflammation and cellular adhesion molecules. Levels of markers of inflammation and cellular adhesion molecules were measured in a gender-stratified random sample of subjects representative of the participants of the Rotterdam Study. We do assume that the sampling of subjects will not depend on the associations between insulin and levels of markers of inflammation and cellular adhesion molecules, making selection bias unlikely.

The results of our study are in line with recent results from the Insulin Resistance Atherosclerosis Study, in which CRP, fibrinogen, and white cell count were found to be associated with fasting insulin in nondiabetic subjects.[7] In healthy, middle-aged subjects, CRP was found to be related to insulin resistance as well.[8] Also, in subjects with type 2 diabetes mellitus, an elevated acute-phase response was particularly marked in those with features of the metabolic syndrome.[6] Factor analysis of data on healthy, elderly people from the Cardiovascular Health Study, however, found inflammatory variables only weakly linked to insulin resistance.[31]

The etiology of the clustering of metabolic factors in the insulin resistance syndrome remains controversial. A common view is that insulin resistance, with its compensatory hyperinsulinaemia, is the underlying mechanism.[1] Alternatively, abdominal obesity may be the primary defect of the clustering.[2] Our data and those of others[6-8] give support to the hypothesis that raised concentrations of proinflammatory cytokines, originating from various cells, and the resultant acute-phase response may underlie much of the metabolic clustering.[32] Furthermore, a key role for the cytokine tumor necrosis factor-α (TNF-α), which induces hepatic synthesis of acute-phase proteins,[33] has been suggested in the pathogenesis of insulin resistance. TNF-α increases serum triglycerides and very-low-density lipoprotein; stimulates insulin-independent glucose use, while inhibiting stimulated glucose uptake by fat and muscle; and causes an increase in counter-regulatory hormones.[34] Moreover, TNF-α plays a role as a mediator of peripheral insulin resistance in obesity by inhibiting the tyrosine kinase activity of the insulin receptor and its substrate.[35] The cross-sectional nature of the design of our study complicates etiological interpretation of the results. However, prospective data showed markers of inflammation to be associated with the development of diabetes mellitus, probably reflecting the pathogenesis of type 2 diabetes.[36]

An alternative explanation for the association between insulin and levels of markers of inflammation might be the presence of atherosclerosis, which is associated with both insulin resistance and markers of inflammation.[37] In our population, however, associations between insulin and levels of markers of

inflammation were still present after adjustment for presence of cardiovascular disease (Table 2, model 2) and after exclusion of subjects with prevalent cardiovascular disease (data not shown). This suggests that atherosclerosis did not induce the association between insulin resistance and markers of inflammation. However, because we adjusted only for presence of cardiovascular disease, the assessment of degree and extent of atherosclerosis might lack accuracy in this respect. Furthermore, we have to consider the possibility that decreased insulin sensitivity leads to, rather than is the consequence of, raised concentrations of inflammatory mediators. Insulin inhibits acute-phase protein synthesis in human hepatoma cell lines,[38] suggesting that insulin resistance might amplify the cytokine effect on the liver.

We are the first to describe an association between insulin and levels of the cellular adhesion molecule sICAM-1, which has been found to be associated with an increased risk of future coronary events.[39,40] In our population, the cellular adhesion molecule sVCAM-1 was not associated with insulin. Previous results in healthy men participating in the Physician's Health Study describe associations of sICAM-1 with several metabolic cardiovascular risk factors encompassed in the insulin resistance syndrome, such as triglycerides, HDL cholesterol, fibrinogen, and hypertension.[41] In dyslipidemic patients, increased levels of sICAM-1 and sVCAM-1 were found as well.[42]

Levels of sICAM-1 were associated with levels of markers of inflammation. Adjustment of the association between insulin and sICAM-1 for CRP, ACT, or IL-6, however, did not materially affect the results. These results may indicate that inflammation is not the principal mechanism linking insulin and endothelial activation in our population.[15,43] Heterogeneity of markers of low-grade inflammation may have played a role in these findings as well. Another mechanism explaining the association between insulin and levels of sICAM-1 may be increased oxidation of LDL cholesterol.[44] Moreover, a direct effect of glucose or insulin on the expression of cellular adhesion molecules has been demonstrated in rabbits.[45] On the other hand, we have to consider the possibility that the association between insulin and sICAM-1 may be induced by atherosclerosis, which has been shown to be associated with higher levels of sICAM-1.[39] Associations, however, remained after adjustment for the presence of cardiovascular disease (Table 2, model 2) and were equally present in subjects without prevalent cardiovascular disease (data not shown). Further studies should determine whether our observation can be confirmed. An understanding of the role of cellular adhesion molecules in insulin resistance may lead to a potential target for prevention or treatment of atherosclerosis. Recently, for example, antibodies to ICAM-1 have been shown to reverse atherogenesis in hypercholesterolemic rabbits.[46]

In summary, our results indicate that insulin is strongly and independently

associated with markers of inflammation and the cellular adhesion molecule sICAM-1, suggesting that subclinical inflammation and endothelial activation are an integral part of the insulin resistance syndrome. These factors may contribute to the well-known relationship between insulin resistance and cardiovascular disease risk. Moreover, anti-inflammatory treatment and strategies aimed at antagonizing effects of cellular adhesion molecules may possibly gain clinical importance in the treatment of insulin resistance and its complications.

Acknowledgements

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CHAPTER 3

Menopause, sex steroids, and cardiovascular disease risk

3.1

The increase in cholesterol with menopause is associated with the apolipoprotein E genotype

A population-based longitudinal study

Abstract

During menopause, a sharp increase in cholesterol concentration occurs with a wide variation in change. It is not known why some women have no or only a slight increase in cholesterol, whereas others exhibit a large cholesterol increase. Possibly, this difference can be explained by genetic variation.

In the Eindhoven Perimenopausal Osteoporosis Study, we examined the effect of the apolipoprotein E (APOE) genotype on the change in cholesterol level with menopause among 1116 Dutch women experiencing natural menopause. Women with the APOE3E3 genotype were regarded as the reference category and changes were adjusted for age at baseline, years of follow-up, years since menopause, and body mass index.

Mean age of the women at baseline was 50.4 years. After 5.9 years of follow-up, the women were on average 4.3 years (SD ± 1.5 years) postmenopausal. Both at premenopausal and at postmenopausal assessments, cholesterol levels were associated with the APOE genotype. The relative contribution of the APOE genotype to the total phenotypic variation of cholesterol was 3.8% at the premenopausal assessment, whereas at the postmenopausal assessment it was 5.8%. The mean increase in cholesterol with menopause in women with the APOE3E3 genotype was 0.67 mmol/L (95% CI, 0.61-0.72 mmol/L). In women with the APOE2E3 genotype the increase in cholesterol was 0.44 mmol/L (CI, 0.32-0.56 mmol/L). The increase in cholesterol in women with the APOE3E4 genotype did not differ from the increase in women with the APOE3E3 genotype. Additional adjustment for smoking and alcohol use did not materially affect the results.

Our results show that the increase in cholesterol level with menopause is 30% lower in women with the APOE2E3 genotype when compared with women with the APOE3E3 genotype, indicating that the APOE genotype contributes to the variation in the increase in cholesterol with menopause.

Introduction

The incidence of cardiovascular disease in women rises sharply after middle age. Menopause is thought to be a determinant of this increase.[1-3] Studies consistently show that total and low-density lipoprotein (LDL) cholesterol are the primary cardiovascular risk factors affected by menopause.[4-13] Longitudinal studies show an average increase in total cholesterol with menopause of 0.5 mmol/L, with a wide variation in change.[14-17] It is not known why some women have no or only a slight increase in cholesterol, whereas others exhibit a large increase. Possibly, this difference can be explained by genetic variation.

An important polymorphism associated with cholesterol level is the apolipoprotein E (APOE) genotype.[18] The heterogeneity in APOE genotype is responsible for different isoforms of apolipoprotein E (apoE), which is mainly present on chylomicrons and very-low-density lipoproteins (VLDLs). When associated with these lipoproteins, apoE serves as a ligand for the hepatic lipoprotein receptors. It has been firmly established that the APOE polymorphism affects plasma cholesterol level. Compared with the APOE*3 homozygotes, the most common genotype, the APOE*2 allele is associated with lower levels of cholesterol, whereas the APOE*4 allele has opposite effects.[18,19] In a cross-sectional study, the association between the APOE genotype and cholesterol concentration has been found to be weaker in premenopausal compared with postmenopausal women[20] suggesting that estrogen affects the influence of the APOE genotype on cholesterol level.

In a Dutch population-based cohort of women, the Eindhoven Perimenopausal Osteoporosis Studies, we examined prospectively among 1116 women experiencing natural menopause whether the variation in increase in cholesterol with menopause may be explained by the APOE genotype.

Methods

Study population

The Eindhoven Perimenopausal Osteoporosis Study is a population-based cohort study originally designed to examine determinants of bone mass, with special emphasis on gynecological parameters, in perimenopausal women.[21] The baseline examination was conducted between September 1994 and September 1995. All women living in the city of Eindhoven, The Netherlands, and born between 1941 and 1947 were invited by the Diagnostic Center Eindhoven, a diagnostic center for general practitioners, and the Department of Municipal Public Health Services Eindhoven for screening of their bone mineral density. Of the

8503 eligible women, 6700 (79%) participated and gave informed consent to be invited for future research.

In the year 2000, we selected the population for the current study. To prevent admixture we restricted our population to the 6448 white Dutch women. Of these, we selected the 2892 women who were premenopausal, defined as last menses less than 1 year ago, at the baseline examination (1994-1995). We excluded women using hormone replacement therapy or oral contraceptives (n=244) and women using cholesterol-lowering therapy (n=21) at baseline because these medications influence cholesterol levels. Four women used both types of medication, leaving 2631 women. Of the 2631 women, baseline serum samples were present in 2457, 208 of whom moved outside the area, leaving 2249 subjects to be invited for the follow-up study, which was conducted between November 2000 and May 2001. The study protocol was approved by the medical ethics committee of the Erasmus MC, Rotterdam, The Netherlands.

Of the 2249 invited women, 318 did not respond to the invitation, 68 refused to participate, 8 moved outside the area after the selection of women to be invited, 12 were not able to participate because of physical or mental illness, 7 had died, and 7 responded after the ending of the study, resulting in 1829 participating women, which corresponds with a participation rate of 81%.

Interview and clinical examination

At the baseline examination (1994-1995), women were invited to the Diagnostic Center Eindhoven or the St. Joseph Hospital in Veldhoven, a suburb of Eindhoven, where information on menstruation pattern, menopausal state, and medication use was obtained through an interview by a trained research assistant. Subsequently, weight and height were measured, body mass index (BMI, weight divided by height squared) was computed, and nonfasting blood samples were taken. Serum samples were obtained and stored at -80^{0}C for future use. Bone mineral density of the lumbar spine was measured by dual energy X-ray absorptiometry. After the visit, participants were asked to fill-in a questionnaire on menopausal complaints, smoking habits, and alcohol use, and return this to the Diagnostic Center Eindhoven within 1 week (response 92%).

At the follow-up examination (2000-2001) at the Diagnostic Center Eindhoven, women were interviewed by a trained research assistant. Menopausal state was ascertained by questioning whether the menses had stopped, and if so, at what age and the reason for its cessation (natural or artificial). The type of artificial menopause was subsequently registered. Information on smoking habits and alcohol use was obtained. Participants were asked to bring their current medication to the research center, where preparation names were noted (oral contraceptives, hormone replacement therapy, and cholesterol-lowering medication).

Length and weight of the participants was assessed, BMI was computed, and nonfasting blood samples were taken by venapuncture.

Cholesterol

Serum samples of the baseline investigation were retrieved from storage, defrosted at room temperature, and subsequently vortexed. Total cholesterol levels of baseline and follow-up serum samples were assessed in the same batch to prevent interassay variation contributing to differences between baseline and follow-up cholesterol levels, with an automatic enzymatic procedure[22] at the laboratory of the Diagnostic Center Eindhoven. The interassay coefficient of variation was 0.49% and the intraassay coefficient of variation was 0.99% at a level of cholesterol of 7.40 mmol/L.

DNA isolation and APOE genotyping

EDTA samples obtained at follow-up were frozen at -20°C until DNA-isolation and genotyping were performed at the genomic laboratory of the department of Internal Medicine, Erasmus MC. Genomic DNA was isolated from peripheral leukocytes using PUREGENE® DNA isolation kit of Gentra Systems (Minneapolis, USA) with slight modifications of the provided protocol. The extracted DNA was amplified using a duplex polymerase chain reaction (PCR) generating a 244 bp PCR fragment of APOE using oligonucleotide primers:

Forward: 5'- TAAGCTTGGCACGGCTGTCCAAGGA -3'

Reverse: 5'- AGAATTCGCCCCGGCCTGGTACAC -3'

PCRs were carried out in 10 µl reaction volumes containing 60 ng of genomic DNA, 10*PCR buffer [(Promega) containing 20 mM Tris-HCl (pH 8.0), 100 mM KCl, 0.1 mM EDTA, 1 mM DDT, 50% glycerol, 0.5% Nonidet®-P40, and 0.5% Tween®20], 1.5 mM $MgCl_2$, 0.2 mM deoxy-NTP, 9 pmol of each ApoE primer, and 1 U of Taq polymerase (Promega). The reactions were performed in 96-well format in a thermocycler (MJ-tetrad). Each reaction mixture was denatured for 5 min at 95°C and subjected to 35 cycles of amplification by primer annealing (59°C for 45 sec), extension (72°C for 45 sec), and denaturation (94°C for 45 sec). Subsequently, APOE genotyping was performed using the SNaPshot procedure using primers:

codon 112: 5' - $(T)_{12}$ GGGCGCGGACATGGAGGACGTG - 3'

codon 158: 5' - $(T)_{18}$ CGATGCCGATGACCTGCAGAAG - 3'

The SBE reaction was performed according to details provided by the manufacturer (ABI Prism® SNaPshot™ ddNTP Primer Extension Kit of PE Biosystems) with slight modifications of the provided protocol. Samples were analyzed in a random fashion and the laboratory technician carrying out the genotyping procedures was blinded for the cholesterol levels of the samples concerned.

Population for analysis

Of the 1829 women participating at follow-up, 133 women still had a normal menstruation pattern, 357 women had an irregular menstruation pattern, and 1339 women reported 1 year of amenorrhea. Of these 1339 women, cessation of the menses had occurred in 70 women after surgery of the womb and/or ovaries, in 5 women after treatment with chemotherapy for breast cancer, and in 1264 women spontaneously. We excluded women using hormone replacement therapy (n=76) or anti-estrogens (n=4), and women using cholesterol-lowering medication (n=56) at the time of blood drawing. Two women used two types of medication, leaving 1130 women. Due to logistic reasons, cholesterol levels were missing for 6 women at baseline and for 3 women at baseline and at follow-up, leaving 1121 women. DNA isolation was not feasible in blood samples of 5 women, resulting in a population for analysis of 1116 women.

Statistical analysis

Initially, we used a paired t-test to compare continuous characteristics measured at baseline and at follow-up, and the McNemar test for paired comparisons of dichotomous variables.

We used the formula of Boerwinkle et al[23] to calculate the average allelic effects of APOE on cholesterol levels at premenopausal and postmenopausal assessments. Because age and BMI influence cholesterol level we adjusted for these variables.

We used a general linear model to compute and compare mean values of cholesterol at baseline (premenopausal state) and at follow-up (postmenopausal state) as well as changes in cholesterol during follow-up (change with menopause = follow-up level - baseline level) in strata of the APOE genotype. In these analyses, the APOE3E3 genotype was used as the reference category. In the analyses at the premenopausal and postmenopausal state, we adjusted for age and BMI at the moment concerned. In subsequent models, we additionally adjusted for smoking (yes-no) and alcohol use (yes-no). In models regarding change in cholesterol with menopause, we adjusted for age at baseline, years of follow-up, years since menopause, BMI at baseline, and change in BMI during follow-up. In subsequent models, we additionally adjusted for smoking (yes-no) and alcohol use (yes-no) at baseline and at follow-up. For missing data on smoking at baseline, a missing value indicator was used,[24] whereas for missing data on body mass index the mean value as calculated from the study population was imputed.

By using analysis of variance (ANOVA) we estimated the contribution of the APOE genotype to the phenotypic variation of cholesterol. ANOVA was done on residual values after adjustment for age and BMI at premenopausal and post-

menopausal assessments. For the analysis regarding the change of cholesterol, ANOVA was done on residual values after adjustment for age at baseline, years of follow-up, years since menopause, BMI at baseline, and change in BMI during follow-up. The genotypes of APOE were entered as dummy variables in the analyses.

We considered 2-sided probability-values < 0.05 to be statistically significant. SPSS 10.0 for Windows (SPSS Inc., Chicago, Illinois) was used for all analyses.

Results

Table 1 shows the baseline and follow-up characteristics of the study population. The mean period of follow-up was 5.9 years (SD ± 0.3 years) and ranged from 5.3 to 6.6 years. The mean age at menopause of the 1116 women was 52.1 years and the women were on average 4.2 years postmenopausal (SD ± 1.5 years) at the follow-up visit. During follow-up, women lost averagely 0.8 cm of their height (SD ± 1.3 cm) and gained 4.0 kg (SD 5.3 kg). At follow-up, fewer women smoked, whereas the proportion of women drinking alcohol had increased compared with the baseline examination. The mean serum cholesterol level increased with 0.64 mmol/L (95% CI, 0.60-0.69 mmol/L) during follow-up. Figure 1 shows the distribution of the change in cholesterol levels in the 1116 women experiencing

Table 1. Baseline and follow-up characteristics of 1116 women experiencing natural menopause during follow-up

Characteristic	Premenopausal (baseline)	Postmenopausal (follow-up)
Age, y	50.4 ± 2.2	56.3 ± 2.1‡
Height, m	1.65 ± 0.06	1.64 ± 0.06‡
Weight, kg	68.5 ± 11.7	72.5 ± 12.8‡
Body mass index (BMI), kg/m^2	25.3 ± 4.3	27.0 ± 4.8‡
Smoking, %*	30	26‡
Alcohol use, %†	59	62§
Cholesterol, mmol/L	5.72 ± 0.98	6.36 ± 1.06‡

Values are unadjusted mean ± SD or percentages.
At baseline, height was missing in 1 woman, weight and BMI were missing in 4 women, and information on smoking was missing in 129 women (12%).
At follow-up, weight and BMI were missing in 1 woman.
* More than 1 cigarette per day.
† More than 1 glass per week.
‡ $P < 0.001$ compared with baseline measurement.
§ $P < 0.05$ compared with baseline measurement.

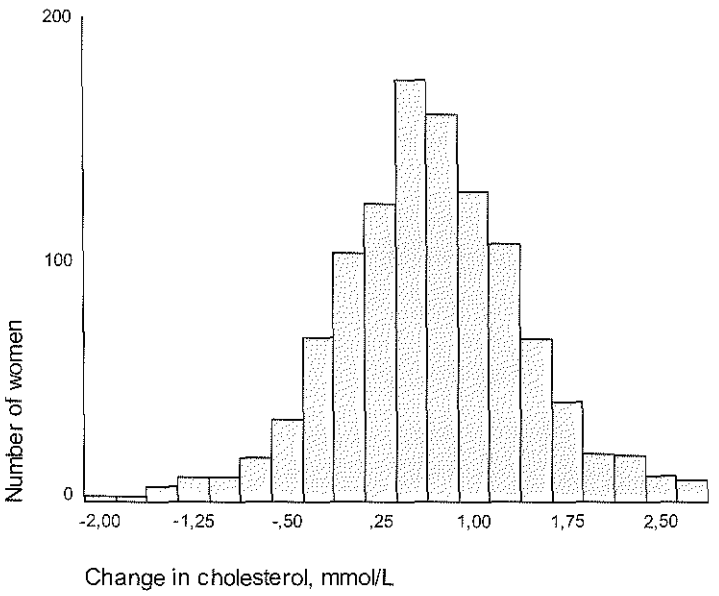

Figure 1. Change in cholesterol level (mmol/L) in 1116 women experiencing natural menopause during 5.9 years of follow-up

natural menopause during follow-up.

The distribution of the APOE polymorphism in our study population was in Hardy-Weinberg equilibrium (χ^2=3.26; df=3; P=0.35, Table 2). Both at premenopausal and postmenopausal assessments, the E*3 allele hardly affected the grand mean of cholesterol of the population. At the premenopausal assessment, the E*2 allele was associated with a reduction of the cholesterol level of -0.52 mmol/L, whereas the E*4 allele was associated with a +0.20 mmol/L increased cholesterol level. At the postmenopausal assessment, these values were -0.48 mmol/L and +0.25 mmol/L, respectively.

In Table 3, the mean levels of cholesterol according to menopausal state and changes in levels of cholesterol during follow-up are shown in strata of the APOE genotype. Both at premenopausal and at postmenopausal assessments, age-adjusted cholesterol levels were intermediate in women with the APOE3E3 genotype, lower in women with the APOE2E3 genotype, and higher in women with the APOE3E4 genotype. At the postmenopausal assessment, cholesterol levels in women with the APOE2E2 genotype were no longer different from cholesterol levels in women with the APOE3E3 genotype. By using ANOVA, age and BMI accounted for approximately 3% of the variance of cholesterol

Table 2. Distribution of APOE genotypes and allele frequencies in 1116 women participating in the study

APOE genotype*	No of women	Relative frequency (%)
E2E2	9	0.8
E2E3	147	13.2
E2E4	25	2.2
E3E3	687	61.6
E3E4	221	19.8
E4E4	27	2.2
Allele	Frequency	
APOE*2	0.085	
APOE*3	0.78	
APOE*4	0.13	

This study includes Dutch white women only.
* χ^2 Hardy-Weinberg distribution is 3.26; df=3; P=0.35.

at the premenopausal and the postmenopausal assessment. The APOE genotype explained 3.8% of the total phenotypic variation of cholesterol at the premenopausal assessment ($F_{5,1110}$=8.78, $P < 0.001$), whereas at the postmenopausal assessment it explained 5.8% ($F_{5,1110}$=13.60, $P < 0.001$), adjusted for age and BMI.

The mean increase in cholesterol level during menopause in women with the APOE3E3 genotype was 0.67 mmol/L (CI, 0.61-0.72 mmol/L), adjusted for age at baseline, years of follow-up, years since menopause, BMI at baseline, and change in BMI during follow-up. Women with the APOE2E3 genotype showed a 30% smaller increase of 0.44 mmol/L (CI, 0.32-0.56 mmol/L) with menopause. The increase in cholesterol with menopause in women with the APOE2E2 genotype was 1.45 mmol/L (CI, 0.96-1.94 mmol/L), although the number of women was low (n=9). The increase in cholesterol with menopause in women with the APOE3E4 or APOE4E4 genotype did not differ from the increase in women with the APOE3E3 genotype. The change in cholesterol level during follow-up according to the most common APOE genotypes is visualized in Figure 2. By using ANOVA, age at baseline, years of follow-up, years since menopause, BMI at baseline, and change in BMI during follow-up explained 8.2% of the change in cholesterol level with menopause. Adjusted for these variables, the APOE genotype explained 2.6% of the variation of cholesterol increase with menopause ($F_{5,1110}$=5.99, $P < 0.001$).

Table 3. Mean levels of cholesterol (95% CI) (mmol/L) according to APOE genotype in 1116 women

APOE genotype	n	Premenopausal* (baseline)	Postmenopausal* (follow-up)	Menopausal increase† (absolute)	Menopausal increase† (relative)
All women	1116	5.72 (5.66 ; 5.77)	6.36 (6.30 ; 6.42)	0.64 (0.60 ; 0.69)	12.3% (11.4% ; 13.1%)
E2E2	9	4.97 (4.33 ; 5.60)‡	6.31 (5.62 ; 6.99)	1.45 (0.96 ; 1.94)‡	34.0% (24.9% ; 43.1%)‡
E2E3	147	5.37 (5.21 ; 5.52)‡	5.81 (5.64 ; 5.97)‡	0.44 (0.32 ; 0.56)‡	9.2% (6.9% ; 11.4%)‡
E2E4	25	5.37 (4.99 ; 5.74)	5.92 (5.51 ; 6.33)‡	0.55 (0.26 ; 0.84)	11.8% (6.5% ; 17.2%)
E3E3 (*reference*)	687	5.73 (5.66 ; 5.80)	6.39 (6.32 ; 6.47)	0.67 (0.61 ; 0.72)	12.6% (11.6% ; 13.7%)
E3E4	221	5.91 (5.79 ; 6.04)‡	6.62 (6.49 ; 6.76)‡	0.71 (0.61 ; 0.80)	12.6% (10.8% ; 14.4%)
E4E4	27	6.18 (5.81 ; 6.54)‡	6.87 (6.48 ; 7.26)‡	0.71 (0.43 ; 0.99)	13.1% (7.8% ; 18.3%)

* Adjusted for age and body mass index.
† Adjusted for age at baseline, years of follow-up, years since menopause, body mass index at baseline, and change in body mass index during follow-up.
‡ Statistically significantly different from APOE3E3 ($P < 0.05$).

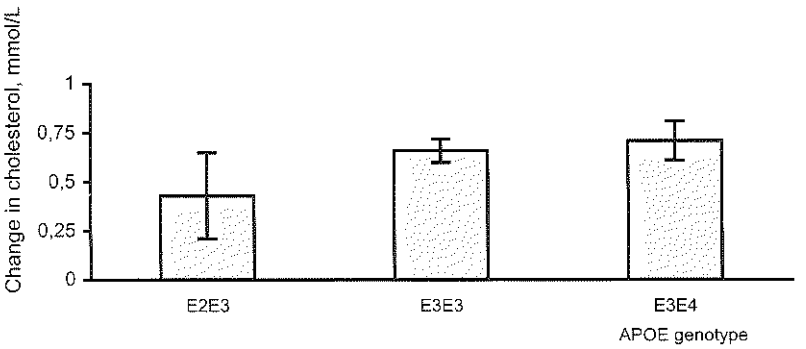

Figure 2. Change in cholesterol level (mmol/L)* according to APOE genotype in 1055 women† experiencing natural menopause during 5.9 years of follow-up

* Values are adjusted for age at baseline, years of follow-up, and years since menopause.
† Women with the E2E2 (n=9), E2E4 (n=25), or E4E4 (n=27) genotype are excluded.

Discussion

Our results among 1116 women experiencing natural menopause show that the contribution of the APOE genotype to the phenotypic variation of cholesterol is higher in postmenopausal women than in premenopausal women. The increase in cholesterol level during menopause is 30% lower in women with the APOE2E3 genotype when compared with women with the APOE3E3 genotype.

In the current large population-based study, we were able to measure intra-individual changes in cholesterol levels in women experiencing natural menopause. The increase in cholesterol was similar to previously described changes in early postmenopausal women.[17] The largest increase in cholesterol with menopause occurs in the perimenopausal years.[17] Because we included women on average 1.7 years before the cessation of their menses we were able to adequately monitor the menopausal increase in cholesterol. The increase in cholesterol level with menopause is most pronounced for LDL cholesterol.[16] Because measures of LDL cholesterol were not available in our study, we used total cholesterol, which we consider to be a valid proxy. We excluded women using lipid-lowering medication at premenopausal or postmenopausal assessments. APOE*4 carriers are known to have higher cholesterol levels,[18,19] therefore, we may preferentially have excluded women carrying this allele.

Whereas all women in our study experienced menopause, the increase in cholesterol level was different among strata of the APOE genotype, indicating that

the APOE genotype contributes to the variation in change of cholesterol with menopause. From cross-sectional data among premenopausal and postmenopausal women it was inferred that the increase in cholesterol with menopause would be 9% among women with the APOE2E3 genotype,[20] which is similar to our results. Also, the increase in cholesterol with menopause was inferred to be similar in women with the APOE3E3 or APOE3E4 genotype.[20] In the Healthy Women Study, no effect of the APOE genotype on differences in changes in cholesterol level was observed between women who became postmenopausal and age-matched women who stayed premenopausal during 3.5 years of follow-up.[25] However, only 12 and 18 postmenopausal women, respectively, were present in the APOE2E3 and APOE3E4 genotype groups. Furthermore, cholesterol concentration start to increase from perimenopause onward. Comparing postmenopausal women with age-matched premenopausal women,[25] of whom some will be perimenopausal, may therefore lead to an underestimation of the effect of menopause. Also in this study,[25] the lower values of cholesterol for women with the APOE2E3 genotype were maintained through menopause despite an increase of cholesterol levels.

In the Framingham Offspring Study, the association between the APOE genotype and cholesterol concentration was absent in premenopausal women, whereas it was present in postmenopausal women,[20] suggesting that the decrease in estrogen level at the time of menopause fully unmasks sensitivity to the effects of the APOE genotype. In our study, we found the APOE genotype to be associated with cholesterol level at both the premenopausal and the postmenopausal assessment. Therefore, our results do not entirely support the hypothesis that menopause unmasks genetic susceptibility to the effects of the APOE genotype. However, also in our study the contribution of the APOE genotype to the total phenotypic variation of cholesterol was higher in postmenopausal women than in premenopausal women, indicating that the effect of the APOE genotype on cholesterol level is amplified by menopause.

Although the number of women with the APOE2E2 genotype in our study was small (n=9), women with this genotype displayed a very large increase in cholesterol level with menopause. Homozygosity for the APOE*2 allele is a very common, albeit not sufficient, cause for type III hyperlipoproteinemia (type III HLP), which is characterized by both hypercholesterolemia and hypertriglyceridemia.[26] Even though the frequency of the APOE2E2 genotype is about 1 in 100 in the general population, as in our study population, the disorder occurs only about 1 in 5000.[26] Additional metabolic factors are usually required for full clinical expression.[27] Menopause is considered to be a factor contributing to the expression of this disorder,[28,29] which gives support for the hypothesis that estrogen modifies the effect of the APOE genotype on cholesterol level. Furthermore,

our data support the interaction between the APOE2E2 genotype and menopause in the expression of this disorder.

