Artery Wall Imaging and Effects of Postmenopausal Estrogen Therapy

KENNY A. RODRIGUEZ-MACIAS WALLBERG
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Abstract
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Postmenopausal estrogen therapy, initiated early in the menopause, seems to protect against development of atherosclerosis and cardiovascular diseases. This thesis concerns studies of artery wall thickness and arterial stiffness estimated by noninvasive ultrasound techniques in long-term estrogen treated postmenopausal women who initiated therapy at the time of the menopause.

A noninvasive 25 MHz high-frequency ultrasound technique was validated in the imaging of superficial arteries by using an animal model. Ultrasound estimates of the artery wall layers obtained in vivo in the pig were compared to ex-vivo histomorphometry. Valid estimates of total artery wall and media thickness were found for the most superficial arteries. Adventitia thickness was underestimated and intima thickness overestimated in this animal model when non-atherosclerotic vessels were imaged.

To validate the clinical usefulness of separately estimating the artery wall layers in the human, the carotid artery wall was imaged in elderly subjects. Separate estimates of intima thickness, media thickness and intima/media ratio differed significantly between subjects with and without atherosclerosis and CVD, indicating that this noninvasive high-frequency ultrasound method might be a strong tool in monitoring changes in artery wall morphology associated with aging and development of atherosclerosis.

The investigation of intima thickness, media thickness and intima/media ratio of the carotid and femoral arteries in long-term estrogen treated postmenopausal women showed a maintenance of a thin intima and a preservation of media thickness and intima/media ratio at values similar to those obtained in women of fertile age. By comparing estrogen-users with age-matched postmenopausal nonusers, long-term estrogen therapy initiated at the time of the menopause seemed to counteract the increase in intima and decrease in media thickness associated with aging and development of atherosclerosis. The preservation of the artery wall morphology into older age might be a mechanism for the well-documented cardioprotective effects of estrogen when therapy is initiated early after menopause. However, long-term estrogen therapy showed no substantial effects on the age-related changes in arterial stiffness estimated at the aorta, carotid and femoral arteries, suggesting that any long-term cardioprotective effect that estrogen therapy may have is unlikely to be mediated by an impact on arterial stiffness.

Keywords: artery wall thickness, tunica media, tunica intima, arterial stiffness, carotid, aorta, cardiovascular disease, atherosclerosis, menopause, estrogen, hormone therapy, ultrasound

Kenny A. Rodriguez-Macias Wallberg, Department of Women's and Children's Health, Akademiska sjukhuset, Uppsala University, SE-75185 Uppsala, Sweden

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Coronary heart disease is the leading cause of morbidity and mortality for women in most developed countries: 55% of deaths in women in Europe. (European Cardiovascular Disease Statistics, AHA, 2003)

In developing countries it will be the leading cause of death in the next 20 years. (Brister, 2001)

To my family
List of publications

This thesis is based on the studies reported in the following original papers, referred to in the text by their respective Roman numerals:

I. Rodriguez-Macias KA, Naessen T, Bergqvist D
   Validation of *in vivo* noninvasive high-frequency ultrasonography of the arterial wall layers.

II. Rodriguez-Macias KA, Lind L, Naessen T.

III. Naessen T, Rodriguez-Macias KA.
    Menopausal estrogen therapy counteracts normal aging effects on intima thickness, media thickness and intima/media ratio in carotid and femoral arteries. An investigation by noninvasive high-frequency ultrasound. Submitted.

IV. Rodriguez-Macias KA, Naessen T, Boström A, Bergqvist D
    Arterial stiffness is not improved in long-term use of estrogen.

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Abbreviations

CVD  Cardiovascular disease
CHD  Coronary heart disease
MI   Myocardial infarction
ET   Estrogen therapy
HT   Hormone Therapy (including both ET and combined estrogen and progestogen therapy)
MPA  Medroxyprogesterone Acetate
HR   Hazard Ratio
CI   Confidence Interval
OR   Odds Ratio
ER   Estrogen receptor
VSMC Vascular smooth muscle cells
IMT  Intima-Media Thickness
CCA  Common carotid artery
LCCA Left common carotid artery
LCFA Left common femoral artery
SBP  Systolic blood pressure
DBP  Diastolic blood pressure
Ep   Stiffness elastic modulus
β Index Stiffness β index
E2   Estradiol
SHBG Sex-hormone binding globulin
FSH  Follicle-stimulating hormone
CV   Coefficient of variation
BMI  Body Mass Index
PWV  Pulse Wave Velocity
DC   Distensibility Coefficient
PI   Pulsatility Index
RI   Resistance Index
Introduction

Cardiovascular disease in women

According to WHO estimates, 16.6 million people around the globe die of cardiovascular diseases (CVD) each year (1). Coronary heart disease (CHD) alone accounts for about half of deaths from CVD and nearly one third are from stroke. Due to an aging population, the absolute numbers of deaths related to CVD in both sexes are increasing and this trend is especially noticeable among women, who have a longer life expectancy than men and who account for the majority of cardiovascular disease deaths (53.5%) (1, 2).

The specific issue of cardiovascular disease in women is, however, only recently highlighted and still complex. For many years the predominantly male-focused cardiovascular research has been generalized to women and, probably, as a result of this and despite significant advances in diagnosis and treatment of CVD, the prognosis of CHD in women is poorer than in men (2). Upon presentation women are older than men and have a higher prevalence of known cardiovascular risk factors such as diabetes and hypertension (3). Other factors that may also play a role are delayed presentation to the emergency unit, reduced perception of risk of myocardial infarction (MI) by both women and health care providers and presence of atypical symptoms and signs (4). However, women with the same symptoms as men are less likely to be admitted to the coronary care unit and seem to be less aggressively treated than men, although women seem to have more severe infarcts with higher mortality after admission than men (3, 4). For women, primary prevention oriented to reduce cardiovascular risk factors is likely to be the only practical solution (5).

