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CLINICAL STUDY

Endogenous sex hormone levels in postmenopausal women undergoing carotid artery endarterectomy

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Abstract

Objective: To study the endogenous sex hormone levels in natural postmenopausal women and their association with the presence of internal carotid artery (ICA) atherosclerosis.
Design: Case-control study
Methods: We compared 56 patients with severe ICA atherosclerosis referred for carotid artery endarterectomy (CEA) with 56 age-matched control subjects free of severe atherosclerotic disease. The presence of atherosclerosis was determined by high-resolution B-mode ultrasound. Metabolic parameters and sex hormones were measured or calculated: total cholesterol, high-density lipoprotein, low-density lipoprotein, triglycerides, glucose, insulin, quantitative insulin sensitivity check index, insulin resistance index, IGF-I, DHEA, DHEA sulfate (DHEA-S), free testosterone, total testosterone, estrone, estradiol, androstenedione, and sex hormone-binding globulin.
Results: The cases had statistically significant lower levels of both total testosterone (0.23 ± 0.12 vs 0.31 ± 0.20 μg/l, $P=0.043$) and free testosterone (3.42 ± 1.94 vs 4.59 ± 2.97 ng/l, $P=0.009$) and significantly lower levels of androstenedione (625.3 ± 168.7 vs 697.0 ± 211.9 ng/l, $P=0.017$) when compared with controls. Multivariate linear regression analysis, adjusted for traditional cardiovascular risk factors, baseline and physiologic characteristics, showed a significant inverse relationship between both serum free testosterone ($\beta=-0.234$, $P=0.028$) and androstenedione ($\beta=-0.241$, $P=0.028$) levels with the presence of severe atherosclerosis of ICA.
Conclusions: The study provides evidence of a positive association between low serum androgen levels and severe ICA atherosclerosis in postmenopausal women. It suggests that higher, but physiological, levels of androgens in postmenopausal women have a protective role in the development of atherosclerosis of ICA.

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Introduction

The incidence of cardiovascular disease (CVD) is much lower in premenopausal women than in men with the same age but the menopause initiates a phase of increased risk for CVD (1). The rate of CVD rises steadily after menopause, approaching but not attaining the levels observed in age-matched men (2). These observations suggest that the physiological hormonal changes associated with menopause might be responsible for the higher risk for CVD. The possible relationship of endogenous androgens and atherosclerosis in men has been widely evaluated (3). The possible atherosclerotic protection of hormone replacement therapy in postmenopausal women has also become a popular research topic (4). Nevertheless, studies concerning the evaluation of endogenous sex hormone levels and the presence of atherosclerosis in postmenopausal women are rather limited. Prospective longitudinal studies (5–8) of women could not demonstrate a relationship between the levels of androgens and the development of peripheral arterial disease or risk of CVD mortality. On the other hand, it was shown in two cross-sectional studies that higher DHEA sulfate (DHEA-S) (9), androstenedione (10), and androgen concentrations (9, 10) in women are related to lower carotid wall thickness. Golden *et al.* found, in their cross-sectional case–control study (11), higher total testosterone and sex hormone-binding globulin (SHBG) to be inversely related to carotid atherosclerosis in postmenopausal women.

In view of these findings, we conducted a case–control study to compare the concentrations of endogenous sex hormones of natural postmenopausal women undergoing CEA for high-grade stenosis of the internal carotid artery (ICA) with those of postmenopausal women without or with mild ($<10\%$) atherosclerotic narrowing of the ICA.

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Subjects and methods

Patients

Selection of the case group Natural postmenopausal women referred to our department for carotid artery endarterectomy (CEA) from June 2004 to September 2006 were included. The indications of CEA were asymptomatic $\geq 75\%$ stenosis or symptomatic $\geq 50\%$ stenosis of the ICA. Patients needing intervention for post-CEA restenosis, postirradiation lesions, and kinking of the ICA were excluded. Natural menopausal women were considered those without menstruations in the last two years, and nonmenstruating women 55 years of age or older, who had hysterectomy and at least one intact ovary (12). Women with surgical menopause (bilateral oophorectomy), hormone replacement therapy, thyroid disorders, digitalis and corticoid intake, autoimmune or systemic disease, gonadal disorder, polycystic ovary syndrome (PCOS), active malign process, recent (<1 month) severe infection, and recent (<1 month) stroke were excluded. Women taking lipid-lowering agents or antihypertensives that might have an influence on the arterial intima media thickness (IMT) were not excluded because the extremely limited decrease of IMT observed in several studies (13, 14) did not significantly influence the grade of ICA stenosis.