The beneficial response of cholesterol to hormone replacement therapy in early postmenopausal women has also been found to be related to the APOE genotype.[30,31] In Finnish[30] and Japanese[31] postmenopausal women, the cholesterol-lowering effect of hormone replacement therapy, as studied in a randomized controlled trial design, was absent in women carrying the APOE*4 allele.[30,31] In the Japanese study,[31] results were presented separately for women with the APOE2E3 or the APOE3E3 genotype. The cholesterol-lowering effect of hormone replacement therapy was most pronounced in women with the APOE2E3 genotype.[31] Also in our study, women with the APOE2E3 genotype showed statistically significantly different changes in cholesterol during follow-up when compared with women with the APOE3E3 genotype. Together with our results, these results suggest that estrogen modifies the effects of the APOE genotype on cholesterol level.

The mechanism relating menopause to the increase in cholesterol level is primarily thought to be due to a reduction in LDL receptor number or activity in response to the decline in blood estrogen level.[32] Although our data indicate that the APOE genotype contributes to the variation in increase in cholesterol with menopause, the variation is far from completely explained by the APOE genotype. Other factors, such as expression of estrogen receptors, which mediate the activation of the LDL receptor in the liver,[33] may be involved in the increase of cholesterol with menopause.

Studies on the association between the APOE genotype and either atherosclerosis or cardiovascular disease have shown inconsistent results.[34-37] However, few population-based investigations including women have been performed on this topic. In a Dutch population-based study, the APOE2E3 genotype was inversely related to carotid artery atherosclerosis in elderly men and women.[38] This result agrees with the results of our study, which showed the increase of cholesterol level during follow-up to be lowest in women with the APOE2E3 genotype. Although cholesterol level was not an intermediate in the association between APOE genotype and carotid atherosclerosis,[38] it seems reasonable to speculate that the amount of change of cholesterol with menopause would have an impact on the development or progression of atherosclerosis and cardiovascular disease. In the Healthy Women Study, the amounts of coronary and aortic atherosclerosis measured shortly after menopause were not found to be related to changes in levels of LDL cholesterol with menopause.[39] However, a longer follow-up time may be necessary for effects of higher cholesterol levels on atherogenesis to become detectable.

In conclusion, our results in 1116 women experiencing natural menopause

show that the increase in cholesterol level with menopause is 30% lower in women with the APOE2E3 genotype when compared with women with the APOE3E3 genotype, indicating that the APOE genotype contributes to the variation in the increase in cholesterol with menopause.

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3.2

Increased plasma homocysteine levels after menopause

Abstract

Besides genetic defects in the enzymes involved in homocysteine metabolism and nutritional deficiencies in vitamin cofactors, sex steroid hormones may modulate plasma homocysteine levels. The postmenopausal state has been found to be associated with higher plasma homocysteine levels, but data are inconsistent and studies published so far did not adjust for age, which is an important confounding factor in studying the effect of menopause. We measured total plasma homocysteine levels in a meticulously selected population in which the contrast in estrogen status between premenopausal and postmenopausal women of the same age was maximized. The study comprised 93 premenopausal and 93 postmenopausal women of similar age (range 43 to 55 years). Women were selected from respondents to a mailed questionnaire on menopause, which was sent to all women aged 40 to 60 years in the Dutch town of Zoetermeer (n=12,675). Postmenopausal women who were at least 3 years after menopause or whose menses had stopped naturally before age 48 were age-matched with premenopausal women with regular menses and without menopausal complaints. Plasma homocysteine levels in the fasting state were related to menopausal status: the age-adjusted geometric mean level was 10.7 μmol/L in premenopausal women and 11.5 μmol/L in postmenopausal women [difference 7% (95% CI, 0.3%-14%); P=0.04]. Additional adjustment for plasma creatinine, body mass index, smoking habit (yes, no), and alcohol intake did not influence this difference. The results of this population-based study indicate that plasma homocysteine is affected by menopause.

Introduction

Plasma homocysteine has been found to be an independent risk factor for cardiovascular disease.[1,2] Elevations in plasma homocysteine level are typically caused either by genetic defects in the enzymes involved in homocysteine metabolism or by nutritional deficiencies in vitamin cofactors.[3] There are indications that plasma homocysteine may also be influenced by sex steroid hormones. Homocysteine levels are generally lower in women than in men[4-6] and have been found to be lower during pregnancy[7,8] and during hormone replacement therapy.[9,10]

Menopause, which is associated with a decrease in estrogen levels, is thought to be a major determinant of the rising incidence of cardiovascular disease in women after middle age.[11-13] Only a few studies are available on the effect of menopause on homocysteine levels.[14-17] Results of these studies are inconsistent. Several studies report an increase of homocysteine levels with menopause,[14,15,17] another study, however, does not confirm this.[16] All of these studies are limited in design because they did not adjust for age, which is an important confounding factor in studying the effect of menopause.

In the present study, we examined the relationship between natural menopause and plasma homocysteine level in a highly selected population in which the contrast in estrogen status between premenopausal and postmenopausal women of the same age was maximized.

Methods

Study population

Selection of participants in this study was aimed at maximizing the contrast in estrogen status in healthy premenopausal and postmenopausal women of the same age (Figure 1). A questionnaire including questions about menopausal status, medical history, medication use, and smoking behavior was sent by mail to all women aged 40-60 years and living in the town of Zoetermeer, The Netherlands (n=12,675). The response rate was 54%. Selection of premenopausal and postmenopausal women was based on the questionnaire. Women with a hysterectomy and/or unilateral or bilateral ovariectomy (n=1551) and women with missing information on type or date of menopause (n=233) were excluded.

Women were considered premenopausal if they had 1 or more bleedings in the past 12 months (n=3829). Premenopausal women who reported no longer having monthly bleedings (n=938) and women who reported the presence of climacteric symptoms, defined as perspiration and/or hot flushes (n=1645), were

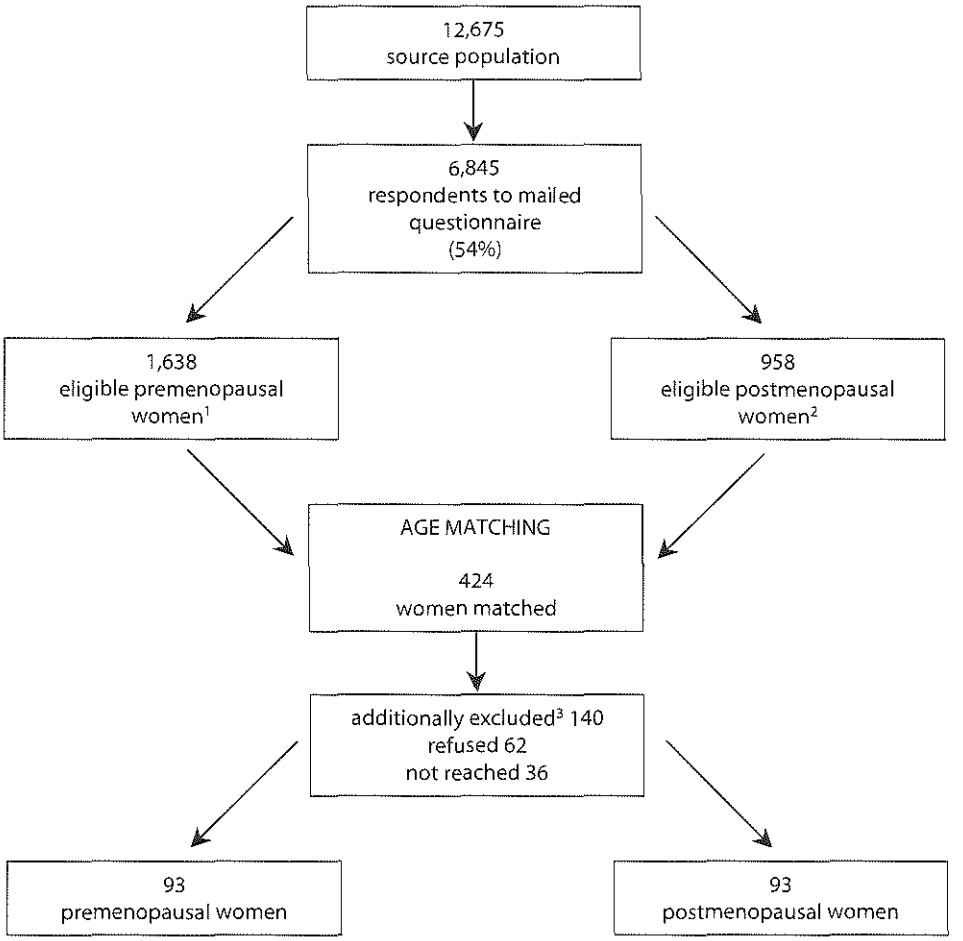

Figure 1. Schematic presentation of the selection procedure of the study population

1. Eligible were women with regular menses and no climacteric symptoms who had not used hormone replacement therapy or oral contraceptives in the past 6 months. Subsequently, women who smoked, who had diabetes mellitus, or used antihypertensive or cholesterol-lowering drugs were excluded.
2. Eligible were women whose menses had ceased naturally > 12 months ago and who had not used hormone replacement therapy. Subsequently, women who smoked, who had diabetes mellitus, or used antihypertensive or cholesterol-lowering drugs were excluded.
3. Women who no longer fulfilled the criteria at the moment of examination were excluded.

excluded from the present study. Furthermore, premenopausal women who reported use of hormone replacement therapy or use of oral contraceptives within 6 months prior to the clinical examination were excluded (n=423). The total number of premenopausal women excluded for these reasons was 2191, leaving 1638 eligible premenopausal women.

Women were considered to have had natural menopause if their menses had ceased naturally for at least 12 months (n=1242). Women who reported a history of hormone replacement therapy for over 6 months or use of female hormones within 6 months prior to the clinical examination and women who reported cessation of bleeding immediately upon stopping hormones were excluded (n=241). The total number of postmenopausal women excluded, including those with missing values on hormone use, was 284, leaving 958 eligible postmenopausal women.

Of the remaining women, we additionally excluded women reporting diabetes mellitus [13 (0.8%) premenopausal and 16 (1.7%) postmenopausal women], use of antihypertensive medication [31 (1.9%) and 35 (3.7%)], use of cholesterol-lowering drugs [3 (0.2%) and 20 (2.1%)], and current smoking of 5 or more cigarettes per day [302 (18.4%) and 218 (22.8%)].

In order to create a sharp contrast in estrogen status, we selected women with either an early or a late natural menopause. Postmenopausal women who were at least 3 years after menopause or whose menses had stopped at least 3 years before the average age of menopause (51 years) were age-matched with premenopausal women with regular menses and without menopausal complaints. If it was not possible to find a match within the same year of age, a match was taken from an adjacent year. If 1 of a matched pair was unwilling to participate, a new match was sought. Women were invited for study participation on average 15 months after return of the questionnaire. Out of 424 invited women, 140 were excluded because they did no longer fulfill the inclusion criteria (regular menses, no climacteric symptoms, no hormone replacement therapy or cardiovascular disease) or no proper replacement match could be found. Sixty-two women (15%) were unwilling to participate and 36 could not be reached. This left 93 premenopausal and 93 postmenopausal women, aged 43-55 years, who participated in the study. All women gave written informed consent, and the study was approved by the medical ethics committee of the Erasmus University Medical School.

Measurements

A medical history was taken by a physician during a visit at our research center. Height, weight, and waist and hip circumferences were measured while the subjects wore indoor clothes without shoes. Body mass index (BMI, weight divided by height squared) and waist-to-hip ratio (WHR) were computed. Alcohol drinking habits and cigarette smoking history were obtained by a standardized questionnaire. Blood pressure was assessed with a DINAMAP automatic blood pressure recorder (Critikon, Inc.). After a 5-minutes rest in the supine position, blood pressure was read 4 times at the right upper arm with an appropriately

sized cuff, and the mean was used in our analyses.

Venous blood samples were drawn from each subject after a 12-hour fast and were centrifuged within less than 60 minutes, which is sufficient to prevent an increase in plasma homocysteine resulting from ex vivo generation of homocysteine by erythrocytes.[18] The processing of the blood samples was identical in all subjects. Plasma was stored at -80°C and total homocysteine was measured as described in detail previously.[19] The intraassay and interassay coefficients of variation were 3.8% and 4.3%, respectively. Total cholesterol was measured with an automated enzymatic method,[20] using the CHOD-PAP high performance reagent kit from Boehringer Mannheim. High-density lipoprotein (HDL) cholesterol was measured by the phosphotungstate method according to Burstein[21] with a minor modification as described by Grove.[22] Low-density lipoproten (LDL) cholesterol was computed with the Friedewald formula.[23] Plasma creatinine was determined with a modified Jaffé method.

Statistical analysis

We used linear regression analysis to estimate the age-adjusted differences in characteristics between premenopausal and postmenopausal women. Differences in frequencies of smoking status were tested using the χ^2 test. Since the distribution of homocysteine was skewed, it was natural-log transformed for the analyses. Renal function, muscle mass, smoking, and alcohol intake are known to influence plasma homocysteine levels.[3,24] Therefore, we made additional adjustments for plasma creatinine, BMI, smoking habit (yes, no), and alcohol intake by including these parameters as independent variables in the regression model.

We considered 2-sided probability values < 0.05 to be statistically significant. SPSS 7.0 for Windows was used for all analyses.

Results

General characteristics of the premenopausal and postmenopausal women are outlined in Table 1. Postmenopausal women were slightly older (mean age 51.1 years, range 43.3 to 54.7 years) than premenopausal women (mean age 50.6 years, range 44.1 to 55.3 years). Among postmenopausal women, the mean number of years since menopause was 5.4 (Standard Deviation [SD] ± 3.0 years) and ranged from 1.3 to 12.8 years. Age, height, weight, BMI, WHR, alcohol intake, blood pressure, HDL cholesterol, plasma creatinine, and smoking habits did not differ between the 2 groups. Age-adjusted levels of total cholesterol and LDL cholesterol were significantly higher in postmenopausal women.

The age-adjusted geometric mean level of total plasma homocysteine in

Table 1. General characteristics of premenopausal and postmenopausal women

	Premenopausal (n=93)	Postmenopausal (n= 93)
Mean ± SD		
Age, y	50.6 ± 2.4	51.1 ± 2.2
Height, cm	166.8 ± 5.7	165.5 ± 7.3
Weight, kg	68.8 ± 11.1	68.6 ± 11.5
Body mass index (BMI), kg/m^2	24.7 ± 3.8	25.0 ± 4.0
Waist-to-hip ratio (WHR), cm/cm	0.77 ± 0.05	0.77 ± 0.05
Alcohol, grams/wk	45 ± 57	45 ± 57
Systolic blood pressure, mmHg	120.7 ± 15.7	120.6 ± 13.0
Diastolic blood pressure, mmHg	67.7 ± 9.5	68.7 ± 10.3
Total cholesterol, mmol/L	5.9 ± 1.0	6.5 ± 0.9†
LDL cholesterol, mmol/L	3.8 ± 1.0	4.3 ± 0.8†
HDL cholesterol, mmol/L	1.6 ± 0.4	1.6 ± 0.4
Creatinine, µmol/L	84.2 ± 9.1	84.0 ± 9.3
Percentage (n)		
Current smoking, % (n)*	6 (6)	6 (6)
Past smoking, % (n)*	42 (39)	39 (36)

* Subjects who smoked 5 or more cigarettes per day were excluded from study participation.
† $P < 0.001$, adjusted for age.

Table 2. Geometric mean plasma homocysteine levels (µmol/L) in premenopausal and postmenopausal women

Premenopausal (n=93)	Postmenopausal (n=93)	Difference	
Mean level (95% CI)	Mean level (95% CI)	Mean % difference (95% CI)	*P*-value
10.7 (10.2 ; 11.2)*	11.5 (11.0 ; 12.0)*	+ 7% (0.3% ; 14%)*	0.04
10.7 (10.2 ; 11.2)†	11.5 (11.0 ; 12.0)†	+ 8% (1.5% ; 14%)†	0.02

CI = Confidence Interval.
* Adjusted for age.
† Adjusted for age, creatinine, body mass index, smoking habit (yes, no), and alcohol intake.

premenopausal women was 10.7 µmol/L (range 6.7-20.7 µmol/L), whereas in postmenopausal women it was 11.5 µmol/L (range 7.2-25.5 µmol/L). When comparing premenopausal women with postmenopausal women, a significant 7% (95% Confidence Interval [CI], 0.3%-14%) difference in total homocysteine levels was observed, adjusted for age (P=0.04) (Table 2). Additional adjustment for plasma creatinine, BMI, smoking habit (yes, no), and alcohol intake did not influence this difference (P=0.02) (Table 2). Subsequently, additional adjustment was made for total cholesterol and HDL cholesterol levels, which did not change the results (data not shown).

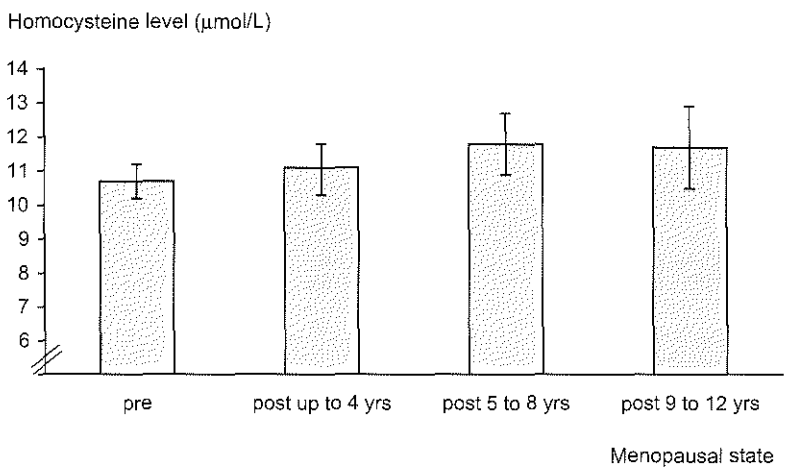

Figure 2. Plasma homocysteine levels* in 93 premenopausal and 93 postmenopausal women, categorized in 3 groups by time since menopause

* Shown levels (µmol/L) are geometric mean values (95% CI), adjusted for age, creatinine, body mass index, smoking habit (yes, no), and alcohol intake.

When women were categorized into 3 groups by time elapsed since menopause, the geometric mean value of total homocysteine was 11.1 µmol/L (CI, 10.3-11.8 µmol/L) in women up to 4 years after menopause (n=39), 11.8 µmol/L (CI, 10.9-12.7 µmol/L) in women 5 to 8 years after menopause (n=36), and 11.7 µmol/L (CI, 10.5-12.9 µmol/L) in women 9 to 12 years after menopause (n=18), adjusted for age, creatinine, BMI, smoking habit, and alcohol intake. These results show that homocysteine levels increased from premenopausal state till 5-8 years after menopause (test for trend P=0.20) and thereafter stabilized (Figure 2).

Discussion

The results of the present study, which is the first study on menopause and plasma homocysteine levels in which subjects were carefully matched for age, show that total homocysteine is significantly higher in postmenopausal women than in premenopausal women, indicating an increase of homocysteine levels with menopause.

In studying the effect of menopause, age is an important confounding factor. By a rigorous selection procedure in the present study, we composed a population of healthy age-matched premenopausal and postmenopausal women from the general population. Because of our stringent inclusion and exclusion criteria

the effect of misclassification of menopausal status is likely to be small. Some misclassification of age of menopause might have occurred, as these assessments were self-reported. The slight age difference between the study groups after age matching was dealt with by further adjustment in the analyses.

To ensure that the results are due to true associations between natural menopause and homocysteine, bias due to other factors also has to be considered as a possible explanation. We excluded women with current or recent use of hormone replacement therapy or oral contraceptives. Moreover, after age-matching and exclusion of women currently smoking 5 cigarettes per day or more, residual confounding by age, renal function, BMI, smoking, and alcohol drinking habits was dealt with by adjustment in the analyses. Some other determinants of early menopause such as socio-economic status were not measured in our study. Although we have no data on socio-economic status and plasma homocysteine levels, this might have affected our results. Elevations in plasma homocysteine are typically caused either by genetic defects in the enzymes involved in homocysteine metabolism or by nutritional deficiencies in vitamin cofactors.[3] We did not measure genetic nor nutritional factors. Because it is unlikely that either genetic defects involved in homocysteine metabolism or food or supplement intake are differently distributed between premenopausal and postmenopausal women, we do not think that this lack of information has affected the validity of our results. If increase of supplementation use had occurred after menopause, this would mean, if anything, that we underestimated the effect of menopause on plasma homocysteine levels.

The women in our study were selected from responders to a mailed questionnaire (response 54%). We assume, however, that the results from our study are generalizable to the general population even if some selection has taken place, as we have no reason to assume that the relation between menopause and homocysteine will be different in responders and non-responders.

Previous studies on the relation between natural menopause and homocysteine levels are limited, comprised only a small number of subjects and showed inconsistent results.[14-17] The efficiency of methionine metabolism in premenopausal women aged 14 to 42 has been found to lead to lower homocysteine concentrations as compared with postmenopausal women aged 45 to 59.[14] Other studies showed that homocysteine concentrations, both fasting and after methionine loading, are significantly higher in postmenopausal than in premenopausal women with reported age differences of 16[15] and approximately 10 years.[17] A sharp increase in fasting homocysteine levels in females after 50 years of age also suggested a relationship of menopause with homocysteine,[25] although this was not confirmed by others.[26] Another study, however, reported no difference in homocysteine levels between premenopausal women under the age of 50 and

postmenopausal women over the age of 50.[16] Age may have been a confounder in the abovementioned studies, as they included women in a broad age range and did not adjust for age in the analyses.

The mechanisms through which estrogens may modulate plasma homocysteine levels are largely unknown.[24] Possibly, lower homocysteine levels in premenopausal women may be due to higher methionine transamination.[27] Several observations suggest a homocysteine-lowering effect of estrogens. Homocysteine concentration was observed to be lower during the high hormonal (=high estrogen) than the low hormonal phase in women using oral contraceptives,[28] although this was not confirmed by others.[29,30] Furthermore, hormone replacement therapy in postmenopausal women led to a decrease of homocysteine levels in subjects with initially high fasting homocysteine levels.[9,10] Unfortunately, these intervention studies lacked a control group and therefore their results may have been influenced by regression to the mean. Another study in postmenopausal women with breast cancer showed that treatment with tamoxifen resulted in a decrease in fasting plasma homocysteine levels.[31] In a recent study in transsexual subjects, homocysteine levels decreased after estrogen and antiandrogen administration to male-to-female transsexuals, and levels increased after androgen administration to female-to-male transsexuals.[32]

In conclusion, our findings in a carefully selected population suggest that plasma homocysteine levels increase with natural menopause. This strengthens the hypothesis that estrogen influences homocysteine levels and proposes one of the mechanisms through which menopause adversely affects cardiovascular disease risk in women after middle age.

Acknowledgements

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3.3

The effect of hormone replacement therapy on serum homocysteine levels in perimenopausal women

A randomized controlled trial

Abstract

Serum homocysteine levels may be lowered by hormone replacement therapy, but randomized controlled trial data are scarce. We performed a single center randomized placebo-controlled trial to assess the 6 months effect of hormone replacement therapy compared with placebo on fasting serum homocysteine levels in 121 perimenopausal women free of cardiovascular disease and recruited from the general population. The trial was double-blind with respect to a sequential combined regimen of oral 17β-estradiol and desogestrel ($17\beta E_2$-D) and the placebo group, and open with respect to a combination of conjugated equine estrogens and norgestrel (CEE-N). At baseline and after 6 months, fasting serum homocysteine levels were measured. Differences in 6 months serum homocysteine levels from baseline between treatment and placebo groups were calculated and expressed as a percentage of the 6 months placebo level. After 6 months, the difference in serum homocysteine levels between women receiving $17\beta E_2$-D and placebo was -6.3% (95% CI, -12.4%; 0.0%, P=0.06). The difference between women receiving CEE-N and placebo was -10.1% (CI, -16.7%; -2.9%, P<0.01). The difference between the combined group of both types of hormone replacement therapy users and placebo was -7.8% (CI, -13.2%; -2.0%, P=0.01). No significant difference was observed between the two active regimens. Our results indicate that hormone replacement therapy decreases homocysteine levels in perimenopausal women.

Introduction

Observational studies have shown a lower risk of coronary heart disease among postmenopausal users of estrogen supplements compared with nonusers.[1-3] Postulated mechanisms for a potential beneficial effect of estrogen on coronary heart disease risk include changes in lipid and hemostatic variables and direct effects on the arterial wall,[4] but mechanisms are not completely elucidated. Homocysteine, which is suggested to be an independent risk factor for atherosclerotic vascular disease,[5-7] may also be influenced by estrogen. Homocysteine levels are generally lower in women than in men[8,9] and have been found to be lower during pregnancy[9,10] and in premenopausal compared with postmenopausal women.[11] In a recent study in transsexual subjects, homocysteine levels decreased after estrogen and antiandrogen administration to male-to-female transsexuals, and levels increased after androgen administration to female-to-male transsexuals.[12] Earlier studies have been conducted on the effect of hormone replacement therapy (HRT) on homocysteine levels.[13-20] Some of these studies, however, lacked a control group,[13-15] and most studies that did include a control group were rather small.[16-18]

The present study was conducted to assess the effect of Org 32818, a 24-day active, 28-day sequential combined regimen of oral 17β-estradiol and desogestrel ($17\beta E_2$-D), and Prempak®, a combination of conjugated estrogens and norgestrel (CEE-N) on several cardiovascular disease risk factors and structural and functional characteristics of the carotid artery.[21,22] Here, we present the results of the effects of HRT on change in fasting serum homocysteine levels compared with placebo after 6 months of therapy in perimenopausal women.

Methods

Study protocol

The design of the study was randomized, group-comparative, double blind with respect to $17\beta E_2$-D and placebo groups and open with respect to CEE-N. The study was conducted in one center, included 121 perimenopausal women and comprized 6 consecutive cycles of 28 days. Participants were recruited from the general population in the town of Zoetermeer (The Netherlands). They completed a questionnaire on menopause and gynecological issues that was sent to all women between 40 and 60 years of age. Women who were eligible based on the questionnaire were invited for the screening procedure. The study was performed from October 1992 to July 1995. The study was approved by the medical ethics committee of the Erasmus University Medical School, and written

informed consent was obtained from all participants.

Subject selection was based on the following criteria, age between 40 and 60 years; not hysterectomized; climacteric symptoms (hot flushes and/or outbreaks of sweating), body weight between 80% and 130% of the ideal body weight (Metropolitan Life Insurance Company Tables for Women, 1983). The main exclusion criteria were absence of spontaneous vaginal bleeding for more than 5 years; use of sex-steroids currently or within the last 2 months or ethinyl-estradiol or injectable sex steroids within the last 6 months or hormone implants at any time earlier; smoking > 10 cigarettes per day; history or presence of any malignant disorder; history or presence of cardiovascular or cerebrovascular disease or thromboembolism/thrombosis; history or presence of hepatic or renal disease, uncontrolled hypertension (systolic blood pressure > 170 mmHg and/or diastolic blood pressure > 105 mmHg); significant hyperlipidemia (fasting total cholesterol > 9.5 mmol/L and/or fasting triglycerides > 2.5 mmol/L). A cervical smear and a mammography were done unless results were available dated less than 1 and 2 years earlier, respectively.

At baseline, information was obtained about smoking. Body weight and body height were measured and body mass index (BMI, weight divided by height squared) was calculated. Blood pressure was measured twice at the right upper arm in sitting position using a Hawksley random-zero sphygmomanometer. The mean of the 2 measurements was taken as the subjects reading. Fasting blood samples were obtained between 8.00 and 10.00 h a.m. After clotting for 1 h, serum was isolated after centrifugation at 2500 g for 10 minutes. Fasting serum aliquots were frozen and stored at -80°C for subsequent analysis. Cholesterol and triglycerides were assayed enzymatically with a Hitachi 747 automated analyzer with kits from Boehringher-Mannheim, currently Roche Diagnostics. High-density lipoprotein (HDL) cholesterol was measured after precipitation with phosphowolfram/phosphotungstic acid and 2 mmol of manganese chloride per liter. The low-density lipoprotein (LDL) concentration was calculated with the Friedewald formula.[23] Creatinine was measured with the Jaffé method on the Hitachi 747 automated analyzer. Fasting blood samples were taken at baseline and on cycle 6 during intervention on day 21 ± 2 of the cycle for the 17βE_2-D group and on day 25 ± 2 for the CEE-N group. Serum homocysteine concentration (i.e., serum total homocysteine, measured as the sum of all homocysteine subfractions in serum including free and protein bound forms) was measured with high-performance liquid chromatography (HPLC).[24] The interassay and intraassay coefficients of variation were 4.0% and 2.3%, respectively. In studies of HRT, blinding is difficult to maintain throughout the study because of the clear effects on menstrual cycle (participants were asked to complete a bleeding diary). Therefore, samples were analyzed in random fashion and laboratory

technicians were blinded for the intervention group.

Individual randomization to treatment with $17\beta E_2$-D, CEE-N, or placebo was performed in a ratio of 3:2:2 using a computerized allocation algorithm. The treatment allocation of 3:2:2 was chosen to assure the gain of sufficient information on the relatively new combination $17\beta E_2$-D. Code numbers were assigned to subjects in the order of their enrollment into the study. The allocation schedule was prepared by the sponsor and unknown to the study physician who dispended the study medication.