Can postmenopausal hormone therapy (HT) help to prevent CVD?

Evidence from observational data and randomized trials

In the Framingham Study, one of the few prospective longitudinal epidemiologic investigations that had included women from its onset, it was shown that coronary events occurred in men before age 50 years but occurred rarely in women before age 55 years. The age-related increase of CHD was ob-
served approximately 10 years later in women than in men and for the more serious clinical manifestations like MI and sudden death women lagged also behind men by 20 years (6, 7). The delayed onset of CVD in women has been attributed to a protective effect of estrogens during reproductive life. This theory has been supported by results of observational studies. Carefully documented onset of menopause in the women from the Framingham cohort revealed a four times increased risk of a coronary event in postmenopausal women when compared with women of the same age who remained premenopausal (7). In the largest cohort of women (116,258 subjects) investigated with respect of menopause and heart disease, the Nurses’ Health Study cohort, it was found that women who had undergone surgical menopause and did not receive hormone therapy (HT) had a 2-fold higher incidence of CHD when compared with those having received HT after adjusting for age and smoking (8). Studies considering age at menopause in the Netherlands have also shown that the risk of cardiovascular mortality is higher for women with early menopause than for those with late menopause. Moreover, for each year's delay in the menopause the cardiovascular mortality risk decreases by 2% (9), suggesting that longer exposure to endogenous estrogens protects against cardiovascular disease.

In women, it has been shown that the atherosclerotic process accelerates after menopause (10). Thus, with advancing age, the rate of increase in atherosclerotic lesions is higher in women than in men, the proportion of CHD events as MI and sudden death increases more sharply in women and the risk of CHD increases substantially, eliminating the advantage over men (7).

Large prospective population-based cohort studies seem to indicate that HT given during the postmenopausal years may prevent CVD. In the USA, the Nurse’s Health Study reported a RR of 0.56 for CHD events in HT users after 10 years follow-up of 48,470 postmenopausal women (11). In Sweden, Falkeborn et al. reported a significant 30% reduced risk for myocardial infarction in HT users by following the entire female population of the Uppsala Health Care Region (23,174 women) (12). Epidemiological studies collecting a considerable amount of data have estimated that the use of HT may account for a reduction of the risk of myocardial infarction by 50% (13, 14). Although the evidence from observational studies indicating a beneficial effect of HT on CHD is consistent, there are several potential sources of bias that may account for these findings (15). Matthews et al. reported that women who elect to take estrogen after the menopause tended to be more educated and had a better cardiovascular risk factor profile than untreated women (16). However, recent data from the Swedish cohort, revealed a more reduced RR for MI for women taking medium-potency estrogens than for those in low-potency estrogen therapy, which is in favor of a real beneficial
effect of estrogens and not only as result of selection bias or adherence to therapy (17).

The recent reports from two prospective randomized studies in the USA, the HERS study (18) and the WHI study (19) showed, however, no overall cardiovascular benefit and a possible early increased risk of CVD events when HT at high doses is initiated in women with documented atherosclerosis (HERS) and at advanced ages (WHI). At older ages, a lower dose is usually recommended and the results of HERS have been considered as not very surprising (20). Post-hoc analyses from HERS showed, however, after the early increase in CVD events, a statistically significant time trend for lowered risk over the study period, \( P = 0.009 \) for the trend (18). Both the early increase in CVD events after starting HT in women with known atherosclerosis, as well as their decrease after long time use of HT had been also shown in observational studies (21). The final analysis of the WHI study, seem to present similar results to those in observational studies: the authors reported a non-significant 24% increased risk for CHD which was highest after 1 year of therapy and decreased over time. The use of HT for 6 years was associated with a 30% decrease in CHD risk (Hazard Ratio, HR 0.70 95% CI, 0.42-1.14) (22). The recent published results of the WHI estrogen-only trial reported, similarly, a smaller, nonsignificant increase of cardiovascular events in the estrogen therapy (ET) group in the first year of ET that diminished over time, suggesting a possible modest benefit with longer-term use. Analyses showed that the women in early postmenopause (50-59 years of age) appeared to respond to estrogen more favorably than older women with reduced Hazard Ratios for CHD events (HR 0.56, 95% CI 0.30-1.03), mortality (HR 0.73, 95% CI 0.47-1.13) and improved global index (HR 0.80, 95% CI 0.62-1.03) (23, 24).

According to many authors, the results from HERS and WHI have been extensively misinterpreted and wrongly extrapolated to younger postmenopausal women with no CVD risk factors and to other HT doses and combinations (20, 25-32). In a recent meta-analysis, Salpeter et al. reported a significant reduction in total mortality in younger postmenopausal women under 60 years participating in controlled clinical trials (Odds Ratio, OR, 0.61; CI 0.39-0.95) whereas no change in mortality was seen in women in the older age groups (OR, 1.03; CI 0.90-1.18) (33). By design, the WHI population was 10-fold underpowered to show cardioprotection of women starting HT in the early postmenopausal years. Thus, observational studies that have shown cardiovascular protection in younger postmenopausal women remain the only applicable clinical approach to this issue (34).

Cross sectional clinical studies in postmenopausal women seem to support the observational findings that estrogen are cardioprotective. By analyzing angiographic studies from 1444 women with substantial coronary occlusion and 744 controls with normal coronary arteries, Sullivan et al. found that the only factor significantly associated with the absence of coronary
disease was estrogen therapy (35). A similar study conducted by Gruchow et al. in 933 postmenopausal women found that occlusion scores by angiography increased significantly with age in non-hormone users but not in estrogen users (36). In this study, the inclusion of HDL-cholesterol in an statistical model reduced the negative association between estrogen use and coronary occlusion, suggesting that the effect of estrogen on coronary disease was partially mediated by HDL-cholesterol. Observational studies have confirmed that the protective effect of estrogen is substantially mediated through estrogen-induced HDL-cholesterol increase (37).