Selection of the control group Natural postmenopausal women with the same conditions as that for the selection of the case group, but without or with only mild (here defined as $<10\%$) stenotic atheromatous plaque of the extracranial carotid arteries were included in the control group. Women with a history of coronary artery disease, stroke, peripheral arterial disease or abnormal ankle/brachial index (ABI) were excluded. These control subjects were matched for age and were recruited from partners, family or friends of the members of the surgical staff, from partners of men undergoing CEA, from women undergoing varicose vein surgery or hernia repair and from patients of the one day geriatric centre. The study was approved by the local ethics committee and all subjects gave informed consent.

Methods

Baseline characteristics of cases and controls All participants underwent a structural interview and clinical examination. Comorbidities (cardiac, renal, and pulmonary) and cardiovascular risk factors (diabetes, tobacco use, hypertension, and hyperlipidemia) were graded using the scheme proposed by the Subcommittee on Reporting Standards for Cerebrovascular Disease (15). Height and weight were measured in patients in the standing position without shoes. Waist circumference was measured halfway between the costal arches and the iliac crest. Body mass index (BMI) was calculated as weight in kilograms divided by the square of height in meters. Blood pressure and heart beat were measured on the dominant arm in the supine position after 5 min rest.

Instrumental evaluation A high resolution B-mode ultrasound was used to evaluate the presence of atheromatous plaques in the carotid arteries. The Doppler determinations were carried out by two trained sonographers, who were blind to all clinical and laboratory characteristics. Scanning of the distal common carotid artery, the carotid bifurcation, and the ICA was conducted bilaterally at longitudinal and transverse projection for the measurement of IMT and plaques. A plaque was defined as a visually distinct area with an IMT greater than that of the neighboring sites (16). The degree of ICA stenosis was determined according to the maximum flow velocity and local narrowing in percent diameter reduction at the maximum of the stenosis, calculated according to the formula of NASCET: $(A-B/A)\times 100\%$, where A represents the diameter of the distal ICA and B the tightest diameter of stenosis (17).

In order to evaluate the state of the peripheral limb arteries, the Basic Doppler appliance (Atys Médical, Saint Genis Laval, France) was used. Subjects were allowed to rest for at least 5 min before measuring the blood pressures at both upper arms and ankles to obtain the ABI.

Sample collection and assay Early morning (between 0730 and 0830 h) fasting blood samples were taken from each control and case (the day before CEA). The serum levels of total cholesterol, high-density lipoprotein (HDL), triglycerides, and glucose were immediately assessed by means of enzymatic methods, low-density lipoprotein (LDL) was calculated according to the Friedewald formula (LDL = total cholesterol − HDL − triclycerides/5). Serum insulin was measured using a commercially available monoclonal IRMA (CIS bio, Gif-sur-Yvette, France). The insulin assay, calibrated against the MRC Insulin World Health Organization 66/304, had an analytical sensitivity of 0.25 mIU/l and within- and between-run coefficients of variation of <4 and $<8\%$ respectively. The assay had no cross-reaction with proinsulin ($<0.001\%$). The quantitative insulin sensitivity check index (QUICKI) was defined by the following formula: QUICKI = 1/(log (fasting insulin + log (fasting glucose)) as described by Katz *et al.* (18). The insulin resistance index (IRI) was calculated as 1/QUICKI. Serum insulin-like growth factor-I (IGF-I)