$17\beta E_2$-D and placebo tablets were supplied in identical looking push-through-strips. CEE-N was supplied in the original, commercially available, strips. Each strip of $17\beta E_2$-D contained 12 tablets with 1.5 mg 17β-estradiol (micronized), 12 tablets with 1.5 mg 17β-estradiol (micronized) + 0.15 mg desogestrel, and 4 placebo tablets (Org 32818; NV Organon). Placebo was $17\beta E_2$-D matched and contained 28 placebo tablets. Each strip of CEE-N contained 28 tablets with 0.625 mg conjugated estrogens and 12 tablets with 0.15 mg norgestrel (Prempak®; Novo Nordisk). Subjects in the $17\beta E_2$-D or $17\beta E_2$-D-matched placebo groups took one tablet per day on a continuous basis. Subjects treated with CEE-N took one tablet per day from day 1 to 16 and two tablets per day from day 17 to 28 for each cycle. Tablets were taken after breakfast. Sex steroids other than the study medication, hydantoins, barbiturates, primidone, carbamazepine, rifampicin, griseofulvin, and lipid-lowering agents were not allowed during the study. Drug compliance was assessed by tablet count and diary-checks. Non-compliance was defined as missing 2 successive tablets from day 1 of the cycle and/or missing on average 1 tablet per week till the day of assessment of that cycle.

Statistical analysis

Assuming a mean homocysteine concentration on the natural-log-scale of 2.41 μmol/L (geometric mean = 11.1 μmol/L)[11] and a drop-out rate of 5%, the sample size calculations for the present trial showed that with 86 subjects in the active treatment groups, a standard deviation (SD) of serum homocysteine on the natural-log-scale of 0.20 μmol/L, a 2-sided α of 0.05, and a power of 0.80, a percentual change in serum homocysteine levels of 11.6% between the treated and placebo group could be detected. In a recent prospective nested case-control study in postmenopausal women conducted as part of the Women's Health Study,[7] cardiovascular disease cases had a 12.1% higher homocysteine level compared with controls.

Data from subjects of whom levels of serum homocysteine at baseline and at 6 cycles were available were used for the analysis on the effect of treatment after 6 months (intention-to-treat analysis with incomplete data). For this analy-

sis, we constructed 2 dummy variables: the first with placebo (value 0), 17βE_2-D (value 1), and CEE-N (value 0) and the second with placebo (value 0), 17βE_2-D (value 0), and CEE-N (value 1). Linear regression analysis with ln-homocysteine after 6 months of treatment as the dependent variable and the 2 dummy variables as the independent variables was used to determine whether serum homocysteine levels differed among treatment groups after 6 months of treatment. In addition, the effect of both treatment groups combined compared with placebo was computed by linear regression analysis with ln-homocysteine after 6 months of treatment as the dependent variable and a variable indicating treatment with HRT (17βE_2-D and CEE-N) or placebo as the independent variable. Moreover, we compared the effects of treatment with 17βE_2-D relative to CEE-N on serum homocysteine levels using linear regression analysis with ln-homocysteine after 6 months of treatment as the dependent variable and a variable indicating treatment with 17βE_2-D or CEE-N as the independent variable. We adjusted all analyses for baseline levels of serum homocysteine. Results are presented as differences in the geometric mean of the 6 months level of serum homocysteine between HRT and placebo group, expressed as a percentage of the 6 months value of the placebo group with 95% Confidence Intervals (CI). All analyses were performed using SPSS 8.0 for Windows.

Results

Participating Subjects

In total, 121 subjects were successfully screened, entered the study, and received study medication. The 121 subjects were between 40 and 57 years of age, with a mean age of 47.2 years. Median time since last menstruation was 2 months (range 1-11 months) in the perimenopausal women, and was similar in the 3 study groups. Out of the 121 women, 13 were postmenopausal, defined as cessation of menses for more than 1 year. Age, weight, BMI, blood pressure, lipids, creatinine, and homocysteine levels at baseline were well balanced between the three groups (Table 1). Women receiving 17βE_2-D tended to smoke less and women receiving CEE-N tended to smoke more than the overall mean smoking percentage of the combined groups (Table 1).

Withdrawn subjects, adverse events, and non-compliance

The total number of subjects that withdrew within 6 months was 23; 11 (21%) in the 17βE_2-D group, 8 (24%) in the CEE-N group, 4 (11%) in the placebo group (Figure). Only small differences in cardiovascular disease risk factors were seen between women who completed the study (n=98) and women who withdrew

Table 1. General characteristics of the 121 women randomized at baseline

	17βE$_2$-D* (n=52)	CEE-N* (n=34)	Placebo (n=35)	All (n=121)
Age, y	46.9 ± 3.9	47.5 ± 3.9	47.2 ± 4.1	47.2 ± 3.9
Weight, kg	66.4 ± 9.4	66.1 ± 9.1	65.1 ± 8.0	66.0 ± 8.9
Body mass index (BMI), kg/m^2	23.4 ± 2.8	23.9 ± 2.9	23.7 ± 2.9	23.7 ± 2.9
Current smoking, %	13.5	32.4	20.0	20.7
Systolic blood pressure, mmHg	112 ± 12	112 ± 15	116 ± 16	113 ± 14
Diastolic blood pressure, mmHg	73 ± 8	73 ± 10	75 ± 11	74 ± 9
Total cholesterol, mmol/L	5.7 ± 1.0	5.8 ± 0.9	5.9 ± 0.9	5.8 ± 0.9
LDL cholesterol, mmol/L	3.6 ± 0.9	3.8 ± 0.8	3.9 ± 0.8	3.8 ± 0.8
HDL cholesterol, mmol/L	1.5 ± 0.3	1.4 ± 0.3	1.5 ± 0.3	1.5 ± 0.3
Triglycerides, mmol/L	1.1 ± 0.5	1.4 ± 0.6	1.2 ± 0.4	1.2 ± 0.5
Creatinine, μmol/L	81.7 ± 8.2	83.3 ± 9.1	82.9 ± 8.3	82.5 ± 8.4
Homocysteine, μmol/L†	11.5 ± 3.6	12.1 ± 2.9	11.7 ± 3.8	11.7 ± 3.2

Values are mean ± SD or percentages.
* 17βE$_2$-D = 17β-estradiol and desogestrel; CEE-N = conjugated estrogens and norgestrel.
† Shown value = geometric mean (interquartile range).

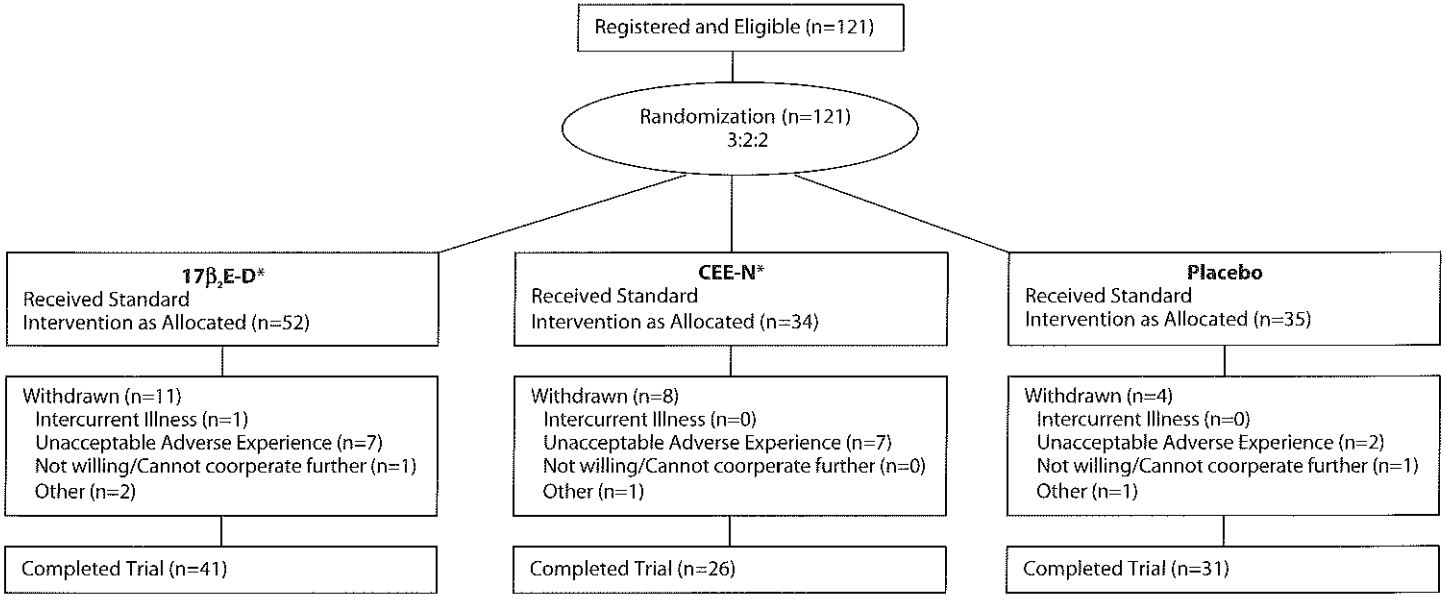

Figure. Flow diagram of the progress of subjects throughout the trial

* $17\beta E_2$-D = 17β-estradiol and desogestrel; CEE-N = conjugated estrogens and norgestrel.

(n=23). In the 3 allocated treatment groups, women who withdrew tended to smoke less than women who completed the study. The occurrence of unacceptable adverse events was the main reason for withdrawal. The type and severity of the adverse experiences recorded were consistent with those more often seen with this type of therapy, such as headache, depressive feelings, abdominal pain, and nausea. During treatment, 4 of the adverse experiences recorded were classified as serious, but probably not drug-related (1 myocardial infarction in the placebo group, and cases of epileptic seizure, syncope, and hysterectomy due to uterus myomatosis and prolapse uteri in the 17βE_2-D group). 96 women had at least one adverse experience in this period, 42 in the 17βE_2-D group (81%), 29 in the CEE-N group (86%), and 25 in the placebo group (72%). In the Org 32818 group 11 subjects were non-compliant with the study drug (21%), in the Prempak group 1 subject (3%), and in the placebo group 6 subjects (17%).

Differences in serum homocysteine levels

Because the distribution of homocysteine was skewed we natural-log-transformed it for analysis. Of the women receiving 17βE_2-D and CEE-N and completing the study, 25 (61%) and 18 (69%) showed a decline in homocyseine levels after 6 months of treatment, respectively. In the women receiving placebo and completing the study the corresponding percentage was 35 (n=11). Table 2 shows the percentual differences in homocysteine levels between the treatment and placebo groups and between both active treatment groups after 6 months of treatment, adjusted for baseline values of homocysteine. The difference in serum homocysteine levels between women treated with 17βE_2-D and placebo was -6.3% after 6 months of treatment, a difference which was borderline sig-

Table 2. Percentual differences* in serum homocysteine levels after 6 months of treatment

	Difference*	(95% CI for the difference)	*P*-value
17βE_2-D† (n=41) versus placebo (n=31)‡	-6.3%	(-12.4% ; 0.0%)	0.06
CEE-N† (n=26) versus placebo (n=31)‡	-10.1%	(-16.7% ; -2.9%)	<0.01
17βE_2-D† + CEE-N† (n=67) versus placebo (n=31) §	-7.8%	(-13.2% ; -2.0%)	0.01
17βE_2-D† (n=41) versus CEE-N† (n=26)#	4.0%	(-3.7% ; 12.3%)	0.32

* Differences in the geometric mean of the 6 months level of serum homocysteine between HRT and placebo, expressed as a percentage of the 6 months value of the placebo group, all adjusted for baseline values of serum homocysteine.

† 17βE_2-D = 17β-estradiol and desogestrel; CEE-N = conjugated estrogens and norgestrel.

‡ Treatment groups separately compared with placebo.

§ Treatment groups combined compared with placebo.

Treatment groups compared with each other.

nificant. Serum homocysteine levels differed -10.1% between women receiving CEE-N and placebo after 6 months of treatment. When both treatment groups were combined, the difference between subjects treated with HRT and placebo was -7.8%. When comparing the $17\beta E_2$-D with the CEE-N treatment group, no significant difference in serum homocysteine level after 6 months of treatment was found.

Despite random allocation of treatment, smoking was unevenly distributed between the treatment groups (Table 1). Because smoking is known to influence homocysteine levels additional analyses were carried out in which we adjusted for smoking (mean number of cigarettes smoked per day) at baseline. This adjustment did not materially affect the results (data not shown).

Discussion

In the present study in perimenopausal women from the general population, we demonstrated a decrease in fasting serum homocysteine levels in women using $17\beta E_2$-D or CEE-N compared with women using placebo after 6 months of therapy.

Some issues of our study need to be addressed. Non-compliance to study medication was smaller in subjects randomized to CEE-N than in those allocated to $17\beta E_2$-D or placebo. This might be due to the fact that the study was by design open with regard to CEE-N (Prempak®), a well-known HRT-preparation. The double-blind design with respect to $17\beta E_2$-D and placebo intervention was difficult to maintain because of the clear effects on menstrual cycle and climacteric symptoms. However, homocysteine measurements were performed in a blinded manner. Although more subjects in the active treatment groups than in the placebo group withdrew from the study, women who completed the study had overall similar levels of baseline cardiovascular risk factors and creatinine compared with women who had withdrawn. Therefore, it is not likely that the drop out has influenced the effectiveness of the randomization process to a large extent. Elevations in homocysteine levels are typically caused by genetic defects in the enzymes involved in homocysteine metabolism or by nutritional deficiencies in vitamin cofactors.[25] Because it is unlikely that either these genetic defects or food or supplement intake are differently distributed between women randomized to HRT or to placebo, we do not think that lack of information on genetic or nutritional factors has affected the validity of our results.

Earlier studies on the relation between HRT and homocysteine also found a decrease of homocysteine levels in women receiving HRT.[13-20] Some studies, however, lacked a control group.[13-15] In these uncontrolled studies a reduction,[13,14]

or greatest reduction,[15] of homocysteine was found in postmenopausal women with initially high fasting homocysteine levels, results which may have been influenced by regression to the mean. Most of the earlier studies with a control group were small.[16-18] These studies found slightly higher treatment effects than the effect found in our study, being a decrease of plasma homocysteine levels of 13%, 10%, and 9% in postmenopausal women receiving HRT, respectively. One recent larger study found a decrease of homocysteine levels of 7% in postmenopausal women assigned to HRT, which is compatible with our results.[20] In a sample of the Postmenopausal Estrogen/Progestin Intervention (PEPI) trial, treatment with HRT had a modest, but transient, impact on plasma homocysteine levels during 36 months of follow-up.[19]

The mechanisms through which estrogens may modulate serum homocysteine levels are largely unknown.[26] Possibly, lower homocysteine levels in women using HRT may be due to higher methionine transamination.[27] The strong binding of homocysteine to LDL cholesterol might also be involved,[28] facilitating an increased clearance of homocysteine by the estrogen-induced increase in LDL-receptor expression,[29] which accompanies the HRT-related decrease in LDL cholesterol. Moreover, the methylenetetrahydrofolate reductase (MTHFR) genotype is suggested to influence the homocysteine-lowering effect of HRT.[30]

Recently, a randomized trial on the effects of HRT in women with coronary heart disease showed no effect on the overall risk of coronary heart disease after 4 years of treatment (the Heart and Estrogen/Progestin Replacement Study (HERS)).[31] However, an increased risk of coronary heart disease events was found in the HRT group in the first year of the trial, while the risk decreased subsequently. This time trend might be attributable to an immediate prothrombotic, proarrythmic, or proischemic effect of treatment, which is gradually outweighed by a beneficial effect on progression of atherosclerosis. Recent results from the Estrogen Replacement and Atherosclerosis trial (ERA), however, did not show slowing of progression of angiographically measured coronary artery lesions in women with established coronary heart disease during 3.2 years of treatment with HRT.[32] The HERS and ERA were conducted in women with documented coronary heart disease, had a relatively short follow-up, and HRT was initiated late, an average of 20 years after the cessation of menses. The results of HERS and ERA are thus compatible with the possibility that HRT is effective in preventing the development of atherosclerotic disease and with the hypothesis that long-term use of HRT may slow the progression of disease once it is established.

In the present study, all the women had climacteric complaints and were predominantly perimenopausal. Although in women with symptoms estradiol levels are decreased compared with premenopausal women,[33] the endogenous

estradiol production in these symptomatic women will probably influence the effects of HRT. Because symptomatic women are the target population for HRT, the vast majority of HRT in the Netherlands is prescribed for the indication of menopausal complaints,[34] it is of interest and of clinical importance to know the effects of HRT on cardiovascular risk factors in this population. We, however, have to be careful to extrapolate our findings from this relatively young population to the use in older, postmenopausal women. When studying postmenopausal women, with low endogenous estrogen levels, the contrast between the placebo and the intervention groups might have been larger.

To summarize, in the present study in healthy perimenopausal women from a general population, we demonstrated a decrease in fasting serum homocysteine levels in women using HRT relative to women using placebo during 6 months of observation.

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Disclosure of interest (Lancet 1997;349:1411-12)

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3.4

High-dose testosterone is associated with severe atherosclerosis in postmenopausal women

Abstract

Despite the paucity of data regarding its long-term effects, inclusion of androgens in postmenopausal hormone replacement regimens is not uncommon and is likely to become more widespread.

In a population-based study in 513 naturally postmenopausal women aged 54 to 67 years, we studied the association between self-reported intramuscularly administered high-dose estrogen-testosterone therapy (estradiol and testosterone esters) and aortic atherosclerosis. Aortic atherosclerosis was diagnosed by radiographic detection of calcified deposits in the abdominal aorta, which have been shown to reflect intimal atherosclerosis. Hormone therapy users were compared with never-users.

Intramuscular hormone therapy-use for 1 year or longer was reported by 25 women. In almost half of these women severe atherosclerosis of the aorta was present (n=11), whereas in women without hormone use severe atherosclerosis of the aorta was present in less than 20% (odds ratio [OR] 3.1; 95% CI, 1.1-8.5, adjusted for age, years since menopause, smoking, and body mass index). The association remained after additional adjustment for diabetes mellitus, cholesterol level, systolic blood pressure, or alcohol use. No association was found for hormone use less than 1 year. Women reporting having used daily oral estrogen therapy for 1 year or longer (n=20) tended to be protected against aortic atherosclerosis (OR 0.4; CI, 0.1-1.2).

Our results suggest that testosterone therapy may adversely affect atherosclerosis in postmenopausal women and indicate that androgen replacement in these women may not be harmless.

Introduction

In women, androgens are important for maintaining bone mass, secondary sex characteristics, and libido.[1,2] With increasing age and after menopause, circulating androgen levels decline because of a combination of decreasing adrenal production and ovarian failure.[3] Androgen treatment in postmenopausal women improves psychological well being and sexual function,[4] and has beneficial effects on bone mass.[5,6] With dehydroepiandrosterone being sold in increasing amounts over-the-counter and the availability of new preparations containing testosterone developed specifically for women, its use is likely to become more widespread.[7] Until now, however, no data are available on the long-term effects of androgen treatment in women.

In the present population-based study, we examined the association between self-reported intramuscularly administered high-dose estrogen-testosterone therapy and aortic atherosclerosis in naturally postmenopausal women.

Subjects and Methods

Population

Between 1975 and 1978, a population-based study on risk factors for chronic diseases was conducted in the Dutch town of Zoetermeer. Inhabitants of 2 districts were invited for a medical examination. In 1985, all female participants aged 45 to 64 years at baseline were invited for a follow-up examination. Details of this study have been previously published.[8,9] The response rate of the women at baseline was 77%. Of 1167 women invited for the follow-up study, 71 had died and 87 had moved away. Of the remaining women, 855 (85%) were re-examined.

Menopausal state and use of hormone therapy

Menopausal state was assessed by a self-administered questionnaire that asked whether the menses had stopped, and if so, at what age and the reason for its cessation (natural or artificial). The type of artificial menopause was ascertained during an interview by a doctor. Postmenopausal state was defined as no menstruation for at least 1 year.

Information on hormone therapy use was gathered by interview, during which women were questioned on past and current use of hormones, including route of administration. In the Netherlands, from the late 1950s until 1980s a substantial part of the hormones indicated for menopausal complaints was administered intramuscularly, which in this period comprised combined estrogen-testosterone therapy (2-5 mg estradiol esters, and 50-100 mg testosterone

esters), to be dispensed monthly. Oral replacement therapy in the reference period primarily comprised 0.625 mg unopposed estrogen daily.

Assessment of covariates

Height and weight were measured without shoes and with indoor clothing. Body mass index (BMI) was calculated (weight/height^2). Blood pressure was measured with a random zero sphygmomanometer with the subject seated. The mean of 2 readings was reported. Serum total cholesterol was measured by an automatic enzymatic method (CHOD/PAP high performance, Boehringer-Mannheim). Information on smoking habits, alcohol use, and medical history was obtained by a self-administered questionnaire, which was checked during the interview by the study physician. Diabetes mellitus was considered present when it was reported in the questionnaire and confirmed during the interview with the physician. Subjects were asked to bring their current medication to the research center, where treatments were noted.

Aortic Atherosclerosis

Lateral radiographic films of the lumbar spine (T12-S1) were made from a fixed distance while the participant was seated. Atherosclerosis was diagnosed off-line by detecting calcified deposits in the abdominal aorta, as described previously.[9,10] Calcification was considered present when linear densities were present 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 ($\leq$ 1 cm; 2-5 cm; 6-10 cm; and > 10 cm). In the analyses, we considered the first 2 classes as mild atherosclerosis and the third and fourth classes as severe atherosclerosis.

All films were examined by 2 independent observers who were unaware of the subjects' exposure status (in the present study: use of hormone therapy). Before the scoring, a sample of the films was read by the 2 observers simultaneously so as to reach agreement on the interpretation of the scoring protocol. If there were differences between observers regarding readings, films were read by both observers simultaneously so as to reach consensus. The score that was agreed upon by both observers was recorded.

The validity of radiographic assessment of aortic atherosclerosis has been studied by comparing results of this method with data obtained at autopsy. Radiographic assessment was shown to be highly specific, and in most cases, visible calcification represented advanced intimal atherosclerosis.[11] Intimal calcification was also shown to be clearly distinguishable from medial calcification.[12] A comparison study involving computed tomography (CT) was performed at our department. In 56 unselected elderly persons, aortic calcifications were indepen-

dently assessed by radiography and CT. Calcifications were detected on abdominal radiography in 32 subjects. In all but 1 person, these calcifications were shown to be located in the aorta on the corresponding CT images.[9]

Aortic calcification is known to be associated with risk factors for cardiovascular disease[9,10] and with atherosclerosis at other sites[13] and predicts cardiovascular morbidity and mortality.[14,15] Comparison of roentgenographic aortic calcification with coronary artery calcium as detected by electron beam tomography at our department within 457 subjects showed that aortic calcification was present in 3.9%, 13.7%, and 31.5% of the subjects within the lowest, the middle, and the highest tertile of coronary artery calcium, respectively (P for trend < 0.001, adjusted for age and sex). These results indicate that aortic calcification is strongly related to coronary calcification.

Population for analysis

Of the 855 women who were examined, menstruation had ceased for less than 1 year in 7 women, and for 11 women information on menopausal state was missing. Postmenopausal women were excluded who reported to have reached menopause by oöphorectomy (n=118), hysterectomy (n=104), after stopping of oral contraceptive use (n=36), or after use of other medication, such as chemotherapy (n=6). In 8 women the cause of menopause was unclear. Natural cessation of menses was reported by 565 women. Because films were missing or not readable, information on aortic atherosclerosis was missing in 22 women. In 22 of the 543 remaining postmenopausal women, information on hormone therapy use was missing and 8 women reported having used hormone therapy with routes of administration different from injections or tablets, such as ointments or subcutaneous implants, leaving 513 naturally postmenopausal women for analysis.

Statistical analysis

The age-adjusted baseline characteristics of the study population were computed in strata of type and duration of hormone therapy use by using general linear models. Proportions of dichotomous variables between the described strata were compared using the χ^2 test. We studied the association between hormone therapy use and mild and severe aortic atherosclerosis using a multivariate polytomous logistic regression model. Never-users of hormone therapy were regarded as the reference category. A distinction was made between use of hormones < 1 year and use ≥ 1 year because no effect on the development of atherosclerosis was expected from use < 1 year. Models were initially adjusted for age by entering age as a continuous variable in the regression model. In subsequent models, we additionally adjusted for years since menopause, smoking (current,

former, or never), and BMI. We adjusted in separate models additionally for total cholesterol level, systolic blood pressure, diabetes mellitus (yes-no), or current alcohol use (yes-no).

We considered 2-sided probability values < 0.05 to be statistically significant. SPSS 10.0 for Windows (SPSS Inc., Chicago, Illinois) was used for all analyses.

Results

Data on aortic atherosclerosis and hormone therapy use (no, intramuscular, or oral) were available in 513 naturally postmenopausal women, defined as no menstruation for at least 1 year after natural cessation of the menses. Mean age of these women was 62.9 years, with a standard deviation (SD) of 5.7 years. Their reported mean age at menopause was 50.0 years (SD ± 4.0 years). One hundred seventeen of the 513 women reported ever-use of postmenopausal hormones, of whom 50% intramuscularly (n=59).

In Table 1 the age-adjusted baseline characteristics of the study population are shown stratified by type and duration of hormone therapy use. Women reporting having used intramuscular hormone therapy ≥ 1 year tended to be somewhat older and had higher levels of total cholesterol than women never having used any hormones. Women having used oral hormone therapy tended to be younger than women never having used any hormones.

Intramuscular hormone therapy use < 1 year was reported by 34 women and was not significantly associated with atherosclerosis of the aorta. Age-adjusted odds ratios for mild and severe atherosclerosis of the aorta were 1.2 (95% Confidence Interval [CI], 0.5-2.9), and 1.0 (CI, 0.4-2.5), respectively. Intramuscular hormone therapy use ≥ 1 year was reported by 25 women, with a median duration of use of 2 years (range 1-25 years). In 44% of these women, severe atherosclerosis of the aorta was present (n=11), being equivalent with atherosclerosis involving at least 1/3 of the length of the abdominal aorta (Figure), whereas in women without hormone therapy use, severe atherosclerosis of the aorta was present in less than 20% (Odds Ratio [OR] 3.1; CI, 1.1-8.5, adjusted for age, time since menopause, smoking, and BMI; Table 2). Additional adjustments for diabetes mellitus, systolic blood pressure, or alcohol use (yes-no) did not materially affect the results. Additional adjustment for cholesterol level led to a decrease of the odds ratio to 2.4 (CI, 0.9-6.9).

For 106 of the 118 women reporting having reached menopause by oöphorectomy, information on aortic atherosclerosis and hormone therapy use was available. Four of these women reported having used intramuscular hormone therapy ≥ 1 year, in 2 of whom severe aortic atherosclerosis was present, lead-

Table 1. Age-adjusted baseline characteristics of 513 naturally postmenopausal women stratified by type and duration of hormone therapy use

Characteristic	Never-use	Intramuscular hormone therapy use		Oral hormone therapy use	
	(n=396)	< 1 year (n=34)	≥ 1 year (n=25)	< 1 year (n=38)	≥ 1 year (n=20)
Mean ± SE					
Age, y	63.0 ± 0.3	62.9 ± 1.0	65.5 ± 1.1*	61.1 ± 0.9*	60.0 ± 1.3*
Time since menopause, y	13.0 ± 0.2	12.6 ± 0.7	13.1 ± 0.8	13.5 ± 0.6	11.0 ± 0.9*
Body mass index (BMI), kg/m^2	26.4 ± 0.2	26.1 ± 0.7	26.3 ± 0.8	27.0 ± 0.6	26.2 ± 0.9
Systolic blood pressure, mmHg	145.2 ± 1.0	142.6 ± 3.4	145.5 ± 4.0	146.4 ± 3.3	148.8 ± 4.5
Diastolic blood pressure, mmHg	82.3 ± 0.5	81.9 ± 1.7	83.6 ± 2.0	82.7 ± 1.6	83.3 ± 2.2
Total cholesterol, mmol/L	7.2 ± 0.1	7.2 ± 0.2	7.9 ± 0.2†	7.2 ± 0.2	6.9 ± 0.3
Percentage					
Diabetes mellitus	6	6	4	6	5
Current smokers	25	32	32	26	30
Former smokers	30	30	28	39	30
Alcohol use	64	59	60	63	60

* $P < 0.05$ compared with never-users.
† $P < 0.01$ compared with never-users.
For some women never having used hormone therapy, data were missing on BMI (n=1), blood pressure (n=2), total cholesterol level (n=9), and diabetes mellitus (n=7).
For 1 woman having used intramuscular hormone therapy < 1 year data were missing on total cholesterol level.
For 1 woman having used intramuscular hormone therapy ≥ 1 year and for 1 women having used oral hormone therapy < 1 year data were missing on diabetes mellitus.