Evidence of a cardioprotective effect of estrogen based on animal experiments

Randomized studies in animal models may overcome the general problems faced with the observational human studies and the difficulties carrying out truly randomized studies in women. Data from animal models strongly support the hypothesis that estrogen have antiatherogenic properties (38). Adams et al. reported a significant reduction of about 50% of coronary atherosclerosis in ovariectomized female cynomolgus monkeys randomized to HT and fed with an atherogenic diet (39). In a more recently developed animal model of surgical menopause in ovariectomized micropigs, a similar 51% inhibition in progression of coronary atherosclerosis was found in the animals that received ET compared with controls (40). In monkey models less coronary atherosclerosis has also been found in premenopausal females compared to surgically postmenopausal females or male monkeys (41). Inhibition of progression of atherosclerosis during the hyperestrogenic state of pregnancy and the presence of more extensive atherosclerotic lesions in ovariectomized monkey females than in estrogen-treated females have also been reported (41). The treatment effect in this model appears to be partially mediated by a decrease in the uptake and/or degradation of low density lipoprotein at the arterial wall level (42) and an improvement in endothelium-mediated dilation of coronary arteries (43). Recent studies using this monkey model suggest that estrogens have beneficial effects in the early stages of atherogenesis, but may have little or no beneficial effects at advanced stages of the atherosclerosis disease (44, 45).

Studies of balloon-induced carotid injury in the rat, which provokes endothelium denudation and neointima formation, have shown a greater neointima formation in male rats that in female rats that did not change after orchiectomy in males but was significantly enhanced after ovariectomy in the females. The systemic administration of 17-beta estradiol to achieve physiological circulating hormone levels, significantly attenuated the neointima formation in both male and female animals suggesting that estrogens mediate the sexual dimorphism of neointima formation and can modulate the response to endoluminal vascular injury (46, 47).
Animal models have been criticized for using young animals which may not represent the females in their menopausal years. However, by investigating aged rats, Moien-Afshari et al. reported an increase in endothelial cell apoptosis and loss of endothelial dependent vasodilation after ovariectomy (48). Replacement therapy with 17-beta estradiol pellets in ovariectomized rats prevented the endothelial cell apoptosis and dysfunction (48).

A reduction of these beneficial estrogen-related effects has been reported by addition of progestins both in animal experiments (49-51) and human clinical studies (52).

Possible beneficial effects of estrogen on the artery wall

Although the beneficial changes in serum lipids and lipoproteins are the best known cardioprotective effect of estrogens, it is estimated to account for only 30% of the observed clinical benefits (37). The remaining 70% is still not fully explained and reviews of the data suggest that direct effects of estrogens on the artery wall may contribute substantially to cardiovascular protection (45, 53-55). By investigating post-mortem specimens of coronary arteries from pre- and postmenopausal women, Losordo et al. reported a reduced expression of estrogen receptors (ER) in the vascular smooth muscle cells (VSMC) of atherosclerotic arteries (56). The association between presence of estrogen receptors and absence of coronary atherosclerosis suggests that these receptors may play a functional role in the vascular atheroprotection (56).

Recently identified actions of estrogen on the vessel wall mediated by the estrogen receptors ER-\(\alpha\) and ER-\(\beta\) present in endothelial and VSMC, include both a rapid nongenomic vasodilatory component, mediated primarily by nitric oxide release and membrane phenomena, and a long-term genomic effect involving the classical pathway of steroid action by activation of estrogen receptors and secondary changes in gene expression (55, 57, 58). Both the genomic and the non-genomic actions of estrogen could be involved in retarding/avoiding the development of CVD (59). Moreover, presence of the estrogen synthetase enzyme aromatase has also been demonstrated in human coronary vessels, indicating that estrogen can be also locally produced by the artery wall (60). Estrogen has been shown to restore the endothelial cell function in the animal model of balloon-induced carotid injury in young rats (61), which may be partially attributed to increased local expression of endothelial growth factor (54) but also through effects on modulating the inflammatory response at the vascular wall (51, 62). In the animal model by using mature rats, estrogen has been shown to maintain endothelial integrity retarding cellular apoptosis and prolonging the functional status of the endothelium, likely through increased nitric oxide release (48).
To date it is difficult to ascertain which of those cardiovascular effects of estrogen observed in basic research may actually have clinical relevance. However, although age per se is associated with a progressive decline in endothelial function in humans (63), endothelial dysfunction in men precedes its occurrence in age-matched women (64). The age-related impairment in endothelium-dependent vasodilation in women is only evident after menopause (63) and an impaired endothelial function has been observed in young women with premature ovarian failure, which may be restored by HT (65).

Investigation of effects of postmenopausal estrogen therapy on the artery wall by using noninvasive ultrasound

Artery wall thickness as a marker of atherosclerosis

To assess the early progression of atherosclerosis, it is necessary to visualize the vessel wall itself. The use of peripheral arteries, i.e. the carotids, as vascular endpoints for assessing atherosclerosis, is widely accepted, as the degree of atherosclerosis in those vessels correlates with that in the coronary arteries, these being more difficult to be imaged with noninvasive techniques (66).

Carotid artery intima-media thickness (IMT), estimated by 7-8 MHz frequency ultrasound, is the present golden standard for noninvasive assessment of development of atherosclerosis. The method has been validated in vitro (67, 68) and although the three-layer image is observed at both the near and far artery wall, measurements of IMT are only accurate when limited to the far wall where the structures present with the order intima-media-adventitia (68). The IMT measurement, combines both the intima and media thicknesses (68). Carotid IMT has been shown to associate with major risk factors for atherosclerosis and CHD (69-72) and with prevalent (72) and future CVD (73, 74). Since the middle 90’s carotid IMT is used as a surrogate endpoint of progression of atherosclerosis and as predictor of clinical coronary events (75, 76) and stroke (73).