was measured after acid–ethanol extraction using a commercially available RIA (DSL, Wester, TX, USA). The IGF-I assay had an analytical sensitivity of 0.8 μg/l and within- and between-run coefficients of variation of <4 and $<10\%$ respectively. Serum androstenedione, DHEA, estrone, and testosterone were measured after extraction with ethyl ether using a commercially available radioimmuno assay (Immunotech, France, BioSource, Belgium, Orion Diagnostica, Finland). The analytical sensitivity was 14 ng/l, 0.3 μg/l, 3.2 ng/l, and 0.1 nmol/l respectively; the intra-and inter-assay coefficients of variation were <8 and $<12\%, <8$ and $<12\%, <10$ and $<15\%, <8$ and $<15\%$ respectively. Serum DHEA-S and estradiol were measured with the automated Elecsys immunoanalyser (Roche Diagnostics) with analytical sensitivity of 0.10 μg/dl and 5 ng/l, and intra-and inter-assay coefficients of variation were <5 and $<10\%, <3$ and $<5\%$ respectively. The cross-reaction of estrone is 0.515%. Serum SHBG was measured using an automated electrochemiluminescence assay (Roche Diagnostics), analytical sensitivity was 0.35 nmol/l and within- and between-run coefficients of variation were <3 and $<4\%$ respectively. Free testosterone values were calculated from total testosterone and SHBG using a fixed albumin concentration, according to Vermeulen *et al.* (19).

The results of the analysis were collected in a database by an independent data nurse, blinded to all clinical characteristics.

Statistical analysis

Statistical analysis was carried out using SPSS 14.0 for Windows program package (SPSS Inc., Chicago, IL, USA). Values are expressed as mean $\pm$ S.D. Distributions of continuous variables were tested for normality by one-sample Kolmogorov–Smirnov test. Continuous variables with normal distribution were compared with the independent-samples *t*-test and variables with a not-normal distribution were compared with the Mann–Whitney test. Categorical variables were compared with χ^2 test or Fisher's exact test.

The relationship between baseline variables and particular parameters with the hormone levels was assessed by factorial ANOVA. The variables case/control, hyperlipidemia, and ABI, which were significantly related with hormone levels, were selected for multivariate analysis using stepwise linear regression. Statistical significance was accepted when $P \leq 0.05$.

Results

The continuous variables height, waist, diastolic blood pressure, right and left ABI, number of pregnancies, triglycerides, glucose, insulin, and sex hormones had a skewed distribution, while age, weight, BMI, systolic blood pressure, pulse rate, total cholesterol, HDL, LDL, and IGF had normal distribution.

Baseline characteristics of patients and controls are presented in Table 1. Cases have a statistically significant higher incidence of atherosclerotic traditional risk factors, including diabetes, smoking, hypertension, and hyperlipidemia. Of the fourteen adult onset diabetics in the case group, ten were treated with oral hypoglycemic medications and four with insulin therapy; in the control group three were adult onset diabetics treated with oral medication and one had an insulin therapy. There were no significant differences in serum levels of glucose, insulin, and IGF-I between both groups. QUICKI and IRI were similar in both groups.

There was a higher prevalence of statin therapy in the case group (57.1% vs 25% $P=0.001$). We noted also a higher rate of cardiac, pulmonary, and renal comorbidities amongst the cases.

Of the postmenopausal women who underwent CEA, 18 (31.8%) presented asymptomatic ICA stenosis and 38 (67.9%) were symptomatic. In this group the mean stenosis grade of ICA was $79.5 \pm 10.7\%$. The physiologic characteristics of cases and controls are summarized in Table 2. The means of systolic blood pressure and waist circumference were significantly higher in the CEA patients, while the mean ABI of both limbs were lower in the case group. The lipid, glucose, and sex hormone profiles in postmenopausal women with carotid artery atherosclerosis and controls are shown in Table 3. The controls have significantly higher serum levels of total cholesterol and HDL, while the cases have higher levels of triglycerides. Postmenopausal women suffering from ICA atherosclerosis had significantly lower levels of both total and free testosterone and significantly lower levels of androstenedione than normal controls.

Table 1 Baseline characteristics of case and control groups.