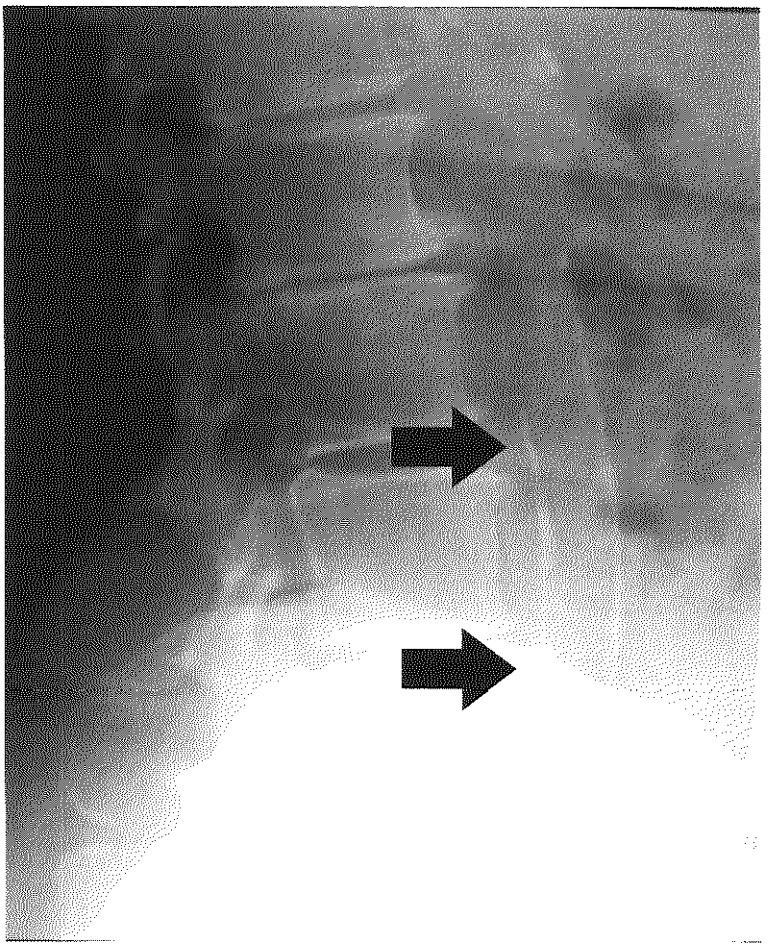

Figure. Severe atherosclerosis of the abdominal aorta (arrows) detected on a lateral X-ray of the abdomen

ing to a an age and multivariate-adjusted odds ratio of 5.7 (CI, 0.5-61.6) relative to never-users of hormone therapy.

Twenty women reported having used oral hormone therapy ≥ 1 year, with a median duration of 3 years (range 1-17 years). In 1 of these women severe atherosclerosis of the aorta was present (5%) and in 3 women mild aortic atherosclerosis was detected. Numbers were too low to present associations between oral hormone use and aortic atherosclerosis in strata of severity of atherosclerosis. Our pooled data indicated that women using oral replacement therapy ≥ 1 year tended to be protected against presence of any aortic atherosclerosis (OR 0.4; CI, 0.1-1.2), Table 3. Oral hormone therapy use < 1 year was reported by 38

Table 2. Odds ratios for mild and severe aortic atherosclerosis associated with intramuscular hormone therapy use ≥ 1 year among naturally postmenopausal women

	Aortic Atherosclerosis							
	No			Mild			Severe	
	n							
Never-use of hormone therapy	224			93			79	
Intramuscular hormone therapy use ≥ 1 year	8			6			11	
Odds ratio* (95% CI)		1.0	reference		1.5	(0.5 ; 4.7)		2.9	(1.1 ; 8.0)
Odds ratio† (95% CI)		1.0	reference		1.5	(0.5 ; 4.6)		3.1	(1.1 ; 8.5)

* Adjusted for age.
† Adjusted for age, years since menopause, smoking (current, former, or never), and body mass index.

Table 3. Odds ratios for aortic atherosclerosis associated with oral hormone therapy use ≥ 1 year among naturally postmenopausal women

	Aortic Atherosclerosis					
	No			Any		
	n					
Never-use of hormone therapy	224			172		
Oral hormone therapy use ≥ 1 year	16			4		
Odds ratio* (95% CI)		1.0	reference		0.4	(0.1 ; 1.3)
Odds ratio† (95% CI)		1.0	reference		0.4	(0.1 ; 1.2)

* Adjusted for age.
† Adjusted for age, years since menopause, smoking (current, former, or never), and body mass index.

women and was not significantly associated with atherosclerosis of the aorta (OR 0.9; CI, 0.5-2.0, adjusted for age).

Discussion

Our findings suggest that testosterone therapy may adversely affect atherosclerosis in postmenopausal women and indicate that androgen replacement in these women may not be harmless.

A limitation of our study is the fact that our results are based on observational data. Women taking hormones tend to have a better cardiovascular risk factor profile than those who do not.[16] Although this "healthy woman effect" may induce the apparently protective effect of oral estrogen on atherosclerosis in observational studies, it would only have diluted the positive association between intramuscular testosterone therapy and atherosclerosis found in our study, implying that the adverse effect of testosterone may even be stronger than our results suggest. Women reporting having used oral replacement therapy ≥ 1 year had a decreased risk of aortic atherosclerosis, which is consistent with earlier observational data.[17-19] Use of hormones was assessed by interview, which might have led to misclassification to a certain extent. This misclassification, however, is unlikely to be differential with regard to atherosclerosis[20] and will therefore only have led us to underestimate the strength of the associations. Our main results were based on analyses in naturally postmenopausal women. A large proportion of androgens, however, is prescribed to women experiencing a surgical menopause, which is accompanied by on average a 50% decline in androgen levels.[21] Our data in women having reached menopause by oöpho-

rectomy suggest that also in these women the adverse effect of testosterone on atherosclerosis is present, although power of the analysis was limited. We examined atherosclerosis of the aorta, which we consider to be a measure of generalized atherosclerosis. More specifically, aortic atherosclerosis is associated with an up to 9-times increased risk of ischemic stroke[22] and predicts cardiovascular mortality.[15]

Adverse effects of androgens on cardiovascular disease risk in women have been suggested before. Hirsutism, a clinical signs of androgen excess, has been found to be associated with an increased risk of coronary artery disease[23] and cardiovascular disease risk factors were found to be increased in women with polycystic ovary syndrome (PCOS).[24] Recently, an association between PCOS and carotid atherosclerosis has been described in middle aged women.[25] In postmenopausal women, endogenous testosterone levels have been found to be associated with atherogenic changes in cardiovascular disease risk factors[26] and the degree of angiographically determined coronary artery disease.[27] A prospective study, however, found no association between endogenous testosterone concentrations and fatal cardiovascular disease in postmenopausal women.[28] In a recent study among premenopausal and postmenopausal women studied together, women with endogenous androgen levels in the highest tertile had significantly lower carotid intimal-medial thickness.[29] We are the first to describe an association between exogenous androgens and atherosclerosis in women. Experimentally induced hyperandrogenism in female cynomologous monkeys led to an increase in the amount of coronary atherosclerosis,[30] which is compatible with our results.

Whether the actions of testosterone in women are predominantly directly mediated via androgen receptors or secondary to conversion to estrogen is not known. Androgen receptors are not entirely specific, therefore part of the effects of supraphysiological amounts of androgens are mediated via estrogen and progestin receptors.[31] Testosterone may adversely affect atherosclerosis due to effects on the lipid profile.[32,33] Our data give support for this hypothesis because the association between intramuscular hormone use and atherosclerosis diluted after adjustment for cholesterol level, probably reflecting the intermediate effect of cholesterol. In women, oral methyltestosterone has been shown to negate some of the beneficial effects of estrogen therapy on lipid levels when combined with it.[32] We studied parenteral testosterone esters, for which effects on lipoprotein levels are less pronounced.[34,35] A recent study on the safety profile of transdermal testosterone patches indicated that this mode of administration did not significantly affect lipid levels.[36] Rather than androgenic potency the mode of administration may influence the effect of testosterone on lipid levels. Orally administered steroids may induce greater lipid changes because of

a first-pass effect.[35] Other factors involved in the potential atherogenic effects of testosterone therapy may be the adverse effects on glucose metabolism. Very high-dose testosterone administered to female-to-male transsexuals resulted in impaired insulin action.[37] However, our understanding of the effects of lower dosages of exogenous androgens on insulin action and glucose metabolism in women is far from complete until now. Furthermore, treatment with testosterone in women may be associated with an increase in visceral fat accumulation,[3] which has consistently been found to be associated with cardiovascular disease.[38,39]

Our findings suggest that intramuscular testosterone therapy may adversely affect atherosclerosis in postmenopausal women. Further studies should determine whether low-dose androgens dispensed with other routes of administration increase the risk of atherosclerosis. If so, this may have implications for decisions whether or not to treat postmenopausal women with androgens.

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3.5

Low levels of endogenous androgens increase the risk of atherosclerosis in elderly men

The Rotterdam Study

Abstract

In both men and women, circulating androgen levels decline with advancing age. Until now, results of several small studies on the relationship between endogenous androgen levels and atherosclerosis have been inconsistent.

In the population-based Rotterdam Study, we investigated the association of levels of dehydroepiandrosteronesulphate (DHEAS) and total and bioavailable testosterone with aortic atherosclerosis among 1032 nonsmoking men and women aged 55 years and over. Aortic atherosclerosis was assessed by radiographic detection of calcified deposits in the abdominal aorta, which have been shown to reflect intimal atherosclerosis.

Relative to men with levels of total and bioavailable testosterone in the lowest tertile, men with levels of these hormones in the highest tertile had age-adjusted odds ratios of 0.4 (95% CI, 0.2-0.9) and 0.2 (CI, 0.1-0.7), respectively, for the presence of severe aortic atherosclerosis. The corresponding odds ratios for women were 3.7 (CI, 1.2-11.6) and 2.3 (CI, 0.7-7.8). Additional adjustment for cardiovascular disease risk factors did not materially affect the results in men, whereas in women the associations diluted. Men with levels of total and bioavailable testosterone in subsequent tertiles were also protected against progression of aortic atherosclerosis measured after 6.5 years (SD ± 0.5 years) of follow-up (*P* for trend=0.02). Levels of DHEAS were not associated with aortic atherosclerosis, neither in men nor in women.

In conclusion, we found an independent inverse association between levels of testosterone and aortic atherosclerosis in men. In women, positive associations between levels of testosterone and aortic atherosclerosis were largely due to adverse cardiovascular disease risk factors.

Introduction

Androgen levels decline with advancing age, both in men[1,2] and women.[3] Although it is not known whether this decline in hormonal activity is causally related to physical changes during aging,[4] exogenous androgens are considered to be an attractive treatment modality to potentially benefit psychological well-being, body composition, and strength in the elderly.[5-7] Dehydroepiandrosterone is being sold in increasing amounts over-the-counter, several androgen replacement therapy modalities are prescribed for men[5] and its use in women is likely to become more widespread.[8] In animal models, treatment with testosterone tended to inhibit the development of atherosclerosis in male rabbits,[9] whereas in female monkeys it induced exacerbation of atherosclerosis,[10] suggesting gender-specific effects of androgens on cardiovascular disease. In humans, the effects of androgen treatment on cardiovascular disease have not been studied, whereas endogenous androgen levels were not found to be related to cardiovascular events in men[11-17] or women.[17,18] Results of several studies on endogenous androgen levels and atherosclerosis have been inconsistent.[19-23] However, most of these studies were relatively small.[19-22]

In the population-based Rotterdam Study, we investigated the association between levels of endogenous androgens and aortic atherosclerosis among a gender-stratified sample of more than 1000 men and women aged 55 years and over.

Methods

The Rotterdam Study

The Rotterdam Study is a population-based prospective cohort study designed to assess the occurrence and the determinants of chronic diseases in an aging population.[24] The study was approved by the medical ethics committee of the Erasmus MC, Rotterdam, The Netherlands. The cohort includes 3105 men and 4878 women aged 55 years and over (78% of the eligible population) living in a defined district in Rotterdam. Written informed consent was obtained from all participants. Baseline data were collected from 1990 until 1993. The third examination phase took place from 1997 until 1999. Between these examinations 1992 persons died and 35 were lost to follow-up. Fifty-five subjects were not invited for the third examination phase because they moved outside the area, resulting in 5901 invited subjects. Of the invited subjects, 1922 men and 2875 women (81%) participated.

Endogenous androgens

At the baseline examination of the Rotterdam Study, blood samples were drawn by venapuncture from nonfasting subjects at the research center between 8.30 AM and 4.00 PM. Levels of steroid hormones were measured in plasma. For the collection of plasma, blood was collected in 5-ml tubes containing 0.5 mL sodium citrate solution. All tubes were stored on ice before and after blood sampling. Platelet-free plasma was obtained by 2-stage centrifugation, first 10 minutes at 1,600 g at 4°C and then for of 30 minutes at 7,000 g at 4°C. Platelet-free samples were immediately frozen in liquid nitrogen and transferred to the laboratory. At the laboratory plasma samples were stored at -80°C until laboratory studies were performed. For the purpose of the present study, plasma levels of dehydroepiandrosteronesulphate (DHEAS), testosterone, and sex hormone binding globulin (SHBG) were estimated in 12 separate batches of samples using coated tube (testosterone) or double antibody radioimmunoassays (DHEAS and SHBG), purchased from Diagnostic Systems Laboratories (Webster, Texas, USA). Because of the relatively small volumes of plasma available, all values reported are single sample estimations. Intraassay coefficients of variation, determined on basis of duplicate results of internal quality control pools with 3 different levels of each analyte, were below 4% for SHBG, 13% for testosterone, and 15% for DHEAS. Because interassay variations were relatively large (14% SHBG, 19% testosterone, and 24% DHEAS) results of all batches were normalized by multiplying all concentrations within a batch with a factor, which equalized results for the internal quality control pools. Assays were performed blind with respect to information on the subject. As a measure of bioavailable testosterone, non-SHBG-bound testosterone was calculated on the basis of hormone, SHBG, and albumin (see below) levels, and respective affinity constants according to the method described by Södergård et al[25] and Van den Beld et al.[26]

Aortic Atherosclerosis

At baseline and at follow-up, lateral radiographic films of the lumbar spine (T12-S1) were made from a fixed distance while the participant was seated. Atherosclerosis was diagnosed off-line by detecting calcified deposits in the abdominal aorta, as described previously,[27,28] by a technician and scored independently of the subjects' exposure status (in the present study: levels of endogenous androgens). Calcification was considered present when linear densities were present in an area parallel and anterior to the lumbar spine (L1-L4). Values for the extent of calcification were scored according to the length of the involved area (< 1 cm; 1-2.5 cm; 2.5-5 cm; 5-10 cm; and ≥ 10 cm). We considered the first 2 classes as mild, the third class as moderate, and fourth and fifth classes as severe atherosclerosis.

Progression of aortic atherosclerosis was defined as the occurrence of new calcifications or enlargement of the calcified area present at baseline. Baseline and follow-up films were examined in pairs. The extent of progression was graded (0.5-1 cm; 1-2.5 cm; 2.5-5 cm; and $\geq$ 5 cm), but because of the relatively small numbers available for analysis, we combined severity grades into 2 groups: progression absent and progression present. No subject showed a decrease in extent of aortic calcification. All films were read by 1 observer who was aware of the date of the radiographs. Before the scoring, a sample of the films was read by 2 observers simultaneously so as to reach agreement on the interpretation of the scoring protocol. Previously determined interobserver agreement on progression scoring (absent versus present) based on 758 pairs of lateral radiographic films of the lumbar spine at our department reached a percentage of agreement of atherosclerotic change of 88, and a κ statistic of 0.74.[27]

The validity of radiographic assessment of aortic atherosclerosis has been studied by comparing results of this method with data obtained at autopsy. Radiographic assessment was shown to be highly specific, and in most cases visible calcification represented advanced intimal atherosclerosis.[29] Intimal calcification was also shown to be clearly distinguishable from medial calcification.[30] A comparison study involving computed tomography (CT) was performed at our department. In 56 unselected elderly persons, aortic calcifications were independently assessed by radiography and CT. Calcifications were detected on abdominal radiography in 32 subjects. In all but 1 person, these calcifications were shown to be located in the aorta on the corresponding CT images.[28]

Aortic calcification is known to be associated with risk factors for cardiovascular disease[27,28] and with atherosclerosis at other sites[31] and predicts cardiovascular morbidity and mortality.[32,33] When aortic calcification (as detected by radiography) was compared with coronary artery calcium (as detected by electron-beam computed tomography) in 457 participants in the Rotterdam Study, aortic calcification was present in 3.9% of participants in the lowest tertile of coronary artery calcium, in 13.7% of those in the middle tertile of coronary artery calcium, and in 31.5% of those in the highest tertile of coronary artery calcium (P for trend < 0.001, adjusted for age and gender).

Covariates

During a home interview at baseline, a trained research assistant gathered information on current and past health, medication, smoking habits, and age of menopause (self-reported age of last menstruation). Participants were subsequently invited to visit the research center, where intake of alcohol was assessed using a food frequency questionnaire.[34] Height, weight, and waist and hip circumferences were measured while each participant was wearing indoor clothing

without shoes. Body mass index (BMI, weight divided by height squared) and waist-to-hip ratio (WHR) were computed. Two blood pressure measurements were taken with a random-zero sphygmomanometer after 5-minutes of rest with the subject in sitting position, and averaged. A venipuncture was performed and nonfasting blood samples were obtained. They were directly put on ice and serum samples were processed within 30 minutes after which they were kept frozen at -20°C. We used an automated enzymatic procedure to determine serum total cholesterol level.[35] High-density lipoprotein (HDL) cholesterol was measured similarly, after precipitation of the non-HDL cholesterol fraction. Albumin was measured using a colorimetric method (KONE Diagnostics, Espoo, Finland). We studied glucose metabolism using a nonfasting oral glucose tolerance test. Diabetes mellitus was defined as the use of glucose-lowering medication or a random or post-load serum glucose level $\geq$ 11.1 mmol/L according to the World Health Organization (WHO) criteria.[36]

Population for analysis

We determined levels of steroid hormones in plasma in a gender-stratified random sample of 1432 subjects (667 men and 765 women). In 1252 subjects (610 men and 642 women) data on aortic atherosclerosis were available. To increase power for the current analyses we additionally sampled plasma from 233 subjects (116 men and 117 women) with moderate to severe aortic atherosclerosis present at baseline. We excluded participants using systemic corticosteroids (16 men and 26 women) or hormone supplements (1 men and 15 women) at time of blood drawing. One woman used both types of medication, leaving 1428 subjects (709 men and 719 women). All women were postmenopausal. To remove residual confounding by current smoking, which influences levels androgens in men[37,38] and women,[39-41] we additionally excluded smoking men (n=205) and women (n=191), leaving 1032 subjects for the current analyses (504 men and 528 women). Due to logistic reasons and insufficient plasma available data on DHEAS and total testosterone were missing for 56 men and 44 women, and 76 men and 58 women, respectively. Due to missing data on binding protein levels, data on bioavailable testosterone were additionally missing for 121 men and 114 women. The sex and age-specific prevalence of cardiovascular disease risk factors and aortic atherosclerosis in subjects with missing data on hormone levels were comparable with the prevalence of these risk factors in the 1032 subjects available for the current analyses.

Statistical analysis

We stratified all analyses by sex to study sex-specific associations. Tertiles of endogenous androgen levels were computed in the randomly selected eligible

population (i.e. without taking the additionally sampled cases with moderate to severe aortic atherosclerosis at baseline into account).

First, we computed age-adjusted levels of cardiovascular disease risk factors according to tertiles of levels of androgens by using general linear models. Tests of significance for the coefficients of the ordered variable of tertiles of androgen levels in subsequent linear regression models with the cardiovascular disease risk factor as dependent variable were considered to be tests for trend.

Second, we used logistic regression models to compute age and multivariate-adjusted odds ratios for severe aortic atherosclerosis according to tertiles of levels of androgens. In these analyses, the number of participants with severe aortic atherosclerosis in subsequent tertiles of androgen levels was compared with the number of participants without any aortic atherosclerosis in these tertiles. Analyses were initially adjusted for age by entering age as a continuous variable in the model. In subsequent models, we additionally adjusted for BMI, systolic blood pressure, cholesterol level, HDL cholesterol level, presence of diabetes mellitus (yes-no), smoking (ever, never), and alcohol intake (in 4 categories: nondrinking; < 1 glass; 1-2 glasses; and > 2 glasses per day). In analyses regarding women, we additionally adjusted for years since menopause and ever-use of hormone replacement therapy (yes-no).

Third, we used logistic regression models to compute age and multivariate-adjusted odds ratios for progression of aortic atherosclerosis during follow-up according to tertiles of androgen level at baseline. These analyses were additionally adjusted for duration of follow-up.

In all multivariate-adjusted models, we used missing value indicators for missing data on categorical covariates,[42] whereas for missing data on continuous covariates we imputed the gender-specific mean value of the respective variable as calculated from the study population of 1032 subjects.

We considered 2-sided P-values < 0.05 to be statistically significant. SPSS 10.0 for Windows (SPSS Inc., Chicago, Illinois) was used for all analyses.

Results

The baseline characteristics of the study population are shown in Table 1. The age of participating men ranged from 55.0 to 89.4 with a mean of 67.9 years. In women age ranged from 55.1 to 89.0 with a mean of 69.5 years. Aortic atherosclerosis was absent in 175 men (35%) and 188 women (36%), whereas severe atherosclerosis was present in 47 men and 46 women (9% in both sexes).

Tables 2A and 2B show the age-adjusted levels of cardiovascular disease risk factors according to tertiles of levels of endogenous androgens. In men, higher

Table 1. Baseline characteristics of the study sample

Characteristic	Men (n=504)	Women (n=528)
	Mean ± SD	Mean ± SD
Age, y	67.9 ± 7.3	69.5 ± 7.9
Weight, kg	79.4 ± 9.9	69.5 ± 10.6
Body mass index (BMI), kg/m^2	26.1 ± 2.9	26.7 ± 3.7
Waist-to-hip ratio (WHR), cm/cm	0.96 ± 0.07	0.87 ± 0.09
Systolic blood pressure, mmHg	138.2 ± 20.3	139.5 ± 21.2
Diastolic blood pressure, mmHg	74.8 ± 10.7	72.8 ± 11.0
Total cholesterol, mmol/L	6.4 ± 1.1	7.0 ± 1.3
HDL cholesterol, mmol/L	1.2 ± 0.4	1.5 ± 0.4
Time since menopause, y	-	20.5 ± 9.1
Albumin, g/L	43.4 ± 2.6	43.1 ± 2.5
SHBG, nmol/L	34.7 ± 14.0	43.8 ± 17.8
DHEAS, µmol/L	4.2 ± 2.5	2.6 ± 2.0
Total testosterone, nmol/L	11.2 ± 3.9	1.4 ± 0.8
Bioavailable testosterone, nmol/L	6.8 ± 2.9	0.7 ± 0.4
	Percentage	Percentage
Diabetes mellitus	8	8
Former smokers	88	34
Alcohol drinkers*	91	74
Ever-use of hormone replacement therapy	-	14
Aortic atherosclerosis		
Mild	32	30
Moderate	24	26
Severe	9	9

* < 1 glass; 1-2 glasses; and > 2 glasses per day.
For some men, data were missing on weight and BMI (n=1), WHR (n=31), blood pressure (n=5), HDL cholesterol (n=2), albumin (n=126), SHBG (n=83), DHEAS (n=56), testosterone (n=76), bioavailable testosterone (n=197), diabetes mellitus (n=14), and alcohol drinking (n=50).
For some women, data were missing on BMI (n=1), WHR (n=40), blood pressure (n=2), total cholesterol (n=1), HDL cholesterol (n=3), albumin (n=113), time since menopause (n=12), SHBG (n=65), DHEAS (n=44), testosterone (n=58), bioavailable testosterone (n=172), diabetes mellitus (n=24), alcohol drinking (n=62), and ever-use of hormone replacement therapy (n=12).

measures of body weight were associated with lower levels of DHEAS and testosterone, and former smokers were overrepresented in the lower tertiles of levels of testosterone (Table 2A). Higher blood pressure levels tended to be associated with lower levels of testosterone, although tests for trend did not reach statistical significance. In women, higher body weight and BMI, and lower levels of HDL cholesterol were associated with higher levels of testosterone (Table 2B). Diabetes mellitus tended to be more prevalent in women with lower levels of DHEAS and higher levels of testosterone.

Table 2A. Age-adjusted cardiovascular disease risk factors according to tertiles of levels of endogenous androgens in 504 nonsmoking men

Characteristic	DHEAS, μmol/L				total T, nmol/L			
	Tertile 1 ≥ 0.1 & ≤ 2.6	Tertile 2 > 2.6 & ≤ 4.6	Tertile 3 > 4.6 & ≤ 15.9	*P*-trend	Tertile 1 ≥ 0 & ≤ 9.8	Tertile 2 > 9.8 & ≤ 12.6	Tertile 3 > 12.6 & ≤ 36.8	*P*-trend
Age, y	70.6 ± 0.6	67.3 ± 0.6	65.8 ± 0.6	<0.001	69.9 ± 0.6	67.9 ± 0.6	66.3 ± 0.6	<0.001
Weight, kg	81.2 ± 0.8	78.9 ± 0.8	78.1 ± 0.8	0.01	81.2 ± 0.9	79.5 ± 0.8	77.8 ± 0.8	0.006
Body mass index (BMI), kg/m^2	26.6 ± 0.2	26.1 ± 0.2	25.6 ± 0.2	0.004	26.5 ± 0.2	26.3 ± 0.2	25.6 ± 0.2	0.006
Waist-to-hip ratio (WHR), cm/cm	0.96 ± 0.01	0.97 ± 0.01	0.96 ± 0.01	0.92	0.97 ± 0.01	0.96 ± 0.01	0.95 ± 0.01	0.07
Systolic blood pressure, mmHg*	137.4 ± 1.8	139.9 ± 1.8	136.8 ± 1.8	0.78	140.2 ± 1.9	137.6 ± 1.8	137.0 ± 1.8	0.25
Diastolic blood pressure, mmHg*	74.8 ± 1.0	75.2 ± 0.9	75.0 ± 0.9	0.90	76.6 ± 1.0	74.4 ± 0.9	75.2 ± 0.9	0.30
Total cholesterol, mmol/L*	6.3 ± 0.09	6.4 ± 0.09	6.4 ± 0.09	0.53	6.3 ± 0.09	6.4 ± 0.09	6.3 ± 0.09	0.79
HDL cholesterol, mmol/L*	1.2 ± 0.03	1.3 ± 0.03	1.2 ± 0.03	0.67	1.2 ± 0.03	1.2 ± 0.03	1.2 ± 0.03	0.89
Diabetes mellitus, %	9	10	6	0.38	8	11	6	0.46
Former smokers, %	91	82	90	0.91	93	88	84	0.013
Alcohol drinkers, %	92	90	93	0.61	90	92	93	0.51

Values are mean ± SD or percentages.
For some men, data were missing on weight and BMI (n=1), WHR (n=31), blood pressure (n=5), HDL cholesterol level (n=2), diabetes mellitus (n=14), and alcohol drinking (n=50).
* 80 men using antihypertensive medication and 17 men using serum lipid-lowering agents were excluded for analyses on blood pressure and cholesterol levels, respectively.

Table 2B. Age-adjusted cardiovascular disease risk factors according to tertiles of levels of endogenous androgens in 528 nonsmoking women

Characteristic	DHEAS, µmol/L				total T, nmol/L			
	Tertile 1 ≥ 0.1 & ≤ 1.5	Tertile 2 > 1.5 & ≤ 2.9	Tertile 3 > 2.9 & ≤ 13.6	*P*-trend	Tertile 1 ≥ 0 & ≤ 1.0	Tertile 2 > 1.0 & ≤ 1.6	Tertile 3 > 1.6 & ≤ 6.9	*P*-trend
Age, y	71.6 ± 0.6	70.2 ± 0.6	66.9 ± 0.6	<0.001	68.7 ± 0.6	69.5 ± 0.6	69.9 ± 0.6	0.17
Weight, kg	69.7 ± 0.8	69.8 ± 0.9	69.0 ± 0.8	0.55	67.2 ± 0.8	69.4 ± 0.8	70.9 ± 0.8	0.001
Body mass index (BMI), kg/m^2	26.6 ± 0.3	26.8 ± 0.3	26.7 ± 0.3	0.89	25.9 ± 0.3	26.6 ± 0.3	27.2 ± 0.3	0.001
Waist-to-hip ratio (WHR), cm/cm	0.88 ± 0.01	0.86 ± 0.01	0.86 ± 0.01	0.02	0.86 ± 0.01	0.88 ± 0.01	0.87 ± 0.01	0.44
Systolic blood pressure, mmHg*	138.0 ± 1.7	138.3 ± 1.8	139.6 ± 1.7	0.51	138.5 ± 1.6	137.1 ± 1.7	140.0 ± 1.7	0.56
Diastolic blood pressure, mmHg*	73.0 ± 0.9	72.3 ± 0.9	72.7 ± 0.9	0.85	72.5 ± 0.9	73.1 ± 0.9	73.3 ± 0.9	0.53
Total cholesterol, mmol/L*	7.0 ± 0.1	6.9 ± 0.1	6.9 ± 0.1	0.57	7.0 ± 0.1	7.0 ± 0.1	6.9 ± 0.1	0.49
HDL cholesterol, mmol/L*	1.5 ± 0.03	1.4 ± 0.03	1.5 ± 0.03	0.91	1.5 ± 0.03	1.4 ± 0.03	1.4 ± 0.03	0.06
Time since menopause, y	20.5 ± 0.4	20.6 ± 0.4	20.2 ± 0.4	0.63	20.7 ± 0.4	20.2 ± 0.4	20.1 ± 0.4	0.32
Diabetes mellitus, %	10	8	5	0.07	7	8	11	0.18
Former smokers, %	32	34	39	0.16	32	34	37	0.32
Alcohol drinkers, %	75	75	74	0.75	76	78	71	0.34
Ever-use of hormone replacement therapy, %	17	14	13	0.32	19	12	13	0.13

Values are mean ± SD or percentages.
For some women, data were missing on BMI (n=1), WHR (n=40), blood pressure (n=2), total cholesterol level (n=1), HDL cholesterol level (n=3), time since menopause (n=12), diabetes mellitus (n=24), alcohol drinking (n=62), and ever-use of hormone replacement therapy (n=12).
* 64 women using antihypertensive medication and 13 women using serum lipid-lowering agents were excluded for analyses on blood pressure and cholesterol levels, respectively.