Study of the artery wall thickness in postmenopausal women

Carotid IMT has been also used in the evaluation of interventions i.e., lipid-lowering medications (77) and postmenopausal estrogen therapy (78-80) in reducing atherosclerosis. By comparing healthy postmenopausal women after natural and surgical menopause in a cross-sectional study, Mack et al. found that time since menopause was related to higher IMT values, i.e., elevated subclinical atherosclerosis (78). In a double-blind randomized controlled clinical trial, Hodis et al. investigated the effect of ET, or placebo, in
222 postmenopausal women of about 60 years of age without known CVD and used the changes in carotid IMT as a surrogate of progression of atherosclerosis during a 2-year follow-up. The results demonstrated that ET significantly reduced the progression of subclinical atherosclerosis, when compared with placebo (79). However, a similar study conducted in older postmenopausal women with known CHD did not show any significant changes on the progression of atherosclerosis after treatment with 17-beta estradiol either alone or in combination with MPA (80). These randomized studies suggest that estrogen replacement therapy may reduce the progression of subclinical atherosclerosis in healthy postmenopausal women without pre-existing cardiovascular disease, but may not offer the same benefits to women with known CHD (81).

Need to develop more accurate noninvasive methods in the investigation of atherosclerosis

Although IMT is being used to assess carotid atherosclerosis in epidemiological studies and clinical trials, the interpretation of IMT measurements made from ultrasound images has been questioned by a validation study showing that IMT, assessed by low-frequency ultrasound, better corresponds to total artery wall thickness than to intima+media thickness per se (82). Further, by comparing IMT estimates and coronary angiography in 350 subjects, Adams at al found a low sensitivity and specificity of IMT to identify patients with or without significant CHD (83). As recently demonstrated, the increase in IMT estimates represents predominantly an increase in intima thickness, at least in individuals in the seventh and eighth decades of life (84). However, morphometric studies in elderly subjects have shown that with advancing age and development of atherosclerosis, changes in intima and media thickness develop in divergent directions, i.e., intima increasing and media decreasing (85). Thus, separate assessments of intima and media thickness might be more appropriate than the combined IMT in assessing/monitoring the artery wall for early changes caused by aging and atherosclerosis and to investigate effects of interventions, i.e., hormone therapy.

Use of high-frequency ultrasound in imaging the artery wall

By using 7-8 MHz frequency ultrasound it is not possible to obtain separate estimates of the intima and media wall layers, in part due to the low resolution associated to the lower frequencies and in part due to that IMT, when estimated at the far wall, produces a systematic overestimation of the intima together with an underestimation of the media layer (86). However, as a large enough difference in acoustic impedance seems to exist between the different tissue structures of the artery wall, each one of the layers could be individually estimated by ultrasound if a high detail resolution is achieved. High-frequency ultrasonography can reach a much higher resolution than low-frequency ultrasound and equipments fitted with probes between 12 and
40 MHz are actually being used for in vitro and in vivo intravascular ultrasonography of the coronary and more peripheral arteries (87-89). Data obtained with intravascular high-frequency ultrasound may illustrate the morphology of the artery wall and it has been suggested that early stages of atherosclerotic disease may be detected by changes on the arterial wall (90, 91). Development of noninvasive methods to obtain that information is suitable.

Interest in investigating separately the thickness of the intima and media layers in postmenopausal women

Human studies have shown that estrogen may preserve skin thickness in postmenopausal women by increasing collagen content (92, 93). An effect of estrogens on the connective tissue and muscular elements of the artery wall may thus be plausible. By using 22.5 MHz frequency noninvasive ultrasound, Baron et al. reported an increase in carotid wall thickness, particularly at the layers with a higher connective tissue component (externa and media layer) in young estrogen-treated postmenopausal women (mean age 52 years) (94). Also by using 22.5 MHz frequency ultrasound, Sator et al. found a significantly thicker media layer in postmenopausal women taking combined HT, when compared to untreated women (mean age 55 years) (95) and in young pregnant women (mean age 22.8 years) when compared to non-pregnant women of similar age (96). A significant reduced intima thickness was also found in pregnant women when compared to non-pregnant women of similar age, suggesting that the mechanism of intimal formation may be estrogen-related (96). No significant differences in intima thickness estimated by 22.5 MHz frequency ultrasound have been reported between those relatively young hormone-treated and untreated postmenopausal women groups (94, 95).

Atherosclerosis is a process that develops with aging. Thus, to investigate a possible atheroprotective effect of estrogen by imaging the arterial wall it is suitable to study postmenopausal women at older ages, with a higher probability of having developed atherosclerotic changes. The use of noninvasive high-frequency ultrasound seems to be appropriate in imaging the artery wall in a clinical setting and it could permit to obtain separate estimates of intima and media thickness. Considering that intima and media layers develop in divergent directions with aging and development of atherosclerosis (85, 97), these separate estimates could give more precise information on the atherosclerosis process than the combined IMT estimated by 7-8 MHz frequency ultrasound. Given the substantial amount of data suggesting that estrogen therapy may be cardioprotective when initiated early after menopause, it should be preferable to investigate those effects on intima and media layer at the artery wall in women who initiated estrogen treatment at the time of the menopause.
The noninvasive high-frequency (25 MHz) ultrasound method intended to be used for imaging the artery wall in this clinical investigation had not been previously validated. Therefore a validation study of the method was first performed. Subsequently, to establish the clinical usefulness of this method in estimating separately the artery wall layers, a group of elderly subjects was investigated. The thickness of media, intima and the intima/media ratio were obtained at the carotid artery, and their values were compared between individuals with and without developed atherosclerosis and CVD.