	Cases *n*=56 number (%)	**Controls *n*=56 number** (%)	***P***
Age (years)[a]	70.4±9.0	70.5±7.6	NS*
Diabetes	14(25)	4(7)	0.01
Smoking	22(39)	5(9)	<0.001
Hypertension	48(86)	29(52)	<0.001
Hyperlipidemia	41(73)	22(39)	<0.001
Cardiac disease	22(39)	5(9)	<0.001
Renal failure	6(11)	0(0)	0.012
Pulmonary disease	13(23)	4(7)	0.018
Hysterectomy	14(25)	14(25)	NS
Unilateral ovariectomy	0(0)	3(5)	NS
Number of pregnancies[a]	2.1±1.7	2.5±1.6	NS**

NS, not significant; *P* values stated are calculated by χ^2 test or *Fisher's exact test, independent-samples *t*-test or **Mann–Whitney test.
[a]All values expressed as mean ± S.D.

Table 2 Physiologic characteristics of case and control.

	Cases $n=56$	Controls $n=56$	P
Weight (kg)	69.6±13.2	69.7±13.1	NS*
Height (cm)	154.5±21.9	159.7±6.2	NS
Body mass index (kg/m^2)	27.6±4.7	27.2±4.9	NS*
Waist circumference (cm)	100.4±14.7	93.6±16.7	0.037
Systolic blood pressure (mm Hg)	148.9±21.2	138.2±19.5	0.007*
Diastolic blood pressure (mm Hg)	76.2±9.0	77.0±11.6	NS
Pulse rate/min	74.3±11.0	73.9±9.2	NS*
Ankle/brachial index right	0.96±0.24	1.14±0.11	<0.001
Ankle/brachial index left	0.98±0.21	1.13±0.10	<0.001

All values expressed as mean±S.D. *P* values stated calculated by independent-samples *t*-test*or Mann–Whitney test. NS, not significant.

The relationship between androgen levels and the significantly different baseline and physiologic characteristics between case and control groups were evaluated by factorial ANOVA. The levels of androstenedione, total and free testosterone did not significantly correlate with diabetes, smoking, hypertension, cardiac disease, renal failure, pulmonary disease, number of pregnancies, waist circumferences, and systolic blood pressure. The levels of total testosterone were higher in the patients group with ABI>1.07(=median); 0.31±0.20 μg/l vs 0.23±0.11 $P=0.028$ and in patients with hyperlipidemia; 0.30±0.21 μg/l vs 0.24±0.12 $P=0.059$. The levels of free testosterone and androstenedione did not differ significantly. The results of the multivariate analyses of case/control, hyperlipidemia and ABI to total testosterone, free testosterone and androstenedione are shown in Table 4. There was no significant correlation between the hormone levels and hyperlipidemia and ABI. The case/control status was inversely associated with free testosterone and androstenedione, in other words the levels of these hormones were significantly higher in control group than in the case group after correction for ABI and hyperlipidemia. The difference of the levels of total testosterone between both groups lost its statistical significance.

Table 3 Results of lipid, glucose, and hormones analyses in case and controls.

	Cases $n=56$	Controls $n=56$	P
Total cholesterol (mg/dl)	192.8±56.15	213.6±32.6	0.018*
HDL (mg/dl)	62.3±15.8	71.9±15.6	0.001*
LDL (mg/dl)	111.9±41.8	121.3±30.5	NS*
Triglycerides (mg/dl)	128.9±62.8	103.3±48.3	0.024
Glucose (mg/dl)	101.5±30.0	100.3±26.2	NS
Insulin (mIU/l)	5.9±3.6	5.5±3.1	NS
QUICKI	3.81±1.86	4.18±3.39	NS
Insulin resistance index	0.282±0.05	0.281±0.06	NS
Insulin-like growth factor 1 (μg/l)	180.4±106.7	201.4±111.1	NS*
DHEA sulfate (mg/l)	0.74±0.57	0.71±0.45	NS
DHEA (μg/l)	2.31±1.96	2.87±4.31	NS
Free testosterone (ng/l)	3.42±1.94	4.59±2.97	0.009
Total testosterone (μg/l)	0.23±0.12	0.31±0.20	0.043
Estrone (ng/l)	24.4±12.8	23.6±13.6	NS
Estradiol (ng/l)	14.7±7.6	17.9±14.3	NS
Androstenedione (ng/l)	625.3±168.7	697±211.9	0.017
Sex hormone-binding globulin (nmol/l)	48.4±20.8	48.3±21.4	NS

All values expressed as mean ±S.D. *P* values stated calculated by independent-samples *t*-test* or Mann–Whitney test.