Table 3A. Odds ratios for severe aortic atherosclerosis* according to tertiles of levels of endogenous androgens in nonsmoking men

	Aortic Atherosclerosis		OR (95% CI)†	OR (95% CI)‡
	Severe, n	No, n		
DHEAS tertiles				
≥ 0.1 & ≤ 2.6 µmol/L	15	42	1 (ref)	1 (ref)
> 2.6 & ≤ 4.6 µmol/L	16	56	1.0 (0.4 ; 2.3)	0.9 (0.4 ; 2.2)
> 4.6 & ≤ 15.9 µmol/L	13	58	0.8 (0.3 ; 2.0)	0.9 (0.3 ; 2.2)
			P-trend=0.68	*P-trend=0.71*
total T tertiles				
≥ 0 & ≤ 9.8 nmol/L	19	38	1 (ref)	1 (ref)
> 9.8 & ≤ 12.6 nmol/L	14	48	0.7 (0.3 ; 1.5)	0.7 (0.3 ; 1.6)
> 12.6 & ≤ 36.8 nmol/L	9	60	0.4 (0.2 ; 0.9)	0.4 (0.1 ; 1.0)
			P-trend=0.03	*P-trend=0.04*
bioavailable T tertiles				
≥ 0 & ≤ 5.6 nmol/L	16	24	1 (ref)	1 (ref)
> 5.6 & ≤ 7.5 nmol/L	8	36	0.4 (0.1 ; 1.0)	0.3 (0.1 ; 0.9)
> 7.5 & ≤ 28.7 nmol/L	5	43	0.2 (0.1 ; 0.7)	0.2 (0.0 ; 0.6)
			P-trend=0.006	*P-trend=0.004*

* Number of men with severe aortic atherosclerosis compared with number of men without aortic atherosclerosis.
† Adjusted for age.
‡ Adjusted for age, body mass index, systolic blood pressure, cholesterol level, HDL cholesterol level, diabetes mellitus (yes-no), smoking (ever, never), and alcohol intake (4 categories).

In Tables 3A and 3B the odds ratios for severe aortic atherosclerosis according to tertiles of levels of androgens are shown. Levels of DHEAS were not associated with the presence of severe aortic atherosclerosis in men or women (Tables 3A and 3B). Men with levels of testosterone in the second and third tertile had lower odds of severe aortic atherosclerosis. Multivariate adjustment did not materially change the results (Table 3A). Women with levels of testosterone in the second and third tertile tended to have higher odds of presence of severe aortic atherosclerosis. Multivariate adjustment diluted the associations (Table 3B). Exclusion of male or female participants using serum lipid-lowering or antihypertensive medication did not affect the results (data not shown).

Of the men with complete data on DHEAS, total testosterone, and bioavailable testosterone, 82% participated in the third examination phase, and in 287, 282, and 208 of these men, respectively, follow-up information of aortic atherosclerosis was available. Of the women with complete data on DHEAS, total testosterone, and bioavailable testosterone, 81% participated in the third examination phase, and in 272, 263, and 197 of these women, respectively, follow-up information of aortic atherosclerosis was available. Progression of aortic atherosclerosis during a follow-up period of 6.5 years (SD ± 0.5 years) was observed

Table 3B. Odds ratios for severe aortic atherosclerosis* according to tertiles of levels of endogenous androgens in nonsmoking women

	Aortic Atherosclerosis		OR (95% CI)†	OR (95% CI)‡
	Severe, n	No, n		
DHEAS tertiles				
≥ 0.1 & ≤ 1.5 µmol/L	16	42	1 (ref)	1 (ref)
> 1.5 & ≤ 2.9 µmol/L	13	51	0.6 (0.3 ; 1.6)	0.5 (0.2 ; 1.5)
> 2.9 & ≤ 13.6 µmol/L	11	67	0.9 (0.3 ; 2.3)	0.7 (0.2 ; 2.2)
			P-trend=0.70	*P-trend=0.33*
total T tertiles				
≥ 0 & ≤ 1.0 nmol/L	5	57	1 (ref)	1 (ref)
> 1.0 & ≤ 1.6 nmol/L	18	57	3.0 (0.9 ; 9.4)	4.4 (1.1 ; 17.5)
> 1.6 & ≤ 6.9 nmol/L	20	54	3.7 (1.2 ; 11.6)	2.8 (0.7 ; 11.5)
			P-trend=0.03	*P-trend=0.19*
bioavailable T tertiles				
≥ 0 & ≤ 0.4 nmol/L	5	38	1 (ref)	1 (ref)
> 0.4 & ≤ 0.8 nmol/L	13	43	2.1 (0.6 ; 7.3)	1.8 (0.4 ; 8.2)
> 0.8 & ≤ 2.9 nmol/L	14	48	2.3 (0.7 ; 7.8)	1.0 (0.2 ; 5.1)
			P-trend=0.21	*P-trend=0.84*

* Number of women with severe aortic atherosclerosis compared with number of women without aortic atherosclerosis.
† Adjusted for age.
‡ Adjusted for age, body mass index, systolic blood pressure, cholesterol level, HDL cholesterol level, diabetes mellitus (yes-no), smoking (ever, never), alcohol intake (4 categories), time since menopause, and ever-use of hormone replacement therapy (yes-no).

in 76% of men and 73% of women. In the Figure the age-adjusted odds ratios for progression of aortic atherosclerosis according to subsequent tertiles of levels of androgens are shown. Men in the second and third tertile of levels of total and bioavailable testosterone were protected against progression of aortic atherosclerosis (Figure A). Multivariate adjustment did not materially affect the results (data not shown). In women, no association between tertiles of levels of androgens and progression of aortic atherosclerosis was found (Figure B).

Discussion

We found an independent, inverse association between levels of endogenous testosterone and severe aortic atherosclerosis in men. In women, higher levels of testosterone tended to be positively associated with severe aortic atherosclerosis, although multivariate adjustment diluted the associations. Levels of DHEAS were not associated with aortic atherosclerosis, in neither women nor men.

When interpreting our results, some methodological issues should be taken

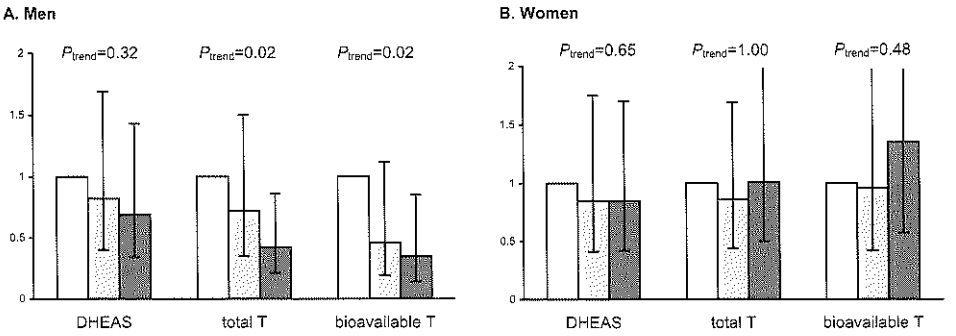

Figure. Age-adjusted odds ratios for any progression of aortic atherosclerosis according to subsequent tertiles of levels of endogenous androgens in nonsmoking men and women

into account. Due to small volumes of plasma available, we were not able to run the assays in duplicate and the single sample measurement will have led to less precise estimations of plasma levels. Furthermore, a relatively large proportion of free testosterone measurements, although random, was missing. Although these factors will have reduced the precision of our risk estimates, they will only have led us to underestimate the strength of the associations.

In our study sample, cardiovascular disease risk factors tended to be more adverse in men with lower levels of testosterone, whereas in women atherogenic changes in cardiovascular risk factors tended to be associated with higher levels of testosterone, which corresponds with previously published data in men[2] and women.[43] The positive association between testosterone and cardiovascular disease risk factors in women largely accounted for the positive association between testosterone and aortic atherosclerosis found in women.

Lower levels of testosterone and free testosterone have been described in 55 male subjects with angiographically measured coronary atherosclerosis.[19] The same author found in 60 postmenopausal women undergoing diagnostic coronary angiography free testosterone levels to be positively associated with degree of coronary atherosclerosis.[20] Results of both described studies[19,20] are in agreement with our results. In a case-control study conducted within the Edinburgh Artery Study among 83 subjects with peripheral arterial disease and a comparable number of controls, however, no association with testosterone was found in neither men nor women.[21] This discrepancy of results may be attributable to the limited sex-specific power of this study and the fact that peripheral arterial disease may encompass subjects with less severe atherosclerosis than the subjects with severe aortic atherosclerosis in our study. Contrary to our results, a recent cross-sectional study in 101 premenopausal and post-

menopausal women found that women in the highest tertiles of testosterone had significantly lower carotid intima-media thickness independent of cardiovascular disease risk factors.[22] Similar results were obtained when analyses were restricted to the 48 postmenopausal women.[22] The apparent discrepancy between our results and the results of studies in which no association between endogenous testosterone levels and coronary heart disease in men was reported[12,13,15] may be attributable to the fact that we studied nonsmokers only and to the fact that the aorta might be more vulnerable to the effects of endogenous sex steroids than other arteries. Aortic atherosclerosis has been found to be associated with an up to 9-times increased risk of ischemic stroke[44] indicating its importance in relation to cardiovascular disease. Mechanisms possibly involved in the association between aortic atherosclerosis and stroke may be pulse pressure or emboli being released from atherosclerotic lesions in the aortic arch.

The mechanisms of the beneficial effect of testosterone on atherosclerosis in males are largely unknown.[45] It has been suggested that testosterone may affect atherosclerosis through modulation of classical cardiovascular disease risk factors.[45] The fact that multivariate adjustment did not influence the association between testosterone and atherosclerosis in men in our study sample does not support this hypothesis. Negative associations between testosterone and the hemostatic risk factors plasminogen activator inhibitor I,[19,46] fibrinogen,[19,46] and factor VII[47] have been reported in men, indicating that testosterone may affect atherogenesis through a modulation of these factors. As suggested by recent animal experiments, direct beneficial effects of testosterone on plaque development, probably mediated by the vascular androgen receptor, may be involved.[48] Another explanation for our results that should be considered, however, is the hypothesis that higher levels of testosterone do not protect against atherosclerosis in men, but are merely a marker of good health.[4]

DHEAS is the most abundantly produced adrenal steroid. It is considered to be a weak androgen, mainly contributing to androgenicity by its peripheral conversion to the more potent androgens testosterone and dihydrotestosterone. It has been suggested that DHEA(S) exerts antiatherogenic effects[49] and reduced levels of DHEAS may, among others, mediate the relation between insulin resistance and atherosclerosis.[50] We found an inverse association between levels of DHEAS and presence of diabetes mellitus, especially in women, which is consistent with previously described inverse associations between DHEAS and insulin.[51,52] We, however, did not find an association between levels of DHEAS and atherosclerosis. Within the prospective population-based Bruneck Study, DHEAS was not found to be associated with development and progression of carotid atherosclerosis among 867 subjects during 5 years of follow-up either.[23]

These results together with the failure to find an association between levels of DHEAS and the onset of cardiovascular disease,[14,16,17] indicate that the suggestion that DHEAS is a 'treatment for aging' lacks a solid scientific basis until now, at least with regard to cardiovascular disease.

In recent years, testosterone replacement strategies have been developed for men[5] and new preparations developed specifically for women are becoming available.[8] Many of their aspects, however, remain controversial and increasing blood hormone levels to those found in 30 to 50-year old individuals has not yet been uniformly proven to be safe and of benefit.[4] We have to be careful to extrapolate our results regarding the association between endogenous androgen levels and aortic atherosclerosis to potential effects of therapeutic application of androgens. Dose, duration, the identification of elderly who might benefit most, and possible effects on the process of atherosclerosis of testosterone suppletion remain subjects for study.[4]

In conclusion, we found an independent inverse association between levels of testosterone and severe aortic atherosclerosis in men. In women, higher levels of testosterone tended to be positively associated with severe aortic atherosclerosis, which was largely accounted for by more adverse cardiovascular disease risk factors. Whether treatment with testosterone may protects against atherogenesis in men remains to be studied.

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CHAPTER 4

Alternative endocrine cardiovascular disease risk factors

4.1

Subclinical hypothyroidism is an independent risk factor for atherosclerosis and myocardial infarction in elderly women

The Rotterdam Study

Abstract

Background: Overt hypothyroidism has been found to be associated with cardiovascular disease. Whether subclinical hypothyroidism and thyroid autoimmunity are also risk factors for cardiovascular disease is controversial.

Objective: To investigate whether subclinical hypothyroidism and thyroid autoimmunity are associated with aortic atherosclerosis and myocardial infarction in postmenopausal women.

Design: Population-based cross-sectional study.

Setting: A district of Rotterdam, The Netherlands.

Subjects: Random sample of 1149 women (mean age ± SD, 69.0 ± 7.5 years) participating in the Rotterdam Study.

Measurements: Data on thyroid status, aortic atherosclerosis, and history of myocardial infarction were obtained at baseline. Subclinical hypothyroidism was defined as an elevated thyroid-stimulating hormone level (> 4.0 mU/L) and a normal serum free thyroxine level (11-25 pmol/L [0.9-1.9 ng/dL]). In tests for antibodies to thyroid peroxidase, a serum level > 10 IU/mL was considered a positive result.

Results: Subclinical hypothyroidism was present in 10.8% of participants and was associated with a greater age-adjusted prevalence of aortic atherosclerosis (odds ratio (OR) 1.7 [95% CI, 1.1-2.6]) and myocardial infarction (OR 2.3 [CI, 1.3-4.0]). Additional adjustment for body mass index, total and high-density lipoprotein cholesterol level, blood pressure, and smoking status, as well as exclusion of women who took β-blockers, did not affect these estimates. Associations were slightly stronger in women who had subclinical hypothyroidism and antibodies to thyroid peroxidase (OR for aortic atherosclerosis, 1.9 [CI, 1.1-3.6], OR for myocardial infarction, 3.1 [CI, 1.5-6.3]). No association was found between thyroid autoimmunity itself and cardiovascular disease. The population attributable risk percentage for subclinical hypothyroidism associated with myocardial infarction was within the range of that for known major risk factors for cardiovascular disease.

Conclusion: Subclinical hypothyroidism is a strong indicator of risk for atherosclerosis and myocardial infarction in elderly women.

Introduction

Overt hypothyroidism, with its accompanying hypercholesterolemia and hypertension, has been found to be associated with cardiovascular disease.[1-3] Subclinical hypothyroidism, defined as an asymptomatic state characterized by normal serum concentrations of free thyroxine and elevated serum concentrations of thyroid-stimulating hormone (TSH),[4] is highly prevalent in elderly women.[5,6] Whether subclinical hypothyroidism is related to risk for cardiovascular disease is controversial. Case-control and cross-sectional studies on the association between subclinical hypothyroidism and cardiovascular disease have been done.[7-11] Results from these studies are not consistent, but many of the studies were small. The same controversy surrounds thyroid autoimmunity. In the late 1960s and early 1970s, autopsy studies[12,13] and studies in hospital inpatients[12,14] suggested that asymptomatic autoimmune thyroiditis was an important risk factor for coronary heart disease. These findings, however, were not confirmed by other studies.[7,8,11,15]

In our population-based study, we examined whether subclinical hypothyroidism and thyroid autoimmunity are associated with aortic atherosclerosis and myocardial infarction in elderly women. We conducted our study in a random sample of 1149 postmenopausal women who were participating in the Rotterdam Study.

Methods

The Rotterdam Study

The Rotterdam Study is a population-based cohort study designed to assess the occurrence and clarify the determinants of chronic diseases in an aging population.[16] The cohort includes 3105 men and 4878 women at least 55 years of age (78% of the eligible population) living in a defined district in Rotterdam, The Netherlands. Baseline data were collected from August 1990 until July 1993. During a home interview, a trained research assistant gathered information on current and past health, medication, lifestyle and risk factors for chronic diseases. Participants were subsequently invited to visit at a research center for clinical examination. The study was approved by the medical ethics committee of the Erasmus University Medical School, Rotterdam, The Netherlands.

Clinical examination and laboratory methods

Height and weight were measured while each participant was wearing indoor clothing without shoes. Body mass index (BMI) was computed as weight divided

by height squared. A trained research assistant measured sitting systolic and diastolic blood pressure with a random-zero sphygmomanometer after 5 minutes of rest, and a standard 12-lead electrocardiogram was obtained (ACTA electrocardiogram recorder, Esoate, Florence, Italy).

Venipuncture was performed, and nonfasting serum samples were collected. The samples were immediately put on ice and were processed within 30 minutes, after which they were kept frozen at -20°C. We used an automated enzymatic procedure to determine serum total cholesterol level.[17] High-density lipoprotein (HDL) cholesterol levels were measured in a similar manner after precipitation of the non-HDL cholesterol fraction. Total protein level was measured by using the biuret method, albumin level was measured by using the bromescol-green method, and creatinine concentration was measured by using an enzymatic colorimetric method. (All products were manufactured by Boehringer-Mannheim, Mannheim, Germany, currently Roche Diagnostics, Basel, Switzerland.) We assayed levels of TSH by using TSH Lumitest (Henning, Berlin, Germany, currently Brahms, Berlin, Germany).[18] When TSH concentrations were abnormal (>4.0 mU/L or <0.4 mU/L), serum free thyroxine levels were measured with an in vitro immunodiagnostic reagent (Ortho-Clinical Diagnostics, Amersham, United Kingdom); values between 11-25 pmol/L (0.9-1.9 ng/dL) were considered normal. Serum antibodies to thyroid peroxidase were assessed by using ELISA (Milenia, DPC, Los Angeles, California); tests results were considered positive if levels were > 10 IU/mL.

Thyroid definitions

Subclinical hypothyroidism was defined as a TSH level > 4.0 mU/L in the presence of a normal free thyroxine level (11-25 pmol/L [0.9-1.9 ng/dL]). Clinical hypothyroidism was defined as a TSH level > 4.0 mU/L and a decreased free thyroxine level (<11 pmol/L [<0.9 ng/dL]).[4] Euthyroidism was defined as a normal TSH level (0.4-4.0 mU/L).

Aortic atherosclerosis

Aortic atherosclerosis was assessed on a lateral radiographic film of the lumbar spine, which was obtained from a fixed distance while the participants were seated. A research assistant who was unaware of the participants' thyroid status diagnosed atherosclerosis off-line by detecting calcified deposits in the abdominal aorta, as described elsewhere.[19,20] Calcification was considered present when linear densities were found in an area parallel and anterior to the lumbar spine (L1-L4). We classified aortic atherosclerosis as mild, moderate, or severe, according to the length of the involved area (≤ 1 cm, 2-5 cm, and > 5 cm, respectively). Because of a relatively small number of participants in the categories of

aortic atherosclerosis, we combined severity grades into 2 categories -"present" or "absent"- for analysis.

The validity of radiographic assessment of aortic atherosclerosis has been studied by comparing results of this method with data obtained at autopsy. Radiographic assessment was shown to be highly specific, and in most cases visible calcification represented advanced intimal atherosclerosis.[21] A comparison study involving computed tomography (CT) was performed at our department. In 56 unselected elderly persons, aortic calcifications were independently assessed by radiography and CT. Calcifications were detected on abdominal radiography in 32 subjects. In all but 1 person, these calcifications were shown to be located in the aorta on the corresponding CT images.[20]

Aortic calcification is known to be associated with risk factors for cardiovascular disease[19,20] and with atherosclerosis at other sites[22] and predicts cardiovascular morbidity and mortality.[23,24] When aortic calcification (as detected by radiography) was compared with coronary artery calcium (as detected with electron-beam computed tomography) in 457 participants in the Rotterdam Study, aortic calcification was present in 3.9% of participants in the lowest tertile of coronary artery calcium, in 13.7% of those in the middle tertile of coronary artery calcium, and in 31.5% of those in the highest tertile of coronary artery calcium (P for trend < 0.001, adjusted for age and sex). These results indicate that aortic calcification is strongly related to coronary calcification.

Myocardial infarction at baseline

The presence of myocardial infarction was assessed by self-report and by analysis of the standard 12-lead electrocardiograms, which were stored digitally and analyzed by using the Modular Electrocardiogram ANalysis System (MEANS).[25,26] For participants who reported myocardial infarction but had no electrocardiographic evidence of it, we collected additional information from their general practitioners or cardiologists. Myocardial infarction was confirmed if the information in the medical records met standard diagnostic criteria. An experienced cardiologist reviewed the electrocardiograms of participants who had not reported myocardial infarction but had electrocardiographic evidence of it. In these participants, absence of symptoms was confirmed by medical records review. When the cardiologist confirmed myocardial infarction (silent myocardial infarction), it was considered present. We combined both types of myocardial infarction into 1 variable for the analyses. No information on the thyroid status of participants was available at assessment of myocardial infarction.

Follow-up procedures

We collected data on incident myocardial infarction from baseline (1990-1993)

until 1 April 1996. Fatal and nonfatal events were reported by general practitioners in the research area (in which 85% of the cohort resides), who cooperate with the Rotterdam Study and provide information through a computerized system. Research physicians verified all information by checking participants' medical records at the general practitioners' offices. In addition, we obtained letters from medical specialists and discharge reports for hospitalized patients. Two research physicians coded events independently according to the International Classification of Diseases, 10th Revision.[27] If the 2 physicians disagreed, they reached consensus in a separate session. Subsequently, a medical expert in the field reviewed all events coded by the research physicians and verified that all coding rules had been applied correctly. When discrepancies were found between the coding of the medical expert and that of the research physicians, the expert's judgement was considered final. Myocardial infarction was defined as a nonfatal or fatal myocardial infarction (ICD-10 codes I21-I23). When we compared our results with data registered by the nationwide morbidity registry of hospitals, we found that 98% of all incident myocardial infarctions that occurred in Rotterdam Study participants before 1 April 1996 had been detected by our follow-up data collection system.

Selection of the sample for analysis

The selection of the population for analysis is shown in the Figure. We determined thyroid status in a random sample of 1149 women after excluding those who took amiodarone, which may nonsystematically alter TSH levels.[28] To obtain a reference category that included only euthyroid women (those whose TSH levels were within the normal range), we excluded women with clinical hypothyroidism (n=13); those with a decreased TSH level (<0.4 mU/L), which indicated clinical hyperthyroidism (free thyroxine level > 25 pmol/L [>1.9 ng/dL]) or subclinical hyperthyroidism (free thyroxine level, 11-25 pmol/L [0.9-1.9 ng/dL]) (n=73); and/or those taking thyroid medication (l-thyroxine or thyrostatic medication [propylthiouracil, carbimazole, or thiamazole]) (n=10).

Of the 1055 women remaining, we excluded those for whom data were missing (n=50) or improper (n=30); therefore, 975 women were included in our analyses of aortic atherosclerosis (Figure, selection 1). Data on myocardial infarction at baseline were available for 994 women (Figure, selection 2). At the time of analysis, 19 women had not been completely followed because of linking problems between their general practitioners' medical records and our computerized registration system. Therefore, until 1 April 1996, completed follow-up for analysis of incident myocardial infarction was available for 1036 women, covering an average period (±SD) of 4.6 ± 0.7 years (Figure, selection 3).

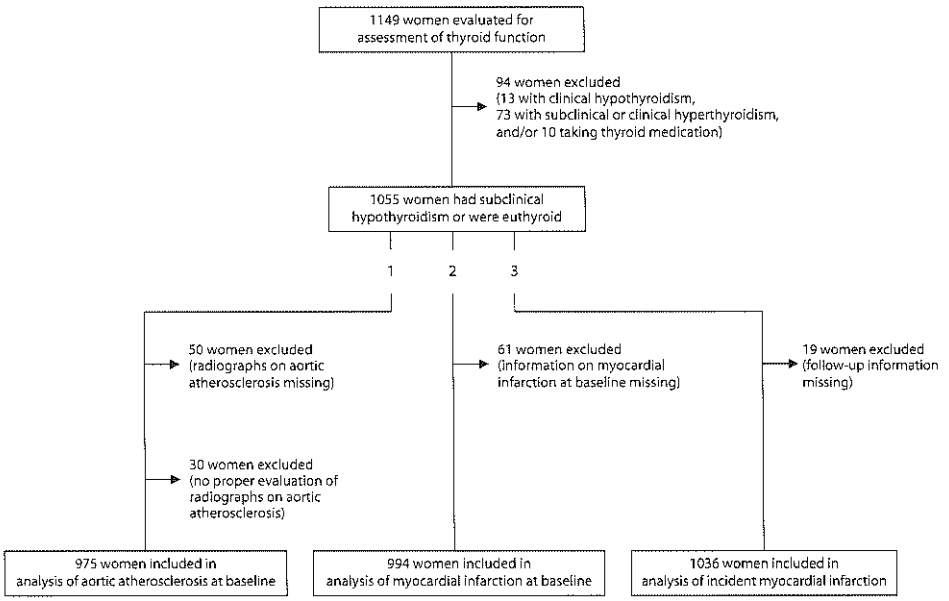

Figure. Selection of sample for analysis

1 = selection of women for analysis of aortic atherosclerosis at baseline; 2 = selection of women for analysis of history of myocardial infarction at baseline; 3 = selection of women for analysis of incident myocardial infarction.

Statistical analysis

We used linear regression analysis to compare the age-adjusted continuous baseline characteristics of euthyroid women and women with subclinical hypothyroidism. The χ^2 test was used to compare proportions of women who smoked in both groups and to compare proportions of women who had subclinical hypothyroidism and antibodies to thyroid peroxidase according to vascular disease status.

Multivariate logistic regression analysis was used to evaluate the association of aortic atherosclerosis and myocardial infarction as assessed at baseline (history of myocardial infarction) with subclinical hypothyroidism. For women with subclinical hypothyroidism, we computed the risk for incident myocardial infarction (both fatal and nonfatal) during follow-up by using Cox proportional hazards regression analysis. In this analysis, we excluded women with a history of myocardial infarction (n=79). We adjusted all analyses for age by entering age as a continuous variable in the regression model; we subsequently adjusted analyses for BMI, cholesterol and HDL cholesterol level, systolic and diastolic blood pressure, and smoking status (never, past, or current). To ensure that

comparisons between models were valid, the age-adjusted models included the number of participants for whom information was available on all of the covariates for which the multivariate model was adjusted.

We performed additional analyses after excluding women who took β-blockers (alprenolol, oxprenolol, pindolol, propranolol, timolol, and sotalol) (n=37) because these drugs may influence TSH levels.[29] In addition, we used logistic regression analysis to compare the associations of aortic atherosclerosis and history of myocardial infarction with subclinical hypothyroidism in women who had subclinical hypothyroidism and antibodies to thyroid peroxidase relative to those in euthyroid women who did not have antibodies to thyroid peroxidase. We also used logistic regression analysis to compare the frequency of aortic atherosclerosis and history of myocardial infarction in women with antibodies to thyroid peroxidase and women without antibodies to thyroid peroxidase, independent of thyroid status.

The attributable risk percentage, or etiologic fraction, and the population attributable risk percentage for subclinical hypothyroidism associated with incident myocardial infarction were calculated.[30] For purposes of comparison, we calculated the attributable risk percentage and the population attributable risk percentage for the 4 major, classic risk factors for cardiovascular disease - hypercholesterolemia (total cholesterol level ≥ 8.0 mmol/L [≥ 309 mg/dL]), hypertension (systolic blood pressure ≥ 160 mmHg and/or diastolic blood pressure ≥ 95 mmHg, and/or antihypertensive medication use), smoking status (current and past compared with never), and diabetes mellitus (use of antidiabetic medication or a random postload glucose level > 11.1 mmol/L [200 mg/dL]) - associated with incident myocardial infarction in all female participants of the Rotterdam Study (n=4878).

All measures of association are presented with their 95% CIs. A 2-sided probability value < 0.05 was considered statistically significant. We used SPSS 8.0 for Windows (SPSS, Inc., Chicago, Illinois) for all analyses.

Results

Before exclusion of clinically hypothyroid women, women with a decreased TSH level, and women using thyroid medication, the prevalence of subclinical hypothyroidism in the study sample was 10.8%. The baseline characteristics of the study sample are shown in Table 1. Women with subclinical hypothyroidism did not differ from euthyroid women with regard to age, BMI, blood pressure, total protein level, albumin level, creatinine concentration, or smoking status but had significantly lower levels of total cholesterol and borderline significantly

Table 1. Baseline characteristics of the study sample

Variable	Euthyroid women (n=931)*	Women with subclinical hypothyroidism (n=124)†
Mean ± SD		
Age, y	68.9 ± 7.4	69.0 ± 7.9
Body mass index, kg/m^2	26.7 ± 4.1	27.1 ± 3.8
Systolic blood pressure, mmHg	138 ± 21	137 ± 22
Diastolic blood pressure, mmHg	73 ± 11	73 ± 11
Total cholesterol, mmol/L‖	7.0 ± 1.2	6.7 ± 1.0‡
HDL cholesterol, mmol/L‖	1.5 ± 0.4	1.4 ± 0.4§
Total protein, g/L	71.0 ± 4.9	71.7 ± 4.7
Albumin, g/L	42.7 ± 2.4	43.0 ± 2.5
Creatinine, µmol/L#	77.6 ± 13.9	77.4 ± 14.7
Percentage (n)		
Smoking		
Never	50 (466)	51 (63)
Past	29 (272)	30 (37)
Current	19 (176)	19 (23)

* For some euthyroid women, data were missing on body mass index (n=7), blood pressure and HDL cholesterol level (n=4), albumin level (n=2), creatinine concentration (n=1), and smoking habits (n=17).
† Data on body mass index, blood pressure, and smoking habits were each missing for 1 woman.
‡ $P < 0.05$, adjusted for age.
§ $P = 0.07$, adjusted for age.
‖ To convert mmol/L to mg/dL, multiply by 38.67.
To convert µmol/L to mg/dL, multiply by 0.0113.