Artery wall stiffness as a marker of atherosclerosis

Arterial stiffness, estimated by noninvasive ultrasound, is a valid indicator of the mechanical properties of the vascular wall at the point of sampling (98). Increased arterial stiffness has been proposed as a marker of the initiation/progression of atherosclerosis and hypertension (99), is associated with CHD (100-102) and cardiovascular risk factors including age (103-106), male gender (107), smoking habits (108), total- and LDL-cholesterol (109, 110), elevated triglycerides, glucose and insulin levels (111) and insulin resistance (112).

Interest in evaluating the mechanical properties of the artery wall in postmenopausal women

An effect of menopausal status on stiffness has been suggested from the steeper age-related increase in carotid stiffness in women, between 45 and 60 years of age, than in men of similar age (113). Higher arterial stiffness has also been demonstrated in postmenopausal women, when compared to premenopausal women of the same age (all 50 years), before and after controlling for smoking and insuline levels (114). HT seems to reduce carotid artery stiffness early after menopause, indicating that this therapy may modify the age/menopause-associated increase in stiffness in women in the short-term perspective (115). A better knowledge of the mechanical properties of the artery wall in older postmenopausal women and after long-term estrogen therapy should be of interest.
Present investigations

Aims
The general aim of the thesis was to investigate, in a clinical setting and by using noninvasive ultrasound methods, possible effects of long-term postmenopausal estrogen therapy on the thickness of the artery wall layers and on arterial stiffness.

The particular aims of the studies upon which this thesis is based were:

To validate a noninvasive high-frequency ultrasound (25 MHz) method in imaging the artery wall and assessing separate estimates of the thickness of the artery wall layers on superficial arteries

To evaluate the clinical usefulness and validity of the high-frequency (25 MHz) ultrasound technique by assessing separate estimates of intima thickness and media thickness at the carotid artery in a cohort of subjects, all of the age of 70 with and without cardiovascular diseases

To investigate possible effects of long-term estrogen therapy initiated at the time of menopause on the thickness of the intima and media layers at the common carotid and common femoral arteries

To investigate possible effects of long-term estrogen therapy initiated at the time of menopause on the arterial stiffness of the common carotid artery, common femoral artery and aorta

Material and Methods
Animals
Thirteen pigs participating in research projects approved by the Local Ethics Committee on animal experiments at Uppsala University were included in study I. The pigs were between 10 and 12 weeks of age and had a mean weight of 27.6 Kg. The animals were under general anaesthesia during the high-frequency ultrasound investigation of superficial arteries.
Subjects

Eleven healthy female volunteers, age range 26-61 years, were recruited from the hospital staff and served as study group in a pilot human study to calculate the coefficient of variation for high-frequency obtained ultrasound estimates of arterial total wall thickness, media and intima thickness at the carotid and femoral arteries.

In study II, one-hundred consecutive subjects (56 men and 44 women) out of a cohort of 1016 randomly selected persons, all aged 70, living in the Uppsala community and participating in the PIVUS (Prospective Investigation of the Vasculature in Uppsala Seniors) study were included. All subjects underwent a physical examination and filled a form including medical history of cardiovascular diseases. Each subject gave informed consent to the study, and the study was approved by the local ethics committee, Uppsala University, Sweden.

In study III and IV the study population included a total of 54 women who were recruited as follows:

Seventeen postmenopausal women were recruited from a cohort of women treated with 17-β estradiol implants for more than 5 years and living in the Uppsala community. Mean age was 68.8 years and mean duration of treatment 20 years. All women had had a hysterectomy and most of them had started with implants at the time of the operation. They received usually 20 mg pellets estradiol implanted subdermally every 6 months.

Seventeen aged-matched nonusers were selected from the population registry of the same municipality as the treated women, matched for age (±1 year) and recruited through mailed invitations. Their mean age was 68.8 years.

Twenty women in the fertile age without any hormonal therapy, mean age of 39.6 years, were recruited from the hospital staff and served as a premenopausal reference group.

The 54 women were investigated regarding to separate estimates of intima and media thickness at the carotid and femoral arteries by using high-frequency (25 MHz) ultrasound (Study III) and arterial stiffness at carotid, femoral and aorta (Study IV). In all women, venous blood samples were obtained on the day of the high-frequency ultrasonography. Each subject gave informed consent and the study was approved by the local ethics committee, Uppsala University, Sweden.
Assessment of total wall thickness, media and intima thickness

Estimates of total artery wall thickness and the thickness of the individual artery wall layers were assessed noninvasively by means of a high-resolution ultrasonographic equipment fitted with a broad-banded probe with 25 MHz center frequency which housed a built-in water chamber (Osteoson® Minhorst GmbH, Meudt Germany). The scan converter enables freezing of the image in a selected scan-time (here 2 sec) and the unit permits two-dimensional data acquisition, presenting the results as A- and B-scans. About 128 lines of echo data were detected as an A-echo signal, sampled by an eight-bit analogue–to-digital converter, converted by scan to a rectangular format and viewed as B-mode images on a 32 colour scale monitor. Image resolution is approximately 0.07 mm axially along the ultrasonographic beam (data provided by the manufacturer) and depth of focus is in the range of 13.5 to 14.5 mm in front of the lead of the probe. The system recognizes objects of the size of about 0.015 mm and the software-driven cursors permit a minimal digital display of 0.02 mm.

The site of scanning was selected by palpation of the pulse of the chosen artery. The near artery wall was identified after the scans revealed a pulsating structure formed by three layers: two echodense zones with an echoluent area in between, followed by the echolucency of the artery lumen. Figure 1 shows a typical ultrasonographic image of the near carotid artery wall noninvasively obtained with this method. In study I and III, twenty B-scans were taken from the near artery wall and measurements of total artery wall thickness, adventitia, media and intima thickness were performed off-line. Ten B-scans from the chosen artery were taken in study II. Total artery wall thickness was measured from the leading edge of the adventitia to the far edge of the intima. Measurements of the adventitia and intima thickness were done considering only the brightest echoes from leading edge to far edge, and the thickness of the media layer was measured as the distance between the two brightest echoes.