Discussion

To our best knowledge, this is the first published case–control study of postmenopausal women comparing the levels of female and male sex hormone between a selected CEA population and 'atherosclerotic free' subjects. Postmenopausal women with proven carotid artery disease were found to have significantly lower serum levels of free testosterone and androstenedione, compared to postmenopausal women with (nearly) normal carotid arteries. On the other hand, the two groups did not differ in mean levels of SHBG, DHEA-S, DHEA, estrone, and estradiol. As expected, the case group had a significantly higher prevalence of cardiovascular risk factors and comorbidities but even after adjustment of these factors, the difference in hormone levels remains significant. It seems also logical that the ABI, an indicator for arterial insufficiency of the limbs, is lower in the cases. The incidence for extracranial carotid artery stenosis rises indeed up to 21.8% in older patients

Table 4 Multivariate analyses of case/control, hyperlipidemia and ABI to total testosterone (TT), free testosterone (FT) and androstenedione (A).

	Case/control	Hyperlipidemia	ABI
TT			
β	−0.153	−0.071	0.167
P	NS	NS	NS
FT			
β	−0.234	−0.013	0.140
P	0.028	NS	NS
A			
β	−0.241	0.021	−0.015
P	0.028	NS	NS

ABI, ankle/brachial index; β, Standardized regression coefficients; *P*, probability.

with peripheral atherosclerotic disease (20). Despite these differences, linear regression analysis did not detect a relationship between ABI and hormone levels.

In this study, the mean waist circumference, and not the BMI, was significantly higher in the cases, but is not related to sex hormone levels. This observation underlines that the so-called 'visceral' adiposity is an independent cardiovascular risk factor (21). The higher prevalence of statin therapy in the case group can explain the lower mean levels of total cholesterol in this group compared with the controls. A significant statin-induced decrease of cholesterol, which is an immediate precursor for steroid synthesis, did not change the serum levels of testosterone, cortisol, or prednisolone (22). The hypertriglyceridemia and the lower HDL concentration in the case group are probably related to the higher mean waist circumference.