Table 2. Characteristics of women according to vascular disease status

	All Women (n=1055)	Women with aortic atherosclerosis (n=560)	Women with a history of myocardial infarction (n=79)
Mean ± SD			
Age, y	68.9 ± 7.5	70.7 ± 7.4	71.1 ± 6.9
Median (25th, 75th Percentile)			
Thyroid-stimulating hormone level, mU/L	1.7 (1.1, 2.7)	1.7 (1.1, 2.8)	2.0 (1.2, 3.4)
Percentage (n)			
Women with subclinical hypothyroidism	11.8 (124)	13.9 (78)*	21.5 (17)†
Women with subclinical hypothyroidism and antibodies to thyroid peroxidase	5.8 (61)	7.1 (40)*	13.9 (11)†
Women with antibodies to thyroid peroxidase	21.6 (228)	21.4 (120)	26.6 (21)

* $P < 0.05$ compared with women without the specific vascular disease status (χ^2 test).
† $P < 0.01$ compared with women without the specific vascular disease status (χ^2 test).

lower levels of HDL cholesterol in age-adjusted comparisons.

Table 2 shows the characteristics of participants according to vascular disease status. Fifty-three percent of participants (n=560) had aortic atherosclerosis at baseline, and 7.5% (n=79) had a history of myocardial infarction. Subclinical hypothyroidism was present in 11.8% of women in our sample for analysis. Women who had aortic atherosclerosis and a history of myocardial infarction had a higher prevalence of subclinical hypothyroidism and subclinical hypothyroidism accompanied by antibodies to thyroid peroxidase than those who did not have these diseases. The prevalence of thyroid autoimmunity independent of thyroid status itself did not differ significantly among the specific subgroups. Among women with subclinical hypothyroidism, concentrations of TSH were higher in those with antibodies to thyroid peroxidase than in those without such antibodies (age-adjusted geometric means, 6.6 mU/L [CI, 6.1-7.1 mU/L] and 5.4 mU/L [CI, 5.0-5.8 mU/L], respectively; $P = 0.001$). Independent of thyroid status, TSH levels were also higher in women who had antibodies to thyroid peroxidase than those who did not (geometric means, 2.4 mU/L [CI, 2.2-2.6 mU/L] and 1.6 mU/L [CI, 1.5-1.7 mU/L], respectively; $P < 0.001$).

Subclinical hypothyroidism was associated with a greater prevalence of aortic atherosclerosis. The odds ratio (OR) for aortic atherosclerosis (1.7 [CI, 1.1-2.6]) was increased in women with subclinical hypothyroidism (Table 3). Women with subclinical hypothyroidism also had a greater prevalence of myocardial infarction than euthyroid women (OR, 2.3 [CI, 1.3-4.0]) (Table 3). Additional adjustment for BMI, total cholesterol and HDL cholesterol levels, systolic and diastolic blood pressure, and smoking status did not affect these associations, nor did exclusion of participants who used β-blockers (data not shown). During an average follow-up of 4.6 years, 16 women had a first incident myocardial infarction. When we used a Cox proportional hazard regression analysis in women with subclinical hypothyroidism, a statistically non-significant adjusted relative risk of 2.5 (CI, 0.7-9.1) was observed for myocardial infarction.

Women with subclinical hypothyroidism and antibodies to thyroid peroxidase had a greater prevalence of aortic atherosclerosis than euthyroid women without antibodies to thyroid peroxidase (OR, 1.9 [CI, 1.1-3.6]) (Table 3). The presence of antibodies to thyroid peroxidase increased the odds ratio for a history of myocardial infarction to 3.1 (CI, 1.5-6.3) in women who had subclinical hypothyroidism compared with euthyroid women who did not have antibodies to thyroid peroxidase (Table 3). Because only 1 woman with subclinical hypothyroidism and antibodies to thyroid peroxidase had a myocardial infarction during follow-up, we were not able to compute the corresponding risk for incident myocardial infarction. No association was found between the presence of antibodies to thyroid peroxidase and aortic atherosclerosis or history of myocardial infarc-

Table 3. Odds ratios for aortic atherosclerosis and history of myocardial infarction*

Variable	Condition		Odds Ratio (95% CI)†	Odds Ratio (95% CI)‡
	present	absent		
	n			
Aortic atherosclerosis				
Women with subclinical hypothyroidism	77	37	1.7 (1.1 ; 2.6)	1.9 (1.2 ; 3.1)
Euthyroid women	474	376	1§	1§
Women with subclinical hypothyroidism and antibodies to thyroid peroxidase	39	16	1.9 (1.1 ; 3.6)	2.2 (1.1 ; 4.3)
Euthyroid women without antibodies to thyroid peroxidase	398	301	1§	1§
History of myocardial infarction				
Women with subclinical hypothyroidism	17	99	2.3 (1.3 ; 4.0)	2.3 (1.3 ; 4.2)
Euthyroid women	61	806	1§	1§
Women with subclinical hypothyroidism and antibodies to thyroid peroxidase	11	52	3.1 (1.5 ; 6.3)	3.5 (1.7 ; 7.4)
Euthyroid women without antibodies to thyroid peroxidase	52	660	1§	1§

* The number of women may not be exactly the same as in Table 2 because data on some covariates were missing.
† Adjusted for present age.
‡ Adjusted for present age, body mass index, cholesterol level, HDL cholesterol level, systolic and diastolic blood pressure, and smoking status (current, past, or never).
§ Reference risk.

tion when thyroid status was not altered (data not shown).

From our data, we calculated an attributable risk percentage of 60 and a population attributable risk percentage of 14 for subclinical hypothyroidism associated with myocardial infarction (Table 4). If subclinical hypothyroidism is assumed to be causally related to myocardial infarction, our findings suggest

Table 4. Attributable risk percentages and population attributable risk percentages for subclinical hypothyroidism and classic risk factors for cardiovascular disease associated with incident myocardial infarction in women in the Rotterdam Study

Risk Factor	Age-Adjusted Relative Risk*	Attributable Risk	Population Attributable Risk
		%	
Subclinical hypothyroidism	2.5	60	14
Hypercholesterolemia	2.4	58	18
Hypertension	1.6	38	14
Smoking	2.0/1.2†	50/17†	15
Diabetes mellitus	2.4	58	14

* Determined by using Cox proportional hazards regression analysis.
† Age-adjusted relative risk and attributable risk percentage for current compared with never smokers, and past compared with never smokers, respectively.

that it contributed to 60% of cases of myocardial infarction among women affected by subclinical hypothyroidism and that it was involved in the pathogenesis of 14% of all myocardial infarctions in the study sample. For purposes of comparison, the attributable risk percentages and the population attributable risk percentages for hypercholesterolemia, hypertension, smoking, and diabetes mellitus associated with myocardial infarction in all female participants in the Rotterdam Study are presented in Table 4.

Discussion

Our results show that subclinical hypothyroidism is highly prevalent among elderly women and is associated with a greater frequency of aortic atherosclerosis and myocardial infarction. Among women with subclinical hypothyroidism, these associations are slightly stronger in those who have antibodies to thyroid peroxidase. Thyroid autoimmunity itself is not associated with aortic atherosclerosis or myocardial infarction.

One limitation of our study is the cross-sectional nature of the design, which necessitates careful interpretation of the results. The relative risk for myocardial infarction in women with subclinical hypothyroidism in the prospective part of our study was similar to the point estimate in the cross-sectional part of our study. However, the CI was wide and included 1.0. Furthermore, we must consider the fact that elevated TSH levels may be caused by nonthyroidal illness.[31,32] However, we excluded women with a low free thyroxine level and observed that women with subclinical hypothyroidism did not differ from euthyroid women in levels of total protein, albumin, and creatinine. Therefore, it is highly unlikely that nonthyroidal illness affected the validity of our results. Serum samples were obtained only from women who visited the research center. We do assume that the nonresponse for the visit to the research center will not depend on subclinical hypothyroidism differently among persons with or without the presence of cardiovascular disease, making selection bias unlikely. Furthermore, follow-up information was not available for all study participants as a result of logistic reasons. Because we have no reason to assume that the relation between subclinical hypothyroidism and myocardial infarction in women with complete follow-up data differs from this association in those without follow-up data, we do not believe that this lack of information influenced the validity of our results.

Approximately 11% of women in our sample had a TSH level > 4 mU/L. This prevalence closely resembles that reported in women in the Wickham Survey,[5] the Framingham Study,[6] and a study in community-dwelling elderly persons.[33]

Among all women in our sample for analysis, 13 (1.1%) had unrecognized overt thyroid failure characterized by an elevated TSH level (> 4.0 mU/L) and an abnormal free thyroxine level (<11 pmol/L [0.9 ng/dL]), which is in agreement with reports of prevalence found during screening.[4] These data suggest that our sample is representative of the general population.

Several studies on the association between coronary heart disease and subclinical hypothyroidism have been done. Our results agree with those of previous case-control studies that also showed an association between subclinical hypothyroidism and coronary heart disease in elderly women.[9,10] However, a Finnish study that presented results of men and women together provided no evidence that latent thyroid failure is associated with coronary heart disease.[7] Female patients with coronary heart disease were shown to have significantly lower serum levels of thyroid hormone than controls; however, subclinical hypothyroidism did not seem to be related to the presence of coronary heart disease.[11] In the Wickham survey, no cross-sectional association with ischemic heart disease was observed, but a weak association between minor electrocardiographic changes and minor degrees of hypothyroidism was found in women.[8]

Data on atherosclerosis and subclinical hypothyroidism are scarce. A case-control study in elderly women suggested an association between subclinical hypothyroidism and peripheral arterial disease.[34] We are the first to describe an association between subclinical hypothyroidism and atherosclerosis as assessed by a noninvasive measurement in a general population sample. Aortic atherosclerosis was diagnosed by radiographic detection of calcified deposits in the abdominal aorta, which has been shown to be a highly specific technique for the measurement of aortic intimal atherosclerosis.[21] False-negative misclassification may have occurred in our study, but it was probably independent of thyroid status and therefore may have affected our results only by causing us to underestimate the association. Because we found that subclinical hypothyroidism was associated with both atherosclerosis and myocardial infarction, our data may indicate that atherosclerosis is involved in the mechanism by which subclinical hypothyroidism and myocardial infarction are associated.

Several mechanisms that may be involved with the association of subclinical hypothyroidism with atherosclerosis and myocardial infarction can be considered. A common cause of thyroid failure in elderly women is autoimmune thyroiditis.[6,35] It has been suggested that pathologic immune reactivity (e.g., immune complex-mediated vascular damage) may be important in the association of autoimmune thyroiditis with coronary heart disease.[36] However, the literature on this association is controversial. Some studies have described an association between thyroid autoimmunity and coronary heart disease,[9,10,12,14,37]

and other studies have not.[7,8,11,15] Different uses of various generations of antibody assays and different definitions of thyroid autoimmunity may have played a role in these discrepant findings. We found no association between the presence of antibodies to thyroid peroxidase itself and aortic atherosclerosis or myocardial infarction, which weakens the notion that a pathologic immune reactivity is important. We found that associations between subclinical hypothyroidism and aortic atherosclerosis or myocardial infarction were slightly stronger when subclinical hypothyroidism was accompanied by antibodies to thyroid peroxidase. This suggests that subclinical hypothyroidism, which is thought to be more severe and lasting in the presence of thyroid antibodies, contributes to the pathogenesis of cardiovascular disease.

Some authors found an atherogenic disturbance in the lipid metabolism in subjects with subclinical hypothyroidism,[38-42] whereas other studies did not.[43-45] Although in our study the total cholesterol level was higher in women with overt hypothyroidism than in euthyroid women (data not shown), we did not find that total cholesterol level was higher in women with subclinical hypothyroidism than in euthyroid women. HDL and total cholesterol levels provided no pathophysiologic explanation for the association of subclinical hypothyroidism with aortic atherosclerosis and myocardial infarction. Other lipids - such as low-density lipoprotein (LDL) cholesterol level, enhanced LDL oxidation,[46] triglyceride level, and lipoprotein(a) level[42] - may be responsible for the association between subclinical hypothyroidism and cardiovascular disease, but we did not measure these factors.

Other mechanisms that may be involved in the association between subclinical hypothyroididsm and cardiovascular disease can be derived from experimental data. In vitro, thyroid hormones inhibit collagen-induced platelet aggregation[47,48] and directly relax smooth muscle.[49] These effects may be important if thyroid hormones have the same effect in adult humans, although in subclinical hypothyroidism, by definition, levels of thyroid hormone are not decreased. Hypothyroidism is accompanied by a hypercoagulable state,[50] increased blood viscosity,[51] and a greater plasma concentration of total homocysteine;[52] if these factors are also seen in subclinical hypothyroidism, they may account for atherosclerostic and ischemic disorders.

In conclusion, we found that subclinical hypothyroidism is highly prevalent in elderly women and is strongly and independently associated with aortic atherosclerosis and myocardial infarction. The population attributable risk percentage for subclinical hypothyroidism associated with myocardial infarction was within the range of that for known major risk factors for cardiovascular disease. Additional research should de done to determine whether this association can be confirmed in a prospective study. If so, subsequent studies may focus on

the effectiveness of possible therapies for subclinical hypothyroidism in elderly women and the desirability of screening such women for this disorder.

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4.2

Progression of aortic calcification is associated with metacarpal bone loss during menopause

A population-based longitudinal study

Abstract

Atherosclerosis and osteoporosis are major causes of morbidity and mortality in postmenopausal women and have been suggested to be associated. No study has examined whether progression of atherosclerotic calcification is associated with bone loss. In the present study, we examined progression of aortic calcification, diagnosed by radiographic detection of calcified deposits in the abdominal aorta, in relation to metacarpal bone loss, as assessed by metacarpal radiogrammetry, during menopause. Initially premenopausal women (n=236), aged 45 to 57 years at baseline, were followed for 9 years. We additionally assessed the cross-sectional association between the extent of aortic calcification and metacarpal bone mass and density in 720 postmenopausal women. Twenty-five percent of women going through menopause showed progression of aortic calcification. The average loss of metacarpal bone mass among women with progression of aortic calcification was 3.2 mm^2, and their loss of metacarpal bone density was 7.2%, whereas in women without progression of aortic calcification, these losses were 2.0 mm^2 and 5.6%, respectively, adjusted for age and years of follow-up ($P<0.05$). Additional adjustment for age at menopause, body mass index, blood pressure, smoking, diabetes mellitus, and use of hormone replacement therapy, thiazide, and loop diuretics did not influence these results. In postmenopausal women, a graded, inverse cross-sectional association between the extent of aortic calcification and metacarpal bone mass and density was found. In conclusion, our results indicate that progression of atherosclerotic calcification is associated with increased bone loss in women during menopause.

Introduction

Cardiovascular disease and osteoporosis are major causes of morbidity and mortality in postmenopausal women[1,2] and are generally considered unrelated. Several studies, however, indicate that atherosclerosis and osteoporosis are associated.[3-10] Calcification is a common feature of atherosclerotic plaques and is regulated in a way similar to bone mineralization.[11-16] The relation of vascular calcification to the pathogenesis of atherosclerosis and plaque rupture is not clear yet, but data indicate that moderate calcification of plaques contributes to vascular morbidity and mortality.[17-20]

Several cross-sectional studies have been conducted on the association between atherosclerotic calcification and osteoporosis among elderly women.[3-8,21-23] Most of these studies found an association,[3-8] although some did not.[21-23] Potential confounding factors other than age have not been taken into account in most of these studies.[3-5,8,21,22] No study examined whether progression of atherosclerotic calcification is associated with bone loss. Because the prevalence of atherosclerosis and osteoporosis increases from menopause onward,[24,25] the change from the premenopausal to the postmenopausal state may be an appropriate period to study this association longitudinally.

In the present population-based study, we examined the association between progression of aortic calcification and metacarpal bone loss during menopause in 236 women. In addition, we studied the cross-sectional association between the extent of aortic calcification and metacarpal bone mass and density in 720 postmenopausal women.

Methods

Population

Between 1975 and 1978, a population-based study on risk factors for chronic diseases was conducted in the Dutch town of Zoetermeer. Inhabitants of 2 districts were invited for a medical examination. In 1985, all female participants aged 45 to 64 years at baseline were invited for a follow-up examination. Details of this study have been previously published.[25,26] The response rate of the women at baseline was 77%. Of 1167 women invited for the follow-up study, 71 had died and 87 had moved away. Of the remaining women, 855 (85%) were reexamined.

Aortic calcification

Aortic calcification was diagnosed by radiographic detection of calcified deposits in the abdominal aorta.[26] At baseline and at follow-up, lateral abdominal films

(T12-S1) were made from a fixed distance while the subject was seated. Aortic calcifications were considered present when linear densities were seen in an area parallel and anterior to the lumbar spine (L1-L4). Baseline and follow-up values for the extent of calcification were scored according to the length of the involved area (≤1 cm; 2-5 cm; 6-10 cm; and >10 cm). In the analyses, we considered the first 2 classes as mild calcification and the third and fourth classes as advanced calcification.

Progression of calcification was defined as the occurrence of new calcifications or enlargement of the calcified area present at baseline. Baseline and follow-up films were examined in pairs. The extent of progression was graded, but because of the relatively small numbers in the categories, we combined severity grades into 2 groups: progression absent and progression present in the analyses. No subject showed a decrease in extent of aortic calcification.

All films were examined by 2 independent observers without knowledge of the metacarpal bone mass and density of the subjects. Before the scoring, a sample of the films was read by the 2 observers simultaneously so as to reach agreement on interpretation of the scoring protocol. Observers were aware of the date of the radiographs. If there were differences between observers regarding readings, films were reviewed by both observers simultaneously so as to reach consensus. The score that was agreed upon by both observers was recorded. The percentage of agreement for absence versus presence of progression was 88 and the κ statistic was 0.74.

The validity of radiographic assessment of aortic intimal calcification was studied by comparisons made on necropsy material. The method was shown to be highly specific, and in most cases, visible calcification represented advanced atherosclerosis.[27] A comparison study with computed tomography (CT) in 56 unselected elderly subjects showed that calcifications that were detected on the abdominal X-ray in 32 subjects were independently shown to be located in the aorta on the corresponding CT images in all but 1 subject.[26] Moreover, aortic calcification is known to be associated with cardiovascular disease risk factors[26,28] and atherosclerosis at other sites[29] and to predict cardiovascular morbidity and mortality.[18,19] Comparison of roentgenographic aortic calcification with coronary artery calcium as detected by electron beam tomography at our department within 457 subjects showed that aortic calcification was present in 3.9%, 13.7%, and 31.5% of the subjects within the lowest, the middle, and the highest tertile of coronary artery calcium, respectively (P for trend < 0.001, adjusted for age and sex). These results indicate that aortic calcification is strongly related to coronary calcification.

Metacarpal radiogrammetry

Anteroposterior radiographs of the hands were used for measurements of the cortical thickness of metacarpals II, III, and IV of both hands. At baseline and at follow-up, measurements of the outer diameter (D) and the medullar diameter (d) of the metacarpal bones were conducted at the midshaft with the use of a x 7 magnifying loupe with an accuracy of 0.01 mm. The metacarpal cortical area (MCA) was calculated as the mean value of D^2-d^2 for 6 metacarpals. As standardization for differences in body size, the relative cortical area (RCA) was calculated. This was achieved by expressing the MCA as a percentage of the size of the metacarpal bone: 100% x (D^2-d^2)/ D^2 for each metacarpal bone.[30,31] The mean value of the 6 metacarpals was used for the analyses. The MCA and RCA can be interpreted as indicators of bone mass and bone density, respectively. For the MCA and the RCA, the total loss during follow-up was calculated by subtracting the baseline measurements from those at follow-up. The observers measuring the metacarpal bone mass and density were unaware of the aortic calcification score of the subjects.

We estimated the measurement precision of metacarpal radiogrammetry in 100 duplicate measurements. The mean intraindividual standard deviation of a duplicate measurement was 1.9 mm^2 (4% of the initial mean value) for MCA and 2.5% (3% of the initial mean value) for RCA, which is sufficient to allow inferences concerning bone loss after a 9-year period. In women, the mineral content of the metacarpals correlates well with that at other peripheral skeletal bone sites (r ranges from 0.75 to 0.96).[32] The accuracy of the measurement was demonstrated by Exton-Smith et al, [33] who found a correlation of 0.85 between the mineral content of the metacarpal cortical area and the ash mineral content of the metacarpal bones.

Menopausal state

Menopausal state was assessed by a self-administered questionnaire that asked whether the menses had stopped, and if so, at what age and the reason for its cessation (natural or artificial). The type of artificial menopause was ascertained during an interview by a doctor. Postmenopausal state was defined as no menstruation for at least 1 year.

Assessment of covariates

Assessment of covariates was similar at baseline and at follow-up. Height and weight were measured without shoes and with indoor clothing. Body mass index (BMI) was calculated (weight/$height^2$). Blood pressure was measured with a random zero sphygmomanometer with the subject seated. The mean of 2 readings was reported. Serum total cholesterol at baseline was measured by an

automatic enzymatic method. During follow-up, a modified reagent was used (CHOD/PAP high performance, Boehringer-Mannheim). Information on smoking habits and medical history was obtained by a self-administered questionnaire, which was checked during an interview by the study physician. Diabetes mellitus was considered present when it was reported in the questionnaire and confirmed during the interview with the physician. Subjects were asked to bring their current medication to the research center, where treatments were noted.

Population for analysis

Of the 855 women examined at follow-up, menstruation had ceased for < 1 year in 7 women, and for 11 women information on menopausal state was missing. Because films were missing or not readable, information on aortic calcification and/or metacarpal bone density was missing in 45 women, leaving 792 postmenopausal women. Of these women, 282 were premenopausal at baseline. Data on progression of aortic calcification or bone loss were missing in 27 women. Age at menopause could not be ascertained for 19 women, leaving 236 women for the analysis considering the association between progression of aortic calcification and bone loss. The mean duration of follow-up for these women was 8.9 ± 0.8 years. For the cross-sectional analysis in postmenopausal women at follow-up, we excluded women with missing information on age at menopause only if their age at follow-up was < 60 years (n=72), because we assumed elderly women to be postmenopausal. This left 720 postmenopausal women for the cross-sectional analysis at follow-up.

Data analysis

Initially, we compared continuous baseline characteristics between premenopausal women with and without progression of aortic calcification during follow-up by use of a general linear model adjusted for age. Dichotomous variables were compared by a χ^2 test.

We used a general linear model to compute and compare adjusted mean values of metacarpal bone loss in categories of progression of aortic calcification. The cross-sectional association between aortic calcification and metacarpal bone mass and density in all postmenopausal women at follow-up was assessed by linear regression analysis with MCA and RCA as dependent variables and the variable indicating the extent of aortic calcification (no, mild, or advanced) as an independent variable. A test of significance for the coefficient of this ordinal variable was considered to be a test for trend. Adjusted mean values of bone mass and density in categories of aortic calcification were computed by use of a general linear model.

A 2-sided probability value < 0.05 was considered statistically significant.

SPSS 8.0 for Windows was used for analyses.

Results

The characteristics of the study population are shown in Table 1. The age of premenopausal women at baseline ranged from 45.0 to 56.8 years. Mild aortic calcification was present in 25 premenopausal women at baseline, whereas only 1 woman showed advanced aortic calcification. Metacarpal bone mass (MCA) and density (RCA) decreased during follow-up, by 4.5% and 7.4%, respectively. The age of all postmenopausal women at follow-up ranged from 53.5 to 76.2 years.

During follow-up, progression of aortic calcification was observed in 59 women going through menopause (25%). No subject showed a decrease in the extent of aortic calcification. Compared with premenopausal women without

Table 1. Baseline and follow-up characteristics of the study population

	Premenopausal at baseline and postmenopausal at follow-up		All postmenopausal women at follow-up
	Baseline (n=236)	Follow-up (n=236)	Follow-up (n=720)
Age, y	49.0 ± 2.5	57.9 ± 2.6	62.9 ± 5.6
Height, m	1.64 ± 0.06	1.63 ± 0.06	1.62 ± 0.06
Weight, kg	67.2 ± 9.7	69.2 ± 11.3	69.0 ± 10.4
Body mass index (BMI), kg/m^2	25.1 ± 3.4	26.1 ± 4.2	26.3 ± 3.9
Systolic blood pressure, mmHg	132 ± 19	141 ± 21	145 ± 21
Diastolic blood pressure, mmHg	82 ± 11	83 ± 9	82 ± 10
Serum cholesterol, mmol/L	5.8 ± 0.9	7.0 ± 1.2	7.2 ± 1.3
Current smokers, %	37	28	24
Former smokers, %	27	36	31
Diabetes mellitus, %	1	4	6
Use of hormone replacement therapy, %	0.4	3	1
Use of thiazide diuretics, %	13	14	15
Use of loop diuretics, %	0.4	2	4
Cardiovascular disease history, %	1.7	3.4	3.5
Mild aortic calcification, %	11	16	23
Advanced aortic calcification, %	0.4	11	20
Metacarpal Cortical Area (MCA), mm^2	51.5 ± 6.6	49.2 ± 6.5	47.9 ± 6.5
Relative Cortical Area (RCA), %	81.2	75.2	71.7

Values are mean ± SD or percentages.

progression of aortic calcification during follow-up, women with progression of aortic calcification had a higher systolic blood pressure (136 versus 130 mmHg, respectively; P=0.03), a higher serum cholesterol level (6.2 versus 5.7 mmol/L, respectively; P<0.001), both adjusted for age, and smoked more (56% versus 31%, respectively; P=0.001) at baseline. No significant differences were seen in other cardiovascular disease risk factors.

Table 2. Bone loss according to progression of aortic calcification in 236 women premenopausal at baseline and going through menopause during follow-up

Bone loss	Aortic Calcification		*P*-value
	Progression (n=59)	No Progression (n=177)	
Change in MCA, mm^2*	-3.2 ± 0.4	-2.0 ± 0.2	0.01
Change in MCA, mm^2†	-3.5 ± 0.4	-2.0 ± 0.2	< 0.01
Change in RCA, %*	-7.2 ± 0.6	-5.6 ± 0.3	0.02
Change in RCA, %†	-7.5 ± 0.6	-5.5 ± 0.3	< 0.01

Values are mean ± SE.
MCA = metacarpal cortical area.
RCA = relative cortical area.
* Adjusted for age and years of follow-up.
† Adjusted for age, years of follow-up, age at menopause, body mass index at baseline, change in body mass index during follow-up, systolic blood pressure at baseline, change in systolic blood pressure during follow-up, smoking at baseline (never, former, or current), stopping and starting of smoking during follow-up, diabetes mellitus at baseline, diabetes mellitus developed during follow-up, and use of hormone replacement therapy, thiazide, and loop diuretics at baseline and at follow-up. Because of missing values, the number of subjects is not exactly the same.

Table 3. Bone mass and density according to aortic calcification in 720 postmenopausal women at follow-up

Bone measure	Aortic calcification			*P*-trend
	No (n=409)	Mild (n=167)	Advanced (n=144)	
MCA, mm^2*	48.1 ± 0.3	48.4 ± 0.5	46.4 ± 0.5	0.02
MCA, mm^2†	48.2 ± 0.3	48.5 ± 0.5	46.5 ± 0.5	0.04
RCA, %*	72.1 ± 0.4	71.5 ± 0.6	70.8 ± 0.6	0.06
RCA, %†	72.2 ± 0.4	71.7 ± 0.6	71.1 ± 0.6	0.15

Values are mean ± SE.
MCA = metacarpal cortical area.
RCA = relative cortical area.
* Adjusted for age.
† Adjusted for age, body mass index, systolic blood pressure, smoking (never, former, or current), diabetes mellitus, and use of hormone replacement therapy, thiazide, and loop diuretics at follow-up. Because of missing values, the number of subjects is not exactly the same.

Among women with progression of aortic calcification, the average loss of initial metacarpal bone mass was 6.1%; their average loss of initial metacarpal bone density was 8.9%. In women without progression of aortic calcification, these losses were 3.9% and 6.9%, respectively. Additional adjustment for potential confounding factors did not influence these results (Table 2), nor did additional adjustment for cardiovascular disease history (data not shown). In women already postmenopausal at baseline, there was no association between progression of aortic calcification and metacarpal bone loss during follow-up (data not shown).

We detected an inverse, graded, cross-sectional association between extent of aortic calcification and metacarpal bone mass and density in all postmenopausal women at follow-up, adjusted for age (Table 3). Again, additional adjustment for potential confounders did not influence the results (Table 3), nor did additional adjustment for cardiovascular disease history (data not shown).

Discussion

Our results show that during menopause, women with progression of aortic calcification lose more metacarpal bone than women without progression of aortic calcification. In postmenopausal women, a higher degree of aortic calcification is associated with a lower metacarpal bone mass and density.

When interpreting our results, some methodological issues should be taken into account. An advantage of the present study is the fact that the association between progression of aortic calcification and bone loss was studied during menopause, the period from which onward the prevalence of atherosclerosis and osteoporosis increases.[24,25] The prevalence of hormone replacement therapy use in our population was low, which was common in the Netherlands during the period the present study was conducted.[34] We measured aortic calcification radiographically. We assume this is intimal calcification, which is clearly distinguishable from medial calcification.[35] A limitation of our measurement of aortic calcification is the fact that it detected progression in a linear manner, whereas in fact it may have been circumferential. However, we assume that errors in the measurement of progression of aortic calcification and bone loss occurred randomly, which means that, if anything, we underestimated the association between progression of aortic calcification and bone loss. Although the density of calcification may be relevant with respect to plaque vulnerability and the subsequent onset of acute coronary events, the present study does not provide data on the density of calcification. No woman showed a decrease in the extent of aortic calcification. However, the fact that readers were aware of date of the

radiographs could have biased them against the detection of decreased calcification. Lack of information contributed to loss of data. We assume that the association between progression of aortic calcification and metacarpal bone loss will not differ between subjects with or without complete availability of data, making selection bias unlikely.