In study I, we investigated the left femoral artery in the pigs in 7 cases and the cutaneous superficial branch of the medial circumflex femoral artery in 12 cases. The gain setting was kept constant throughout the study. In 7 pigs a single artery was imaged and in 6 pigs two arteries were sonographed. In 4 animals, the right and left cutaneous arteries and in 2 animals the left femoral and the left cutaneous were investigated. Mean values were calculated from up to 20 measurements. After the ultrasound scans were obtained, a mark was drawn on the skin over the examined site to indicate where the vessel should be subsequently dissected.

In studies II and III, before beginning the ultrasound imaging, subjects rested quietly for at least 15 minutes. Ultrasound examinations at the left common
carotid artery (LCCA) were performed with the subjects sitting in an upright position and looking straight ahead. The probe was positioned at the point of maximal pulsation in front of the sternocleidomastoid muscle with the axis of the transducer being perpendicular to the skin surface. In study III, the left common femoral artery (LCFA) was examined below the inguinal ligament, midway between anterior superior iliac spine and the symphysis pubis, with the subject in the supine position.

In study II, 10 B-scans (point estimates) were taken and measurements of the arterial wall thickness and its layers were performed off-line. Means of the ten measurements were calculated and used in the analysis. In study III, means of duplicate estimates, each one calculated as the average of 10 consecutive measurements, were used in the analyses.

Histomorphometry

In Study I, after the animals had been sacrificed a 2-cm section of the sonographed artery segment was taken and immediately submerged in freshly prepared Millonig’s constant osmolality phosphate buffered with 2.5% glutaraldehyde. The specimens were stored for fixation at 4°C for 12-18 hours, placed in perforated plastic cassettes for ethanol dehydration, xylene substitute clearing and paraffin embedment. From each vessel 15 slices (4μm thick) were sectioned with a microtome, stained with hematoxylin-eosin to assess general histological features and with Verhoeff’s Iodine Iron stain to identify elastic fibers.

The histological sections were analyzed with a digital video system (Image-Pro® Plus version 3.1, Media Cybernetics, Silver Spring, USA). A television camera linked to a light microscope reproduced the original slide on the analyzer video screen computer. After calibration for the 4x objective, the distance of 1 mm was equivalent to 767.509 pixels, which made possible to measure distances as small as 0.001 mm.

Twenty measurements of intima, media and adventitia layers were obtained from up to 5 different slices of each vessel at those sites where the arterial wall was thinnest, in order to avoid artificial thickening due to tangential cuts. Total arterial wall was calculated as the sums of the three layers measured at a site. Mean values from the 20 measurements for each layer and for total arterial wall were used in the analysis. Histological measurements were determined by the same investigator unaware of the ultrasonographic data.

A calculation of the mean media thickness was also estimated on 3 slices of each artery by using a similar image analysis system (Leica Q500 MC Image analysis system, Leica Microscopie Systèmes S. A. Heerbrug, Suisse). After calibration for the 2.5 objective 1 mm was equivalent to 371.747 pixels. Mean media thickness was calculated as the difference between the radius of
the external circumference (External Elastic Lamina, EEL) minus the radius of the internal circumference (Internal Elastic Lamina, IEL).

A surrogate estimate of shrinkage artifact induced by histological preparation was expressed as the mean of all percentage differences between ultrasonographic and histological estimates of the total arterial wall calculated as: (ultrasound estimates - histology estimates)/ultrasound estimates x 100.

**Diagnosis of cardiovascular diseases**

In study II, all subjects underwent a physical examination and filled a form including medical history of CHD (myocardial infarction (MI), angina pectoris, coronary by-pass or balloon angioplasty), heart failure, stroke, hypertension, diabetes and hyperlipidemia. CVD was defined as presence of CHD, heart failure, stroke or hypertension. Coronary heart disease outcomes were defined by ICD-10 (International Classification of Diseases, 10th revision) codes I20-I25, stroke by ICD codes I60-I69, hypertension by ICD codes I10-I15, diabetes by ICD codes E10-E14 and hyperlipidemia by ICD codes E78. Durations of hypertension, diabetes and hyperlipidemia were estimated as years of treatment for these disorders.

**Arterial stiffness assessments**

In Study IV, arterial stiffness was assessed noninvasively by measuring the pulsatile diameter changes with ultrasonography and relating them to the external blood pressure of the right upper arm. A B-mode real-time ultrasound scanner fitted with a 5.0 MHz linear array transducer (E2U-PL22, Hitachi, Tokyo, Japan) was used. The real-time scanner was attached to a vessel wall moving detector equipped with an electronic echo-tracking instrument (Diamove, Teltec AB, Lund, Sweden) which automatically locked to the lumen-intima interfaces at both the near and far artery walls. A loop circuit restored the position of an electronic gate relative to the moving echo indicating instantaneously any vessel diameter changes. Repetition frequency of the echo-tracking loops was 870 Hz and time resolution approximately 1.2 ms. The smallest detectable movement is 7.8 μm (116). Figure 2 shows a sonographed carotid artery with the echo-tracking instrument locked to the lumen-intima interfaces at the near and far walls.

The arteries were visualized in a longitudinal section on the real time image of the ultrasound scanner: 2 cm proximal to the bifurcation in the left common carotid (LCCA) and in the left common femoral (LCFA) and 3-4 cm proximal to the bifurcation in the aorta. Three sequential vessel diameter curves from each artery were stored on a personal computer. Each reading contained 5-10 cardiac cycles. Systolic (SBP) and diastolic blood pressure (DBP) were measured in the left brachial artery by the auscultatory method,
immediately after estimation of the diameters in each artery and used in the
calculations of stiffness for the corresponding artery, as previously validated
(117).