We are unable to compare our results of different hormone levels in both groups with others because to date no studies have quantified the hormone levels in postmenopausal women undergoing CEA. Kaczmarek *et al.* (23) found, in 108 postmenopausal women referred for diagnostic coronary angiography, a negative association between testosterone levels and the degree of coronary artery disease. On the other hand, Phillips *et al.* (24) found the opposite results i.e., free testosterone was significantly positive related to the degree of coronary artery disease in 60 postmenopausal women undergoing coronary angiography. However, the same authors (25) and others (26) found that men with coronary artery disease have significantly lower levels of androgens than normal subjects. The nested case–control study of Rexrode *et al.* (27) indicated that postmenopausal women with low SHBG and high free androgen index levels were at increased risk of CVD events, but this was not independent of BMI, hypertension, and diabetes. Our findings were in accordance with the results of studies concerning the relationship between endogenous sex hormone levels and IMT of the carotid artery. First, the data of Bernini *et al.* (10) demonstrated that in 44 postmenopausal women serum androstenedione and free testosterone were inversely related to carotid IMT. Second, the cross-sectional case ($n=182$) and control ($n=182$) study in the Atherosclerotic Risk in Communities cohort (11) found higher total testosterone and SHBG to be inversely related to carotid IMT. All these results suggest that higher testosterone levels, but within the normal range, in postmenopausal women have a protective role in the development of atherosclerosis. On the other hand, clinical studies have shown that women with coronary artery disease were affected more frequently than controls by clinical symptoms of androgen excess, such as hirsutism in women with polycystic ovaries (28, 29). It was suggested that the chronically abnormal hormonal status found in women with PCOS, starting from adolescence, may predispose these women to premature atherosclerosis (29). However, metabolic consequences of PCOS such as insulin resistance, hyperinsulinemia, and dyslipidemia (30) are probably more important than hypergonadism in the explanation for the increased risk of CVD in these patients. On the other hand, it is unclear why physiological high levels of androgens in menopause may be a favorable factor in the pathogenesis of atherosclerosis. There are a number of possible mechanisms of testosterone's interaction with the vascular system. Inflammation plays a central role in the initiation and progression of atherosclerosis. Testosterone appears to suppress activation of pro-inflammatory cytokines which are the mediators of local inflammation in the arterial wall (31). Further, dihydrotestosterone, the active derivate of testosterone, modulates DNA synthesis in vascular smooth muscle cells, inducing stimulation at low concentrations and inhibition at high concentrations (32). Dihydrotestosterone has also been shown to decrease tumor necrosis factor-α and lipopolysaccharide-induced inflammatory response in human endothelial cells (33). Furthermore, endogenous androgens in postmenopausal women have been reported to correlate negatively with circulating regulated upon activation, normal T-cell expressed and secreted (RANTES), a potent chemoattractant of T-lymphocytes (34). In addition, testosterone has been found to have direct vasodilatory effects initiated at the smooth muscle cell membrane of the endothelium, which contains testosterone binding sites (35). Another explanation for the atheroprotective effect is the peripheral aromatization of circulating androgens to estrogens. We know that in postmenopausal women the ovaries cease to produce estrogens, resulting in higher levels of testosterone relative to estradiol. Only about 25% of circulating testosterone is derived by direct secretion from the ovaries, the majority derived from circulating precursors such as DHEA-S from the adrenals and androstenedione and DHEA secreted from both adrenals and ovaries (36). In premenopausal women, the ovary is the main contributor to circulating estradiol, but in postmenopausal women, estrogen biosynthesis is mainly peripheral through conversion of testosterone and androstenedione catalyzed by the enzyme complex cytochrome P450 aromatase (37). Local production of estrone and estradiol mediated by aromatase is detected in smooth muscle cells of the tunica media in human aorta (37). These *in situ* estrogens seem to have local anti-atherosclerotic effects, can suppress elastin and/or collagen accumulation in the intima and media (38), and promote production of endothelium-derived relaxing factors (39). Nathan *et al.* (40) demonstrated that anastrazole, an aromatase inhibitor, abolished the protective effect on atherosclerotic lesion formation of exogenous testosterone in orchectomized male mice. Our study further supports this hypothesis since we found significantly higher levels of both free testosterone and androstenedione in normal subjects. We failed to find an association between atherosclerosis of ICA and DHEA and its sulfated ester DHEA-S. Several prospective,

cross-sectional, and retrospective studies have examined the relationship between DHEA, DHEA-S, and CVD. In a recent review (8), the authors noted numerous discrepancies in the results and indicated that plasma DHEAS-S is not a predictor of CVD outcome in women.

Furthermore, our study failed to find a difference in mean serum estrogen levels between cases and controls. This observation is in accordance with previous studies in which estrogen concentration in postmenopausal women was not related to CAD (41) or IMT of ICA (11) and did not predict fatal CVD (5).

Some limitations can be identified in this study. First, the number of cases and controls were small. The small number included are due to strict inclusion criteria and the limited number of postmenopausal women referred for CEA (in our clinical series of CEA 70% are men). Secondly, we did not measure direct free testosterone but Vermeulen *et al.* (17) demonstrated that the free testosterone value, obtained by calculation from total testosterone and SHBG as determined by immunoassay, appears to be a rapid, simple, and reliable index of bioavailable testosterone, comparable with apparent free testosterone concentration obtained by equilibrium dialysis and suitable for clinical routine, except in pregnancy. Thirdly, our findings can represent a selection bias because both cases and controls were highly selected subjects with many different parameters and the results might not be applicable to the general population. Nevertheless, our case–control study confirms and extends previous observations. Additional prospective follow-up studies in women with low androgen levels in the menopause are needed to strengthen the hypothesis that higher androgen levels have a cardiovascular protective effect.

In conclusion, the current study provides evidence of a positive association between low serum androgen (free testosterone and androstenedione) levels and the presence of severe atherosclerosis of ICA. These results suggest that higher, but physiological, levels of testosterone and androstenedione in postmenopausal women have a protective role in the development of atherosclerosis of ICA.

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