We are the first to describe an association between progression of atherosclerotic calcification and bone loss in women during menopause. The results of the present study are in line with those previous studies that showed cross-sectional associations between bone mineral density and aortic calcification,[3-6] carotid plaques,[7] and coronary calcification[8] among elderly women. Most of the reported studies, however, did not adjust for potential confounding factors apart from age.[3-5,8,21,22] Vogt et al[23] found an association between aortic calcification and bone mineral density at 2 of the 5 measured sites, which remained after adjustment for potential confounders. Two studies in elderly women found an adjusted association between bone mass and density at baseline and cardiovascular death[9] and mortality due to stroke[10] during follow-up.

Atherosclerotic calcification and bone mineralization show similarities. The mineral within calcified atherosclerotic plaques is hydroxyapatite, the same mineral found in bone,[11] and matrix vesicles, the initial nucleation sites for hydroxyapatite mineral in bone, are found in atherosclerotic lesions.[12] Calcifying vascular cells appear in many ways similar to osteoblasts,[13] and specific factors and proteins crucial to bone formation are also present within atherosclerotic lesions. The bone differentiation factor bone morphogenetic protein-2a has been found in atherosclerotic lesions,[14] and arterial calcification involves a variety of bone matrix proteins, such as type-I collagen,[15] and the noncollagenous proteins osteopontin[11] and osteocalcin.[16]

The association between progression of aortic calcification and bone loss during menopause may result from a common etiological factor, such as estrogen deficiency. Epidemiological data suggest that estrogen deficiency is a risk factor for cardiovascular disease and osteoporosis.[36,37] Arteries and bone are target organs for estrogen. Estrogen receptors have been demonstrated on vascular endothelial and smooth muscle cells,[38] osteoblasts,[39] and osteoclasts,[40] suggesting a direct effect of estrogen on vascular and bone cells. Whereas all subjects went through menopause, women with progression of aortic calcification had more bone loss than women without progression of aortic calcification, suggesting that there could be a difference in estrogen loss between subjects. On the other hand, it may not be estrogen deficiency per se, but sensitivity to estrogen deficiency (e.g., due to variability of the estrogen receptor gene)[41] that is the common etiologic factor.

Calcium-regulating hormones may be involved in the association between

vascular calcification and osteoporosis. Parathyroid hormone levels increase with aging.[42] Concurrently, estrogen deficiency is suggested to increase the sensitivity of the skeleton to parathyroid hormone[43] and to reduce intestinal calcium absorption.[44] Hyperparathyroidism, which can also be induced in the elderly by vitamin D deficiency, can on the one hand contribute to bone loss[45] and on the other hand add to soft tissue calcium deposition, in particular, vascular calcification.

Alternatively, it may not be calcification itself but progression of the underlying process of atherosclerosis that is associated with bone loss. Estrogen deficiency may have indirect effects on arteries and bone by the production of inflammatory agents, such as interleukin-1 and -6, and tumor necrosis factor,[46] which are involved in atherogenesis[47] and contribute to bone resorption.[48-50] Another common factor to explain the apparent association between atherosclerosis and bone loss may be the presence of oxidized lipids, which promote atherogenesis[51] and inhibit differentiation and mineralization of bone cells.[52] Plasma homocysteine is a cardiovascular risk factor that increases after menopause,[53] and osteoporosis is a common feature in patients with homocystinuria.[54] Although no association between homocysteine and bone density was found in a small group of postmenopausal women,[55] hyperhomocysteinemia might be involved in the association between atherosclerosis and osteoporosis.

In summary, our results indicate that progression of atherosclerotic calcification is associated with bone loss in women during menopause, suggesting a common etiologic factor.

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CHAPTER 5

General discussion

Despite the research that has been carried out in the past decade on cardiovascular disease in women, the gender gap in coronary heart disease occurrence is not completely understood until now.[1] The work presented in this thesis aims at gaining insight into gender specific issues of cardiovascular disease and the cause of the rising incidence of cardiovascular disease in women after middle age by studying putative endocrine and metabolic risk factors. The shortcomings and merits of the presented studies have been discussed in the previous chapters. In this chapter, the findings are placed in a broader context. Subsequently, some methodological considerations are discussed and views on further research regarding gender specific issues of cardiovascular disease are put forward.

Main findings

Classical risk factors for atherosclerosis

Metabolic syndrome

The insulin resistance syndrome attenuates the female advantage with regard to cardiovascular disease occurrence.[2,3] The etiology of the clustering of metabolic factors in the insulin resistance syndrome remains controversial. A common view is that insulin resistance, with its compensatory hyperinsulinemia, is the underlying mechanism.[4] Alternatively, abdominal obesity may be the primary defect of the clustering.[5] Our data described in chapters 2.2 and 2.3 and those of others[6-8] give support to the hypothesis that raised concentrations of proinflammatory cytokines, originating from various cells, and the resultant acute-phase response are an integral part of the metabolic clustering.[9] In follow-up studies, markers of inflammation have been shown to predict diabetes mellitus,[10,11] supporting a role for inflammation in diabetogenesis.

In addition to providing insight into the mechanism of diabetogenesis, the association between insulin resistance and inflammation may further illuminate a feature underlying the well-known relationship between insulin resistance and cardiovascular disease in which inflammation is thought to be crucial.[12] The association between inflammation and atherogenesis is supported by our finding that C-reactive protein (CRP), a marker of low-grade inflammation, is associated with carotid artery intima-media thickness in healthy, middle-aged women, as described in chapter 2.2. In these middle-aged women, carotid artery intima-media thickness explained much less of the variance of CRP than body mass index (BMI), a marker of adiposity, did. One explanation would be that in middle-aged women, in whom the burden of atherosclerosis is low, adipose tissue

contributes more to the inflammatory state than atherosclerosis. Also, the fact that BMI is likely to be more accurately measured than the burden of atherosclerosis, by measuring carotid artery intima-media thickness, may have contributed to this difference.

Obesity

Obesity is considered to be a risk factor for cardiovascular disease,[13] particularly among women.[14] The mechanism through which obesity adversely affects atherogenesis is primarily thought to be due to the effects of the adverse risk factor profile associated with obesity, such as elevated blood pressure, blood lipids, and blood glucose. Our results in middle-aged women as described in chapter 2.2 and those of others[15] indicate that adiposity is strongly related to low-grade inflammation, suggesting an additional mechanism through which adiposity adversely affects cardiovascular disease risk. In our study population, of all indices of adiposity waist circumference showed the strongest relationship with CRP, suggesting that abdominal fat deposition is most important in inducing inflammation.

The association between obesity and cardiovascular disease has been found to be less pronounced among smoking women than among nonsmoking women.[16-19] The dilution of the association between body weight and cardiovascular disease among smokers is often ascribed to the weight-lowering effect of smoking.[20] In the study described in chapter 2.1 among postmenopausal women, we studied an alternative hypothesis, being that the atherogenic effect of smoking may be different among subjects with lower compared with those with higher body weight. We observed that the association between cigarette smoking and progression of atherosclerosis is stronger in lower-weight than in higher-weight postmenopausal women. Our results are in agreement with data showing that lower-weight older women are at increased cardiovascular disease mortality risk[21] and suggest that adipose tissue in postmenopausal women may not only exert hazardous atherogenic effects. We hypothesized that among smoking postmenopausal women with higher body weight the antiestrogenic effects of smoking[22] may, at least partly, be counteracted by endogenous estrogen retrieved from aromatization of adrenal androgens in adipose tissue.[23,24] The extent to which our results are generalizable to cardiovascular events and mortality needs to be determined.

Menopause, sex steroids, and cardiovascular disease risk

Menopause

The assumption that the higher incidence of cardiovascular disease among older

women is due to menopause is long and widely held but still debated.[25-28] Data on the association between menopause and cardiovascular disease are inconsistent. Several studies have shown an inverse association between age at natural menopause and risk of cardiovascular disease,[29-34] whereas others have not.[35-38] The inconsistency of results of studies on the association between age at menopause and cardiovascular disease may be due to a methodological problem, being a lag time of at least 10 years between menopause and the occurrence of coronary heart disease in women, which makes the effect of menopause difficult to disentangle from the effect of age. On the other hand, many studies did not examine the association between age at menopause and cardiovascular disease by smoking status. Smoking may seriously confound this association because it is strongly related to early menopause[39] and increases the risk of cardiovascular disease. In the Nurses' Health Study, the association between younger age at menopause and higher risk of coronary heart disease was found to be present among current and past smokers, but not among never smokers.[40] This observation, however, was based on only a small number of coronary heart disease cases.

Menopause and cholesterol

Cholesterol is the primary cardiovascular risk factor affected by menopause[41-64] with a wide variation in change. Our results in a population-based study among women experiencing natural menopause as described in chapter 3.1, show that the increase in cholesterol level with menopause is 30% lower in women with the APOE2E3 genotype when compared with women with the most commonly occurring APOE3E3 genotype. These results indicate that the APOE genotype contributes to the variation in change in cholesterol with menopause. The variation in increase in cholesterol with menopause is far from completely explained by the APOE genotype. Other factors, such as density or type of estrogen receptors, which mediate the activation of the hepatic lipoprotein receptors in the liver,[55] may be involved in the increase in cholesterol with menopause.

It seems reasonable to speculate that the amount of change of cholesterol with menopause would have an impact on the development or progression of atherosclerosis and cardiovascular disease. In the Healthy Women Study, the amounts of coronary and aortic atherosclerosis measured shortly after menopause were not found to be related to changes in levels of low-density lipoprotein (LDL) cholesterol with menopause.[56] However, a longer follow-up time may be necessary for effects of higher cholesterol levels on atherogenesis to become detectable. Although after menopause women reach higher levels of cholesterol than men, the female advantage with regard to cardiovascular disease occurrence is not erased. This may be attributable to the fact that women have a

larger and less atherogenic LDL particle size than men.[57] Despite higher levels, women may therefore be relatively protected against the atherogenic consequences of increasing cholesterol levels.

Menopause and homocysteine

Apart from the increase in cholesterol level, the mechanisms through which menopause might exert its effect on the cardiovascular system remain largely unknown. In a meticulously selected population of age-matched premenopausal and postmenopausal women, we found that homocysteine levels were 7% (0.8 μmol/L) higher in postmenopausal women than in premenopausal women (chapter 3.2), proposing an additional mechanism through which menopause may adversely affect cardiovascular disease risk. Boushey et al[58] estimated an increase in homocysteine level of 5 μmol/L to be associated with an odds ratio of 1.8 for coronary artery disease in women, indicating that an increase in homocysteine level of 0.8 μmol/L with menopause does exert only a small effect on coronary artery disease risk. However, throughout the analyses of Boushey et al[58] conservative assumptions were used, indicating that the true effect of homocysteine on coronary artery disease risk may be larger. Furthermore, when comparing homocysteine levels between cardiovascular disease cases and controls in The European Concerted Action Project, homocysteine levels were only 1.5 μmol/L higher in cases than in controls.[59]

Hyperhomocysteinemia is considered to be an independent risk factor for atherosclerotic vascular disease.[60] Although the association between homocysteine levels and cardiovascular disease is biologically plausible[61] and generally strong in cross-sectional and retrospective case-control studies, the data from prospective studies are less consistent.[62,63] Possibly, homocysteine may be predominantly a marker of atherosclerosis or a late-stage predictor of cardiovascular disease, as suggested by others.[64] Currently, randomized trials are in progress, also among women,[65] to test whether lowering homocysteine levels by folic acid and vitamin B supplementation will decrease risks of cardiovascular disease. Although results of these trials will not prove that homocysteine is a cardiovascular risk factor, the public health implications of the trial results may be very important given the simplicity and low cost of vitamin therapy.[64]

Hormone replacement therapy: estrogens

Results from observational studies indicate that estrogen therapy reduces cardiovascular morbidity and mortality risk in postmenopausal women by as much as 40%.[66-68] A problem when studying the effect of estrogen suppletion in observational studies, however, is selection bias because healthier women tend to use hormones, which may explain the apparently protective effect of oral estrogen

on cardiovascular disease.[69] Many randomized controlled trials on effects of hormone suppletion on cardiovascular disease risk factors have been conducted, of which the Postmenopausal Estrogen/Progestin Interventions (PEPI) Trial is one of the largest and most famous.[70] The most consistent reported findings of these trials were favorable effects of hormone suppletion on the lipid profile. In the Romeo trial (chapter 3.3), we found that hormone replacement therapy is associated with a small decrease in homocysteine levels in perimenopausal women.

Contrary to the expectation of most, however, the first randomized trials on secondary prevention of coronary heart disease, the Heart and Estrogen/Progestin Replacement Study (HERS),[71] or coronary atherosclerosis, the Estrogen Replacement and Atherosclerosis trial (ERA),[72] did not show cardioprotective effects of hormone replacement therapy. Results of these trials may indicate that the bias in observational studies is larger than thought until now. The discrepancy with the results from observational studies may also be due to the fact that the cardiovascular benefits may emerge only after several years of hormone replacement therapy.[71,73] Therefore, subjects susceptible to adverse atherothrombotic effects of hormone replacement therapy are not detected in observational studies. Furthermore, in the HERS[71] and ERA[72] progestins were part of the therapy regimens, whereas results from observational studies are mainly based on the use of unopposed estrogen, which may exert stronger cardiovascular protective effects as supported by results from a recently conducted trial.[74] Also, the HERS[71] and ERA[72] were conducted in women with documented coronary heart disease and hormone replacement therapy was initiated late, an average of 20 years after the cessation of menses. It can therefore not be ruled out that hormone replacement therapy is effective in preventing the development of atherosclerotic disease. Final answers have to come from primary prevention trials that are currently being carried out.[75,76] The data available up to date do not justify the initiation of use of hormone replacement therapy for the secondary prevention of cardiovascular disease.[77]

Hormone replacement therapy: health effects

Hormone replacement therapy relieves postmenopausal vasomotor and genitourinary symptoms.[78-80] Long-term use of postmenopausal hormone therapy is advocated for prevention of disease and prolongation of life. Next to potential effects on the occurrence of cardiovascular disease, the effects on other disease outcomes such as potential favorable effects on the prevention of fractures[81,82] and adverse effects on breast cancer[83] have to be taken into account when estimating the health effects of long-term postmenopausal hormone replacement therapy use. Using effect estimates of hormone replacement therapy from observational studies (a protective effect on cardiovascular disease, a favorable effect

on the prevention of fractures, and an adverse effect on breast cancer) Dutch women from the general population are expected to achieve only a modest gain in life expectancy by using hormones for 10 or 20 years following menopause.[84] The potential gain in life expectancy to be achieved by postmenopausal hormone therapy use is modest because of the low incidence of coronary heart disease and hip fracture in relation to the relatively high incidence of breast cancer in Dutch women in the first 2 decades following menopause.[84] Among American women, the lifetime use of hormones is expected to exert a more favorable, albeit still limited, effect.[67] The different findings in Dutch and American women arise from differences in relative frequencies of cardiovascular disease and breast cancer in the Dutch and American population.[85] In the described modeling studies,[67,84] no data on potential effects of hormone replacement therapy on dementia[86,87] and colon cancer[88] are incorporated. If these diseases turn out to be favorably affected by hormone replacement therapy, the answer to the question whether or not treating women with hormone replacement on a long-term basis has favorable health effects may sound more positive. However, even then many questions, such as what is the optimal age to start therapy and how long should treatment be continued, will be left unanswered.

Hormone replacement therapy: androgens

Androgen therapy is considered to be an attractive treatment modality to potentially benefit psychological well being, body composition, and strength in the elderly.[89-91] Inclusion of androgens in postmenopausal hormone replacement regimens is not uncommon and is likely to become more widespread.[92] Until now, however, no data are available on its long-term effects. Because of the fact that in the Netherlands from the late 1950s until 1980s a substantial part of the hormones indicated for menopausal complaints consisted of intramuscularly administered combined estrogen-testosterone therapy, we had the opportunity to study effects of androgen suppletion on atherosclerosis in postmenopausal women. The results of our study described in chapter 3.4 suggest that testosterone therapy may adversely affect atherosclerosis in postmenopausal women and indicate that androgen replacement in these women may not be harmless. Although our results are based on observational data, the "healthy women effect"[69] would only have diluted the positive association found in our study, implying that the adverse effect of testosterone in women may even be stronger than our results suggest. The finding in our study, however, is based on intramuscularly administered high-dose testosterone, a mode of administration that is currently not being used anymore. The mode of administration may be crucial for the effect of testosterone on cardiovascular disease risk factors, such as lipids. Therefore, the extent to which our results are generalizable to the effects

of new preparations developed specifically for women needs to be determined.

Endogenous hormone levels

Endogenous hormone levels have been suggested to contribute to cardiovascular disease pathofysiology exerting opposite effects in the sexes: in women estrogens were hypothesized to protect against cardiovascular disease and androgens were thought to exert adverse cardiovascular effects, whereas in men the associations were hypothesized to be the other way around.[93] Ecological studies do not provide support for the hypothesis that endogenous estrogen levels protect against cardiovascular disease in women: Japanese women exhibit the lowest coronary heart disease rates in the world and also have the lowest endogenous estrogen levels.[94] Estrogen levels have also not been found to be related to cardiovascular mortality in women.[95] Only recently, however, assays with extremely low detection limits suitable for measuring estrogen levels in the low postmenopausal range have become available. When using this assay, neither we found an association between endogenous estrogen levels and atherosclerosis among postmenopausal women (unpublished results). The fact that until now no association has been found between estrogen level and cardiovascular disease in postmenopausal women may be due to the fact that estrogen exposure is not accurately reflected in a single measurement of its level and a potential deterioration of hormone values with prolonged storage.[96] When we used bone density as a marker of cumulative estrogen exposure, we found an association between 'estrogen' (=bone density) and atherosclerosis in postmenopausal women and in women experiencing natural menopause (chapter 4.2), which does support the hypothesis. Another possibility is that exposure to estrogen in premenopausal years may be more important in the association with cardiovascular disease, analogous with the finding that risk factors evaluated premenopausally are powerful predictors of atherosclerosis measured after menopause.[97] However, not much data are currently available regarding this hypothesis.

In population studies, endogenous androgen levels were not found to be related to cardiovascular events in men[96,98-102] or women.[95,102] Results of several studies on endogenous androgens and atherosclerosis have been inconsistent.[103-107] Our data described in chapter 3.5 show that higher endogenous testosterone levels in men are associated with a lower prevalence of aortic atherosclerosis and a lower risk of aortic atherosclerotic progression. The apparent discrepancy between our results and the results of studies in which no association between endogenous testosterone levels and coronary heart disease in men was reported[96,98,100] may be attributable to the fact that we studied nonsmok-

ers only and to the fact that the aorta might be more vulnerable to the effects of endogenous sex steroids than other arteries. Aortic atherosclerosis has been found to be associated with an up to 9-times increased risk of ischemic stroke[108] indicating its importance in relation to cardiovascular disease. Mechanisms possibly involved in the association between aortic atherosclerosis and stroke may be pulse pressure or emboli being released from atherosclerotic lesions in the aortic arch. The fact that in our male study population we also found a protective effect of higher endogenous androgen levels for the occurrence of strokes (unpublished results) supports this inference. Until now, it is unclear whether testosterone is causally involved in atherogenesis. Possibly, higher levels of testosterone do not protect against atherosclerosis in men, but are merely a marker of good health.[109] In women participating in our study (chapter 3.5), higher levels of testosterone tended to be positively associated with aortic atherosclerosis, which was largely accounted for by adverse cardiovascular disease risk factors, such as diabetes mellitus. The hyperandrogenicity in postmenopausal women with diabetes mellitus has been described before.[110] This association may provide insight into the mediation of cardiovascular consequences of diabetes in women, as has been suggested previously.[111]

Thus far, our results on endogenous androgen levels (chapter 3.5) and high-dose testosterone suppletion (chapter 3.4) indicate that androgens may lead to adverse cardiovascular disease risk factors and atherosclerosis in women, whereas in men higher endogenous androgen levels may protect against aortic atherosclerosis (chapter 3.5).

Alternative endocrine cardiovascular disease risk factors

Subclinical hypothyroidism

In the Netherlands, the use of thyroid supplementation is very low as opposed to the high frequency of its usage in the USA. Therefore, we were able to study the association between subclinical hypothyroidism[112] and cardiovascular disease in women participating in the Rotterdam Study. Our results indicate that subclinical hypothyroidism is a strong risk indicator for atherosclerosis and myocardial infarction in elderly women, as described in chapter 4.1. A limitation of our study is the cross-sectional nature of its design, which limits the validity of causal interpretation of our results. However, the relative risk for women with subclinical myocardial infarction in the prospective part of our study was similar to the point estimate in the cross-sectional part of our study, although it did not reach statistical significance. Additional research has to be done to determine whether the association between subclinical hypothyroidism and atherosclerosis and myocardial infarction can be confirmed in a prospective study.

Subclinical hypothyroidism is highly prevalent in postmenopausal women[113,114] and the relative risk associated with myocardial infarction as derived from our data is substantial. The population attributable risk percentage for subclinical hypothyroidism with myocardial infarction is therefore considerable and estimated to be within the range of that for known major risk factors for cardiovascular disease, as described in chapter 4.1. Besides, subclinical hypothyroidism is known to progress to overt hypothyroidism with a rate of 5-15% per year, the rate being highest in women in whom thyroid antibodies are present.[115] The manifestations of overt hypothyroidism vary considerably among patients and therefore it is often not recognized. The serum thyroid stimulating hormone (TSH) assay is considered to be an accurate diagnostic test for hypothyroidism, although this point of view is not universally shared.[116] Furthermore, effective treatment therapies are available for thyroid dysfunction. The enumerated issues indicate that thyroid dysfunction meets many criteria justifying population screening,[117] as is already advocated in the USA in women aged 35 years and over.[118] In this recommendation,[118] the possible association of subclinical hypothyroidism with cardiovascular disease was not considered yet.

Bone loss

Next to cardiovascular disease, osteoporosis is a common cause of morbidity and mortality in postmenopausal women.[119] Among women experiencing natural menopause, we found the progression of atherosclerotic calcification to be associated with increased bone loss, as described in chapter 4.2. Moderate calcification of plaques is thought to contribute to vascular morbidity and mortality,[120,121] indicating its importance in the pathofysiology of cardiovascular disease. More insight into the causes and consequences of vascular calcification with regard to coronary heart disease will be provided by studies using noninvasive measurement of calcification in the coronary arteries, such as electron-beam computed tomography.

The association between progression of atherosclerotic calcification and bone loss during menopause may provide insight in the pathofysiology of these diseases and provide clues for prevention and treatment. Interestingly, bisphosphonates, which are used for the treatment of osteoporosis, appear to prevent deposition of calcium in arterial walls in animal experiments,[122,123] suggesting that treatment with bisphosphonates may favorably affect atherosclerotic calcification. On the other hand, changes in vascular wall calcification may render some plaques more prone to rupture and lead to an increased risk of cardiovascular events during bisphosphonate treatment.[124] Reviewing cardiovascular events in a large database from trials evaluating risedronate in the treatment and prevention of postmenopausal and corticosteroid-induced osteoporosis

revealed no evidence that risedronate influences the occurrence of cardiovascular disease,[125] however, studies thus far were not designed to examine the effect of bisphosphonate therapy on cardiovascular disease rates.

Alternatively, it may not be calcification itself but the underlying process of atherosclerosis that is associated with bone loss. A common etiologic factor, such as estrogen deficiency or sensitivity to estrogen deficiency due to variations in the density or type of estrogen receptors, may be involved in the association between atherosclerosis and osteoporosis. Furthermore, statins, which are used for lipid lowering, have been reported to promote bone formation.[126] Triggered by this finding, observational studies followed,[127-130] which found that bone mineral density was increased[130] and fracture incidence was reduced in subjects taking statins. Reanalysis of randomized controlled trial data from a trial designed to address cardiovascular outcomes, however, found no effect of statins on fracture risk.[131] However, only 17% of subjects in this trial were women. A subsequent observational study found no effect of statins on fracture incidence either[132] and a recent study in rats even indicated that statins might inhibit bone formation and produce a net reduction in bone density.[133] Until now, the effects of lipids and statins on bone and fracture risk are not elucidated yet.

Methodological considerations

The methodological considerations of the presented studies have been discussed in the chapters 2, 3, and 4. In the current paragraph, two methodological issues regarding risk estimation in cardiovascular disease research arising from the fact that the risk of coronary heart disease in women lags 10 years behind the risk in men[134] are discussed.

The first issue to be discussed is the effect of the difference in absolute coronary heart disease risk between the sexes on measures of effect. It has been suggested that the relative risk associated with smoking for myocardial infarction is higher in women than in men.[135] The described differentiation of results by sex may be a biological phenomenon, thus providing insight in the etiology of cardiovascular disease, but may also merely reflect the lower absolute risk of cardiovascular disease in women because magnitudes of relative risks are heavily dependent upon the baseline risk of the disease. Also, interaction effects are dependent on the baseline risk. Therefore, the same issue arises when the joint effects of cardiovascular risk factors are compared between the sexes. A large European collaborative project studied interaction effects of homocysteine and classical cardiovascular disease risk factors and found the joint effect of these factors to be most pronounced in women.[59] Again, the difference in effect

between the sexes may be a biological phenomenon or may reflect the lower absolute risk of cardiovascular disease in women. To validly study whether differences in the magnitude of relative risks or interaction effects between the sexes are due to differences in absolute risks, one has to take into account the difference in "cardiovascular age" between the sexes.

The second issue to be discussed is the fact that because of the difference in absolute risk of coronary heart disease between the sexes it is a standard approach in cardiovascular research to stratify analyses by sex, which is a base for potential problems. It may lead to false positive findings of differences between the sexes, especially when the difference in absolute risk between the sexes is not taken into account correctly, as described previously. False positive findings of differences between the sexes may also arise when no a priori hypothesis underlies the stratification, or when only few subjects are available for analyses and effects are more likely to be due to chance. The loss of power due to stratification by sex becomes especially problematic when interaction, towards which attention shifts in recognition of the complex multicausal etiology of cardiovascular disease, is the topic of interest. This issue arises particularly in research regarding genetic factors, in which gene-gene and gene-environment interaction is a key issue.[136,137] Instead of habitually stratifying cardiovascular analyses by sex, it may be worthwhile to consider the usefulness of this approach.

Future research

The last decade, much effort has been put in describing and studying cardiovascular disease in women. Large population-based studies in women such as the Nurses' Health Study,[138] the Women's Health Study,[139] and the Healthy Women Study,[44] and population-based studies in which women participate such as the Cardiovascular Health Study,[140] the Atherosclerosis Risk In Communities Study,[141] the Rotterdam Study,[142] and randomized controlled trials[71,72] have provided information on cardiovascular disease in women. In the current paragraph, views on future research regarding gender specific issues of cardiovascular disease are given.

Effects of classical cardiovascular disease risk factors are generally similar in men and women, with the exception of diabetes mellitus[143,144] and the insulin resistance syndrome,[2,3] which attenuate the female advantage. Factors possibly contributing to gender differences in cardiovascular disease and requiring further attention are LDL particle size, which is smaller and therefore less atherogenic in women,[57] abdominal fat accumulation,[145] and isolated systolic

hypertension, the prevalence of which is higher in women than in men.[146]

Until now, no solid proof of the hypothesis that estrogens would protect against atherogenesis in women is available. The first randomized trials on secondary preventive effects of hormone replacement therapy on cardiovascular disease did not find clear evidence for cardiovascular protection.[71,72] Results from primary prevention trials have to be awaited.[75,76] However, even if these trials indicate that hormone replacement therapy exerts cardioprotective effects, the health effect of long-term postmenopausal hormone replacement therapy use is expected to be limited.[67,84] Possibly, selective estrogen receptive modifiers are more effective in the prevention of cardiovascular disease in women. The Raloxifene Use for The Heart study[147] will provide information about the applicability of these preparations in preventing coronary heart disease. The question whether the decline in estrogen levels with menopause contributes to the rising incidence of cardiovascular disease is still unanswered[25-38,40] and will remain hard to answer because of the problem that no control population of women staying premenopausal is available. No association between endogenous estrogen levels and cardiovascular events in postmenopausal women has been found until now.[94,95] However, the effect of susceptibility to estrogen because of genetic variations in the density or type of estrogen receptors[148] and its interaction with estrogen levels need further clarification.

Other factors possibly contributing to the rising incidence of cardiovascular disease in women after middle age may be autoimmune diseases through immune-complex mediated vascular damage. We did not find an association between thyroid autoimmunity and cardiovascular disease, but more research should be carried out on the association between autoimmunity and cardiovascular disease. In postmenopausal women, iron stores increase and the recent evidence that heterozygosity for the hemochromatosis gene increases the risk of cardiovascular disease[149] brings new life to the hypothesis that increased iron stores are associated with cardiovascular disease. The "iron hypothesis" may ask for further evaluation as a possible explanation for the rising incidence of cardiovascular disease in women after middle age.