Two indices of arterial stiffness were used: the stress-strain elastic modulus
(Ep) and the β index. The elastic modulus was calculated as the ratio of
stress (arterial pulse pressure; SBP-DBP) to strain (relative diameter (D)
change during the cardiac cycle) (118).

\[
\text{SBP - DBP}
\]

\[
\text{EP} = \frac{K \times (D \text{ systolic} - D \text{ diastolic})}{D \text{ diastolic}}
\]

SBP and DBP are expressed in mmHg and systolic and diastolic diameters
(D) in mm. Ep is expressed in \(10^{-5}\) Newton/m\(^2\), K=133.3 is the factor for
converting mmHg to Newton/m\(^2\) (117).

The β index, similar conceptually to the elastic modulus, was calculated
as the natural logarithm of the systolic and diastolic pressure ratio, divided
by strain. It was developed to reduce the impact of pressure on the measure-
ments of stiffness (104).

\[
\text{ln (SBP / DBP)}
\]

\[
\text{stiffness } \beta = \frac{\ln (\text{SBP} / \text{DBP})}{(D \text{ systolic} - D \text{ diastolic}) / D \text{ diastolic}}
\]

**Serum hormone concentrations**

In Study III and IV serum samples were drawn between 8 AM and 10 AM
after an overnight fast and frozen at –70° C until analyzed in batches. In the
estrogen users, serum samples were not standardized to time since last inser-
tion of estradiol implant. Serum E2 levels were measured by fluoroimmune
assay, (AutoDelfia, Wallac Oy, Turku, Finland), with an intra-assay vari-
ation of 2.7%. Sex hormone-binding globulin was measured by radioimmun-
assay at Medilab (Malmö, Sweden), with an intra-assay variation of 3.0%. Serum FSH concentrations were measured consecutively using Delfia hFSH
from Pharmacia, Wallac Oy, Finland. The total coefficient of variation was
3.6%.
Serum lipids

In study IV, serum total cholesterol was assayed by enzymatic techniques (Instrumentation Laboratories, Lexington, MA) in a Monarch 2000 centrifugal analyzer, the inter-assay coefficient of variation of 4%. Serum LDL-cholesterol was calculated using Friedewald’s formula after separation of HDL-cholesterol by precipitation with magnesium chloride/phosphotungstate.

Statistical methods

Data are presented as mean ± standard deviation (SD). Pair-wise differences between long-term estrogen users and age-matched nonusers (studies III-IV) were tested by paired t-test for normally distributed variables, and by Wilcoxon Signed-Rank test for those not normally distributed. Comparison between independent groups (studies II-III-IV) was done by 2-sample t-test or Wilcoxon Rank Sum test, as determined by the test for normality. Normality of the distributions was tested by Shapiro-Wilk W Test. The Chi square test (study II) and the McNemar’s test (studies III-IV) were used for comparing distributions of categorical variables. Spearman-rank correlation tests were used to test correlation between variables (studies I-II-III-IV). A two-tailed p-value of <0.05 was considered as significant.

The coefficient of variation (CV%), based on double measurements, was estimated according to the formula: CV = 100 (SD/x) and SD = \sqrt{\sum d^2 / 2n}, where SD = standard deviation, x = mean of all measurements, d = difference between duplicate measurement values and n = number of duplicate determinations (119).

In study III, ANCOVA, with Group and Matched Pair-number as factors and e.g. blood pressures (SBP and DBP) and BMI as covariates, was performed to adjust for differences in blood pressure or BMI within each matched pair.

In study IV, pair-wise differences in stiffness were regressed on age to test if the age-associated increase in stiffness behaved differently in estrogen users and nonusers. From the tests of correlation, three covariates were selected (SBP, BMI and LDL-cholesterol) and used in a multiple linear regression model for matched samples to test the difference between groups after adjustment. The Bonferroni correction was used in all correlation analyses to insure an overall type I error rate of 0.05. After the Bonferroni correction, only results with P < 0.005 were regarded as significant and 0.005<P < 0.01 as marginally significant. To avoid false interpretations due to low power in study IV, post-hoc power analysis was used to calculate the detectable mean difference for stiffness indices Ep and β with an 80% power at the 0.05 level.

All statistical analyses were performed with the statistical program packages JMP® or SAS® (SAS Institute Inc., Cary, NC, USA).
Results

Validation of a novel noninvasive high-frequency ultrasound technique in imaging the artery wall (study I)

When considering all vessels combined (cutaneous and femoral) we found a significant correlation between ultrasonographic estimates of total artery wall thickness taken in vivo, with those obtained in vitro by histomorphometry, \( r_s = 0.78, P = 0.0001 \). Also for all vessels, a significant correlation was found between ultrasonographic and histological estimates of the media and adventitia layers expressed as a percentage of the wall \( r_s = -0.51 P = 0.03, r_s = -0.49, P = 0.03 \), respectively). However, the surrogate estimate of shrinkage artifact indicated that shrinkage in the cutaneous arteries was only half (16%) of that in the femoral arteries (34%). Therefore, analysis of association were given separately for cutaneous and femoral arteries.

For the superficial cutaneous arteries, a significant correlation was found for total artery wall thickness \( r_s = 0.69, P = 0.01 \) and media layer \( r_s = 0.76, P = 0.004 \), Figure 3. For the femoral arteries, no significant correlations were found for any of the artery layers. Those arteries were more deeply located than the cutaneous arteries and thus, more difficult to image. Adventitial thickness was underestimated by ultrasound in the femoral arteries and intima thickness overestimated in both femoral and cutaneous arteries, when compared with histomorphometry.