The effect of the gender gap in absolute coronary heart disease risk on differences in magnitudes of relative risks and interaction effects between the sexes needs attention. Furthermore, it may be worthwhile to consider the usefulness of the standard approach in cardiovascular research to stratify analyses by sex, especially in the coming era in which gene-gene and gene-environment interactions will be major topics of interest.[136,137]

Next to etiologic research on gender specific issues of cardiovascular disease, attention for sex specific aspects of diagnosis, treatment, and prevention is essential. Research on gender differences in the access to diagnostic services

and treatment of heart disease is being conducted.[150-153] Efforts to increase the participation of women in cardiovascular randomized controlled trials should be continued because women, particularly elderly women, remain underrepresented in cardiovascular trials relative to their disease prevalence.[154,155] However, possibly the most important issue in ultimately lowering cardiovascular disease occurrence in women is to increase awareness among women. Women in Western countries do still not fully recognize that myocardial infarction is their leading cause of death.[156-158] Results from an American Heart Association survey show that most women believe cancer is their greatest health threat and only less than 10% of women perceive heart disease as their greatest threat.[158] The awareness gap calls for translation of the evidence that cardiovascular disease is the major health threat for women to the public.

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CHAPTER 6

Summary / Samenvatting

6.1

Summary

THE FACT THAT cardiovascular disease is the major cause of morbidity and mortality in women has been recognized already for many years and the last decade much effort has been put in describing and studying cardiovascular disease in women. Until now, it is not completely understood why the incidence in cardiovascular disease in women rises after middle age and why coronary heart disease occurrence remains lower in women than in men at all ages. The work presented in this thesis aims at gaining insight into gender specific issues of cardiovascular disease and the cause of the rising incidence of cardiovascular disease in women after middle age by studying putative endocrine and metabolic risk factors. Data from various population-based studies were used to study these issues.

In **chapter 2**, studies on classical cardiovascular disease risk factors attenuating the female advantage with regard to cardiovascular disease occurrence are presented. Obesity is considered to be a risk factor for cardiovascular disease, particularly among women. The association between obesity and cardiovascular disease has been found to be less pronounced among smoking women than among nonsmoking women, which is often ascribed to the weight-lowering effect of smoking. In the study described in **chapter 2.1**, we studied an alternative hypothesis, being that the atherogenic effect of smoking may be different among women with lower compared with those with higher body weight. In 1680 postmenopausal women participating in the population-based Rotterdam Study, we observed that in lower-weight women (BMI < median) smoking was associated with an odds ratio of 4.3 (95% CI, 2.5-7.2) for any progression of aortic atherosclerosis, whereas in their higher-weight counterparts (BMI ≥ median) the odds ratio was 2.1 (CI, 1.2-3.8) during 6.5 years of follow-up (*P* for interaction < 0.05). These results indicate that the association between cigarette smoking and progression of atherosclerosis is stronger in lower-weight than in higher-weight women and suggest that adipose-tissue derived estrogen may ameliorate the atherogenic effects of smoking in postmenopausal women.

The insulin resistance syndrome attenuates the female advantage with regard to cardiovascular disease occurrence. Until now, the etiology of the clustering of metabolic factors in the insulin resistance syndrome remains controversial. Recent data suggest that the insulin resistance syndrome may be accompanied by an increased acute-phase response. We studied this hypothesis in two study populations. In the study described in **chapter 2.2**, we examined the relationship of C-reactive protein (CRP), a marker of low-grade inflammation, with variables of the insulin resistance syndrome among 186 healthy middle-aged women from the general population. In the study described in **chapter 2.3**, we examined the relationship of insulin resistance (measured by post-load insulin)

with several markers of inflammation among 574 nondiabetic elderly men and women participating in the Rotterdam Study. In both studies, low-grade inflammation was strongly associated with measures of insulin resistance. These results give support to the hypothesis that the acute-phase response is an integral part of the metabolic clustering of the insulin resistance syndrome.

Chapter 3 contains studies on sex specific determinants of cardiovascular disease with a focus on sex steroids. Menopause is thought to be a major determinant of the increase in cardiovascular disease incidence among women after middle age. In **chapter 3.1** and **chapter 3.2** studies on associations between natural menopause and cardiovascular disease risk factors are presented. Cholesterol is the primary cardiovascular risk factor affected by menopause with a wide variation in change. Until now, it is not known why some women have no or only a slight increase in cholesterol level, whereas others exhibit a large cholesterol increase. In the study described in **chapter 3.1**, we studied whether the apolipoprotein E (APOE) genotype can explain differences in the increase in cholesterol level with menopause. In 1116 women participating in the population-based Eindhoven Perimenopausal Osteoporosis Study and experiencing natural menopause during 5.9 years of follow-up, we found that the increase in cholesterol level with menopause is 30% lower in women with the APOE2E3 genotype when compared with women with the most commonly occurring APOE3E3 genotype. The variation in increase in cholesterol with menopause, however, is far from completely explained by the APOE genotype. Apart from the increase in cholesterol level, the mechanisms through which menopause might exert its effect on the cardiovascular system remain largely unknown. In the study described in **chapter 3.2**, we measured plasma homocysteine levels in a meticulously selected population in which the contrast in estrogen status between premenopausal and postmenopausal women of the same age was maximized. The study comprised 93 premenopausal and 93 postmenopausal women of similar age (range 43 to 55 year) selected from respondents to a mailed questionnaire, which was sent to all women aged 40 to 60 years in the Dutch town of Zoetermeer (n=12,675). In this study population, we found that homocysteine levels were 7% (0.8 μmol/L) higher in postmenopausal women than in premenopausal women, proposing an additional mechanism through which menopause may adversely affect cardiovascular disease risk.

Hormone replacement therapy has been suggested to exert anti-atherogenic effects. In the study described in **chapter 3.3**, we studied the effects of hormone replacement therapy on homocysteine levels in a randomized intervention trial. The Romeo trial is a single center randomized placebo-controlled

trial, conducted to assess the effect of a sequential combined regimen of oral 17β-estradiol and desogestrel ($17\beta E_2$-D) and a combination of conjugated equine estrogens and norgestrel (CEE-N) compared with placebo on cardiovascular disease risk factors in 121 perimenopausal women. Our results show that after 6 months of therapy, the difference in serum homocysteine levels between women receiving $17\beta E_2$-D and placebo was -6.3% (CI, -12.4%; 0.0%). The difference between women receiving CEE-N and placebo was -10.1% (CI, -16.7%; -2.9%). These results indicate that hormone replacement therapy is associated with a small decrease in homocysteine levels in perimenopausal women. Despite favorable effects of hormone replacement therapy on cardiovascular disease risk factors, however, the first randomized trials on secondary preventive effects of hormone replacement therapy on cardiovascular disease did not find clear evidence for cardiovascular protection. Results from primary prevention trials have to be awaited.

Androgen treatment in postmenopausal women is considered to be an attractive treatment modality to potentially benefit psychological well-being and bone mass. Inclusion of androgens in postmenopausal hormone replacement regimens is not uncommon and is likely to become more widespread. Until now, however, no data are available on its long-term effects. In the Netherlands, intramuscularly administered high-dose estrogen-testosterone therapy (estradiol and testosterone esters) used to be frequently prescribed for menopausal complaints from the late 1950s until 1980s. Self-reported data on intramuscularly administered high-dose estrogen-testosterone therapy and data on aortic atherosclerosis were available in the population-based EPOZ study (Epidemiological Preventive Organization Zoetermeer). Using these data, we found that intramuscular testosterone therapy-use for 1 year or longer was associated with an odds ratio of 3.1 (CI, 1.1-8.5) for severe aortic atherosclerosis in 513 naturally postmenopausal women aged 54 to 67 years, as described in **chapter 3.4**. This result suggests that testosterone therapy may adversely affect atherosclerosis in postmenopausal women and indicate that androgen replacement in these women may not be harmless.

Endogenous hormone levels have been suggested to contribute to cardiovascular disease pathofysiology exerting opposite effects in the sexes: in women androgens were thought to exert adverse cardiovascular effects, whereas in men androgens were hypothesized to protect against cardiovascular disease. Until now, however, endogenous androgen levels have not been found to be related with cardiovascular events in either men or women, whereas results of studies on endogenous androgen levels and atherosclerosis have been inconsistent. In the study described in **chapter 3.5**, we studied the association between endogenous androgen levels and aortic atherosclerosis in 1032 nonsmoking

elderly men and women participating in the Rotterdam Study. Our results show that relative to men with levels of total and bioavailable testosterone in the lowest tertile, men with levels of these hormones in the highest tertile had odds ratios of 0.4 (CI, 0.2-0.9) and 0.2 (CI, 0.1-0.7), respectively, for the presence of severe aortic atherosclerosis. Men with levels of total and bioavailable testosterone in subsequent tertiles were also protected against progression of aortic atherosclerosis measured after 6.5 years of follow-up. In women, positive associations between levels of testosterone and aortic atherosclerosis were largely due to adverse cardiovascular disease risk factors. Levels of dehydroepiandrosteronesulphate were not associated with aortic atherosclerosis, neither in men nor in women. The apparent discrepancy between our results and the results of studies in which no association between endogenous testosterone levels and coronary heart disease in men was reported may be attributable to the fact that we studied nonsmokers only and to the fact that the aorta might be more vulnerable to the effects of endogenous sex steroids than other arteries. Aortic atherosclerosis has been found to be associated with an increased risk of ischemic stroke, possibly through pulse pressure or emboli being released from atherosclerotic lesions in the aortic arch, indicating its importance in relation to cardiovascular disease.

In **chapter 4**, studies on alternative endocrine cardiovascular disease risk factors in postmenopausal women are described. Overt hypothyroidism has been found to be associated with cardiovascular disease. In the study described in **chapter 4.1**, we studied the association between subclinical hypothyroidism and thyroid autoimmunity and cardiovascular disease in a random sample of 1149 postmenopausal women participating in the Rotterdam Study. We found that subclinical hypothyroidism was associated with a greater prevalence of aortic atherosclerosis (odds ratio 1.7 [CI, 1.1-2.6]) and myocardial infarction (odds ratio 2.3 [CI, 1.3-4.0]). Associations were slightly stronger in women who had subclinical hypothyroidism and antibodies to thyroid peroxidase, whereas no association was found between thyroid autoimmunity itself and cardiovascular disease. A limitation of our study is the cross-sectional nature of its design, which limits the validity of causal interpretation of our results. However, the relative risk for myocardial infarction in women with subclinical hypothyroidism in the prospective part of our study was similar to the point estimate in the cross-sectional part of our study, although it did not reach statistical significance. The population attributable risk percentage for subclinical hypothyroidism associated with myocardial infarction as computed from our data was within the range of that for known major risk factors for cardiovascular disease.

Next to cardiovascular disease, osteoporosis is a common cause of morbidity

and mortality in postmenopausal women. In the study described in **chapter 4.2**, we studied the association between progression of aortic atherosclerotic calcification and metacarpal bone loss among women after middle age by using data from the EPOZ study. In 236 women experiencing natural menopause during 9 years of follow-up, the average loss of metacarpal bone mass among women with progression of aortic calcification was 3.2 mm^2 and their loss of metacarpal bone density was 7.2%, whereas in women without progression of aortic calcification, these losses were 2.0 mm^2 and 5.6%, respectively ($P<0.05$). In a cross-sectional analysis in 720 postmenopausal women, we found a graded, inverse association between the extent of aortic calcification and metacarpal bone mass and density. The association between atherosclerotic calcification and bone loss may provide insight in the pathofysiology of these diseases. A common etiologic factor, such as estrogen deficiency or sensitivity to estrogen deficiency due to variations in the density or type of estrogen receptors, may be involved in the association between atherosclerosis and osteoporosis.

In **chapter 5**, the general discussion, the results described in this thesis are placed in a broader context. Two methodological considerations are discussed, being the effect of the gender gap in the occurrence of coronary heart disease on differences in magnitudes of relative risks and interaction effects between the sexes, and the standard approach in cardiovascular research to stratify analyses by sex. In our views on future research regarding gender specific issues of cardiovascular disease we give some suggestions for further etiologic research. Furthermore, we discuss that attention for sex specific aspects of diagnosis and treatment of cardiovascular disease is essential and stress that among women the awareness should be increased that cardiovascular disease is their major health threat.

6.2

Samenvatting

HET FEIT DAT hart- en vaatziekten de belangrijkste oorzaak zijn van ziekte en sterfte onder vrouwen is reeds vele jaren bekend en de laatste tien jaar is veel onderzoek gedaan naar hart- en vaatziekten bij vrouwen. Tot op heden is echter niet volledig duidelijk waardoor de incidentie van hart- en vaatziekten bij vrouwen toeneemt na middelbare leeftijd en waardoor, ondanks deze toename, coronaire hartziekten tot op hoge leeftijd minder vaak vóórkomen bij vrouwen dan bij mannen. Het werk gepresenteerd in dit proefschrift heeft tot doel inzicht te verschaffen in geslachtsspecifieke kenmerken van hart- en vaatziekten en de oorzaak van de stijgende incidentie van hart- en vaatziekten bij vrouwen na middelbare leeftijd middels het bestuderen van mogelijke endocriene en metabole risicofactoren. Voor het bestuderen van deze onderzoeksvragen werden gegevens uit verschillende populatieonderzoeken gebruikt.

In **hoofdstuk 2** worden studies gepresenteerd betreffende klassieke cardiovasculaire risicofactoren welke het vrouwelijke voordeel ten aanzien van het vóórkomen van hart- en vaatziekten deels teniet doen. Overgewicht wordt beschouwd als een risicofactor voor hart- en vaatziekten, met name bij vrouwen. De relatie tussen overgewicht en hart- en vaatziekten is minder sterk bij rokende vrouwen dan bij niet-rokende vrouwen, hetgeen vaak wordt toegeschreven aan het gewichtsverlagende effect van roken. In de studie beschreven in **hoofdstuk 2.1** onderzochten we een alternatieve hypothese, namelijk de veronderstelling dat het atherogene effect van roken verschilt tussen vrouwen met een laag lichaamsgewicht en vrouwen met een hoog lichaamsgewicht. Binnen een groep van 1680 postmenopauzale vrouwen welke deelnamen aan het Rotterdamse ERGO-onderzoek (Erasmus Gezondheid en Ouderen) vonden we dat, gedurende een vervolgperiode van 6,5 jaar, roken gepaard ging met een odds ratio van 4,3 (95% Betrouwbaarheidsinterval [BI]: 2,5-7,2) voor progressie van aorta-atherosclerose bij vrouwen met een laag lichaamsgewicht (quetelet index (QI) < mediaan), terwijl bij vrouwen met een hoog lichaamsgewicht (QI $\geq$ mediaan) de odds ratio 2,1 (BI: 1,2-3,8) was (P voor interactie < 0,05). Deze resultaten geven aan dat het verband tussen het roken van sigaretten en progressie van atherosclerose sterker is bij vrouwen met een laag lichaamsgewicht dan bij vrouwen met een hoog lichaamsgewicht. Mogelijk antagoneren oestrogenen, welke in vetweefsel gevormd worden uit bijnierandrogenen, het atherogene effect van roken bij postmenopauzale vrouwen.

Het insulineresistentiesyndroom vermindert het voordeel dat vrouwen hebben ten opzichte van mannen betreffende het vóórkomen van hart- en vaatziekten. Tot op heden bestaat er onduidelijkheid over de etiologie van de clustering van metabole factoren in het insulineresistentiesyndroom. Recente data geven aan dat het insulineresistentiesyndroom mogelijk gepaard gaat met een

toegenomen acute-fase-reactie. Wij bestudeerden deze hypothese in twee populaties. In de studie beschreven in **hoofdstuk 2.2** bestudeerden we de relatie tussen C-reactief proteïne (CRP), een ontstekingseiwit, en variabelen van het insulineresistentiesyndroom bij 186 gezonde middelbare vrouwen uit de algemene bevolking. In de studie beschreven in **hoofdstuk 2.3** onderzochten we de relatie tussen insulineresistentie (insulineconcentratie gemeten na de orale glucosetolerantietest) en verschillende ontstekingsindicatoren bij 574 ouderen zonder diabetes mellitus participerend in het ERGO-onderzoek. In beide studies vonden we een sterke associatie tussen laaggradige ontstekingsactiviteit en de gehanteerde maten van insulineresistentie. Deze resultaten ondersteunen de hypothese dat de acute-fase-reactie een onderdeel is van de clustering van metabole factoren binnen het insulineresistentiesyndroom.

Hoofdstuk 3 bevat studies naar geslachtsspecifieke determinanten van hart- en vaatziekten, met de nadruk op geslachtshormonen. De menopauze wordt beschouwd als een belangrijke determinant van de stijgende incidentie van hart- en vaatziekten bij vrouwen na de middelbare leeftijd. In **hoofdstuk 3.1** en **hoofdstuk 3.2** worden studies gepresenteerd welke gericht zijn op de relatie tussen natuurlijke menopauze en cardiovasculaire risicofactoren. Cholesterol is de belangrijkste cardiovasculaire risicofactor welke stijgt tijdens de menopauze. De mate van stijging varieert sterk tussen vrouwen. Het is echter vooralsnog onduidelijk waarom het cholesterolgehalte bij sommige vrouwen niet of nauwelijks stijgt, terwijl andere vrouwen een sterke cholesterolstijging laten zien. In de studie beschreven in **hoofdstuk 3.1** bestudeerden we of het apolipoproteïne E (APOE) genotype bijdraagt aan verschillen in mate van stijging van cholesterol tijdens de menopauze. Bij 1116 vrouwen welke deelnamen aan de Eindhoven Perimenopauzale Osteoporose Studie en welke tijdens een vervolgperiode van 5,9 jaar door de menopauze gingen, vonden we dat de stijging van cholesterolconcentratie ten tijde van de menopauze dertig procent lager was bij vrouwen met het APOE2E3 genotype vergeleken met de cholesterolconcentratiestijging bij vrouwen met het meest vóórkomende APOE3E3 genotype. De spreiding in de toename van cholesterolconcentratie tijdens de menopauze werd echter verre van volledig verklaard door het APOE genotype. Naast de stijging in cholesterolconcentratie zijn de mechanismen waardoor de menopauze mogelijke ongunstige effecten op het hart- en vaatstelsel uitoefent grotendeels onbekend. In de studie beschreven in **hoofdstuk 3.2** bepaalden we de plasma-homocysteïneconcentratie in een nauwkeurig geselecteerde populatie waarin het contrast in oestrogeenstatus tussen pre- en postmenopauzale vrouwen van dezelfde leeftijd was gemaximaliseerd. De studiepopulatie bestond uit 93 pre- en 93 postmenopauzale vrouwen van dezelfde leeftijd (spreiding 43 tot 55 jaar) welke werden

geselecteerd uit respondenten van een schriftelijke enquête welke was gezonden aan alle vrouwen in de leeftijdscategorie van veertig tot zestig jaar, wonende te Zoetermeer (n=12.675). In deze onderzoekspopulatie vonden we dat de homocysteïneconcentratie zeven procent (0,8 µmol/l) hoger was bij postmenopauzale dan bij premenopauzale vrouwen. De stijging van homocysteïne met de menopauze is mogelijk één van de mechanismen welke ten grondslag liggen aan de veronderstelde relatie tussen menopauze en het toegenomen risico op hart- en vaatziekten bij vrouwen na de middelbare leeftijd.

Suppletie van oestrogenen heeft mogelijk anti-atherogene effecten. In de studie beschreven in **hoofdstuk 3.3** werd het effect van oestrogeensuppletie op homocysteïneconcentratie bestudeerd in een interventieonderzoek. Het Romeo-onderzoek is een gerandomiseerde placebo-gecontroleerde studie, welke werd uitgevoerd om het effect te bestuderen van een sequentieel gecombineerde therapie van orale 17β-oestradiol en desogestrel ($17\beta E_2$-D) en een combinatie van geconjugeerde oestrogenen en norgestrel (CEE-N) ten opzichte van placebo op diverse cardiovasculaire risicofactoren bij 121 perimenopauzale vrouwen. Na zes maanden therapie was het verschil in homocysteïneconcentratie tussen vrouwen welke $17\beta E_2$-D en placebo ontvingen -6,3% (BI: -12,4%; 0,0%). Het verschil tussen vrouwen welke CEE-N en placebo ontvingen was -10,1% (BI: -16,7%; -2,9%). Deze resultaten geven aan dat oestrogeensuppletie gepaard gaat met een kleine verlaging van de homocysteïneconcentratie bij perimenopauzale vrouwen. Echter, ondanks de gunstige effecten van oestrogeensuppletie op cardiovasculaire risicofactoren, lieten de eerste gerandomiseerde experimentele studies gericht op secundaire preventie van hart- en vaatziekten geen duidelijk cardiovasculair beschermend effect zien. De resultaten van experimentele primaire preventie onderzoeken worden afgewacht.

Behandeling van postmenopauzale vrouwen met androgenen wordt beschouwd als een aantrekkelijke manier om gunstige effecten op psychologisch welbevinden en botmassa te bewerkstelligen. De toevoeging van androgenen aan postmenopauzale hormoontherapie is niet ongebruikelijk en zal waarschijnlijk toenemen. Tot op heden is echter niet bekend wat de langetermijn effecten van androgeentherapie zijn. In Nederland werd vanaf eind jaren '50 tot de jaren '80 intramusculair toegediende hoge dosis oestrogeen-testosterontherapie (oestradiol en testosteron esters) frequent voorgeschreven ter bestrijding van menopauzale klachten. Zelfrapportagegegevens betreffende intramusculair toegediende hoge dosis oestrogeen-testosterontherapie en gegevens betreffende aorta-atherosclerose waren beschikbaar binnen het EPOZ-onderzoek (Epidemiologisch Preventief Onderzoek Zoetermeer). Gebruik makende van deze gegevens vonden we dat intramusculaire testosterontherapie, welke één jaar of langer werd toegediend, gepaard ging met een odds ratio van

3,1 (BI: 1,1-8,5) voor ernstige aorta-atherosclerose bij 513 natuurlijke postmenopauzale vrouwen in de leeftijd variërend van 54 tot 67 jaar, zoals beschreven in **hoofdstuk 3.4**. Dit resultaat duidt op een wellicht ongunstig effect van testosterontherapie op atherosclerose bij postmenopauzale vrouwen.

Een vaak geopperde hypothese is dat endogene geslachtshormonen bijdragen aan de pathofysiologie van hart- en vaatziekten met tegengestelde effecten in de geslachten: androgenen worden verondersteld ongunstige cardiovasculaire effecten te hebben bij vrouwen, terwijl ze mogelijk zouden beschermen tegen hart- en vaatziekten bij mannen. Tot op heden is er echter geen relatie gevonden tussen endogene androgeenspiegels en het optreden van coronaire hartziekten bij mannen noch vrouwen. De resultaten van studies gericht op het verband tussen endogene androgeenspiegels en atherosclerose zijn vooralsnog niet eenduidig. In de studie beschreven in **hoofdstuk 3.5** bestudeerden we het verband tussen endogene androgeenspiegels en aorta-atherosclerose bij 1032 niet-rokende oudere mannen en vrouwen participerend in het ERGO-onderzoek. Onze resultaten toonden aan dat, ten opzichte van mannen met spiegels van totaal en biologisch beschikbaar testosteron in het laagste tertiel, mannen met hormoonspiegels in het hoogste tertiel odds ratios van respectievelijk 0,4 (BI: 0,2-0,9) en 0,2 (BI: 0,1-0,7) hadden voor de aanwezigheid van ernstige aorta-atherosclerose. Mannen met spiegels van totaal en biologisch beschikbaar testosteron in opeenvolgende tertielen waren tevens beschermd tegen het optreden van progressie van aorta-atherosclerose, gemeten na een periode van 6,5 jaar. Bij vrouwen bestond een positief verband tussen spiegels van testosteron en aorta-atherosclerose, hetgeen grotendeels was toe te schrijven aan ongunstige cardiovasculaire risicofactoren. We vonden geen verband tussen spiegels van dehidro-epiandrosteronsulfaat en aorta-atherosclerose bij mannen noch vrouwen. De klaarblijkelijke discrepantie tussen onze resultaten en de resultaten van studies in welke geen verband werd gevonden tussen endogene testosteronspiegels en coronaire hartziekten bij mannen is mogelijk toe te schrijven aan het feit dat wij alleen niet-rokers bestudeerden. Daarnaast is de aorta mogelijk gevoeliger voor de effecten van endogene geslachtshormonen dan andere arteriën. Aorta-atherosclerose verhoogt de kans op een herseninfarct, mogelijk via de polsdruk of door embolieën welke afkomstig zijn van atherosclerotische laesies in de aortaboog, hetgeen het belang van aorta-atherosclerose in relatie tot hart- en vaatziekten aangeeft.

In **hoofdstuk 4** worden studies beschreven betreffende alternatieve endocriene cardiovasculaire risicofactoren bij vrouwen. Het is bekend dat manifeste hypothyreoïdie samenhangt met hart- en vaatziekten. In de studie beschreven in **hoofdstuk 4.1** onderzochten we de relatie tussen subklinische hypothyreoïdie,

schildklier auto-immuniteit en hart- en vaatziekten in een aselecte steekproef van 1149 postmenopauzale vrouwen welke deelnamen aan het ERGO-onderzoek. We vonden dat subklinische hypothyreoïdie samenhing met de aanwezigheid van aorta-atherosclerose (odds ratio 1,7 [BI: 1,1-2,6]) en doorgemaakt myocardinfarct (odds ratio 2,3 [BI: 1,3-4,0]). De verbanden waren iets sterker bij vrouwen welke subklinische hypothyreoïdie én antilichamen tegen schildklier peroxidase hadden, terwijl geen verband werd gevonden tussen hart- en vaatziekten en schildklier auto-immuniteit onafhankelijk van schildklierstatus. Onze studie betreft een dwarsdoorsnede-onderzoek (cross-sectioneel onderzoek), hetgeen de validiteit van oorzakelijke interpretatie van onze resultaten beperkt. Het relatieve risico voor het optreden van een myocardinfarct bij vrouwen met subklinische hypothyreoïdie in het prospectieve deel van onze studie was weliswaar niet statistisch significant, maar kwam overeen met het risico in het cross-sectionele deel van onze studie. Het populatie-attributieve risico-percentage voor subklinische hypothyreoïdie samenhangend met myocardinfarct zoals berekend uit onze gegevens was in dezelfde orde van grootte als de populatie-attributieve risico-percentages van bekende belangrijke risicofactoren voor hart- en vaatziekten.

Naast hart- en vaatziekten is osteoporose een belangrijke oorzaak van ziekte en sterfte bij postmenopauzale vrouwen. In de studie beschreven in **hoofdstuk 4.2** gebruikten we gegevens uit het EPOZ-onderzoek om het verband tussen progressie van atherosclerotische aortaverkalking en metacarpaal botverlies bij vrouwen na de middelbare leeftijd te bestuderen. In een groep van 236 vrouwen welke een natuurlijke menopauze doormaakten gedurende een vervolgperiode van negen jaar vonden we dat bij vrouwen bij wie progressie van aortaverkalking optrad het metacarpale botverlies 3,2 mm^2 bedroeg en het verlies van metacarpale botdichtheid 7,2% was. Bij vrouwen zonder progressie van aortaverkalking bedroegen deze verliezen gemiddeld respectievelijk 2,0 mm^2 en 5,6% ($P<0,05$). In een cross-sectionele analyse bij 720 postmenopauzale vrouwen vonden we een gradueel invers verband tussen de mate van aortacalcificatie en metacarpale botmassa en botdichtheid. De associatie tussen atherosclerotische calcificatie en botverlies kan inzicht verschaffen in de pathofysiologie van deze ziekten. Mogelijk speelt een gemeenschappelijke etiologische factor, zoals oestrogeenverlies of gevoeligheid voor oestrogeenverlies ten gevolge van variatie in dichtheid of type van oestrogeenreceptoren, een rol in het verband tussen atherosclerose en osteoporose.

In **hoofdstuk 5**, de algemene discussie, worden de resultaten welke zijn beschreven in dit proefschrift in een breder kader geplaatst. Twee methodologische overwegingen worden besproken, namelijk ten eerste het effect van het

geslachtsverschil in het vóórkomen van coronaire hartziekten tussen mannen en vrouwen op verschillen in grootte van relatieve risico's en interactie-effecten tussen de geslachten. Ten tweede bespreken we de standaardbenadering in onderzoek naar hart- en vaatziekten om analyses te stratificeren voor geslacht. In onze visie op toekomstig onderzoek betreffende geslachtsspecifieke aspecten van hart- en vaatziekten geven we suggesties voor verder etiologisch onderzoek. Tevens geven we aan dat er aandacht moet blijven voor geslachtsspecifieke aspecten van diagnose en behandeling van hart- en vaatziekten en benadrukken we dat het van groot belang is het bewustzijn onder vrouwen te vergroten dat hart- en vaatziekten ook voor hen de belangrijkste gezondheidsbedreiging zijn.

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About the author

Liesbeth Hak was born on March 1, 1970 in Sliedrecht, The Netherlands. In 1988 she passed secondary school at the "Oude Hoven" in Gorinchem (atheneum). For 1 year, she studied health sciences at the Maastricht University (previously Rijksuniversiteit Limburg). In 1996 she obtained her medical degree from the same university (doctoral and medical degree cum laude). Subsequently, she worked 9 months as a resident in obstetrics and gynecology at the "St Maartensgasthuis", Venlo. In August 1997 she started the work described in this thesis at the Department of Epidemiology & Biostatistics, in close collaboration with the Department of Internal Medicine, Erasmus MC, Rotterdam. During this period she obtained a Master of Science degree in Clinical Epidemiology from the Netherlands Institute for Health Sciences in Rotterdam and worked 6 months as a resident in internal medicine. From April until August 2002 she will be working as a research fellow at the Department of Epidemiology of the Harvard School of Public Health, Boston, USA. In September 2002 she will start her training as an internist at the Erasmus MC in Rotterdam.

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