Precision error for estimates of artery wall thickness obtained in humans by high-frequency ultrasound

In our pilot human study, the precision errors (CV%), calculated from duplicate estimates in 11 women, were 4.1%, 3.4% and 3.9% for carotid total wall, media and intima thickness, respectively. For the femoral artery the corresponding values were 5.5%, 4.0% and 4.8%, respectively.

Clinical usefulness and validity of separate estimates of intima and media thickness and intima/media ratio (study II)

Mean values for carotid total wall thickness, media thickness and intima thickness obtained noninvasively by using 25 MHz frequency ultrasound were significantly higher in men than in women (all \( P < 0.05 \)), Table 1. The prevalence of cardiovascular diseases was similar in both sexes.

Regarding the whole study group, (n=100), subjects with a diagnosis of CVD, CHD, MI or stroke presented with a significantly thicker carotid intima layer (all \( P < 0.0001 \)), thinner media layer (all \( P < 0.05 \)) and substantially (74% to 195%) higher intima/media ratio (all \( P < 0.0005 \)) than healthy subjects. Subjects with hypertension or hyperlipidemia also had a thicker
carotid intima thickness than subjects without these diagnoses (\( P < 0.0005 \) for both). Similar results were obtained in women and men.

Table 1. Mean values (mm) \( \pm \) SD for total artery wall thickness, media, intima and intima/media (I/M) ratio in men and women.

<table>
<thead>
<tr>
<th></th>
<th>Total wall</th>
<th>Media</th>
<th>Intima</th>
<th>I/M ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Men</td>
<td>0.80 ± 0.2</td>
<td>0.46 ± 0.2</td>
<td>0.20 ± 0.1</td>
<td>0.52 ± 0.3</td>
</tr>
<tr>
<td>Women</td>
<td>0.66 ± 0.1</td>
<td>0.37 ± 0.1</td>
<td>0.18 ± 0.1</td>
<td>0.60 ± 0.4</td>
</tr>
</tbody>
</table>

\( *P \) between groups

Carotid intima thickness was positively associated with presence of CVD risk factors, i.e., weight, BMI, waist, hip and waist/hip ratio (all \( P < 0.005 \)), as well as with years of hypertension, years of hyperlipidemia and number of cigarettes/week (all \( P < 0.05 \)). Intima thickness had also borderline significance with years of smoking (\( r = 0.19; P = 0.057 \)). The intima/media ratio was positively associated with BMI, years of hyperlipidemia and number of cigarettes/week (all \( P < 0.05 \)). No significant associations were found between media thickness and CVD risk factors.

Long-term estrogen therapy initiated at the time of menopause and thickness of the artery wall layers (study III)

In studies III and IV, the serum levels of E2 and FSH in the estrogen users were in the normal range for women of fertile age. Long-term estrogen users had a significantly higher BMI than age-matched nonusers and non-significantly lower total cholesterol and LDL-cholesterol levels than nonusers.

Long-term estrogen users had significantly thinner mean carotid intima layer (-25%; \( P = 0.0002 \)), thicker media layer (+74%; \( P = 0.0002 \)) and a substantially lower intima/media ratio (-54%; \( P < 0.0001 \)), compared with age-matched nonusers.

Estrogen users also had significantly higher mean values for femoral media thickness (+51%; \( P < 0.003 \)) but lower femoral intima thickness (-23%; \( P = 0.010 \)) and intima/media ratio (-50%; \( P = 0.0003 \)). After adjustment for differences in SBP, DBP and BMI the above differences remained significant except for femoral media thickness. After exclusion of subjects on anti-hypertensive drugs these differences remained significant except for femoral media thickness.

Mean values of carotid intima, carotid media and femoral intima thickness in estrogen users did not differ significantly from those in women of fertile age (+9%, -13% and 0%, respectively) whereas mean femoral media thickness was lower (-22%; \( P = 0.04 \)). In contrast, postmenopausal estrogen nonusers had significantly thicker intima, thinner media and higher values.
for intima/media ratio compared to women of fertile age. The proportional difference in mean media thickness was similar in the carotid and femoral artery (about -50%).

Long-term estrogen therapy initiated at the time of menopause and arterial stiffness (study IV)

Compared with women in fertile age, both postmenopausal groups had significantly higher stiffness indices $E_p$ and $\beta$ (carotid $P < 0.001$, femoral $P < 0.05$, aorta $P < 0.001$) and significantly higher SBP and DBP levels ($P < 0.05$). The coefficient of variation (CV%) for estimates of stiffness of the carotid artery based on 10 double measurements in 25 women was 5.0% for $E_p$ and 5.8% for stiffness index $\beta$.

Carotid, femoral and aortic stiffness indices were similar in long-term estrogen users and nonusers and did not differ significantly, Figure 4. Further, after adjustment for SBP, BMI and serum LDL-cholesterol, the absence of significant differences for stiffness remained, as well as after exclusion of subjects on antihypertensive drugs. The tests of the slopes for the pair-wise differences in stiffness regressed on age were not significant for any vessel ($P = 0.54$ for carotid, $P = 0.3$ for femoral and $P = 0.88$ for aorta).

Compared with women in fertile age, carotid diameters were significantly larger in both postmenopausal groups whereas aortic diameter was larger only in estrogen users. Carotid and aortic fractional diameter changes (arterial strain) were significantly smaller in both postmenopausal groups ($P < 0.05$ for both) when compared with women of fertile age. The three study groups did not differ significantly in femoral diameters (absolute values or fractional changes).

Increasing age presented no association with arterial stiffness in long-term estrogen-users but had positive although weak associations with carotid stiffness in postmenopausal nonusers (carotid $\beta$, $r = 0.56$; $P = 0.02$) and with aortic stiffness in premenopausal women (aortic $E_p$ and $\beta$, $r = 0.45$; $P = 0.04$), that did not remain after Bonferroni correction. In estrogen users, no significant association was found when stiffness was regressed on estradiol levels or duration of estrogen treatment. After Bonferroni correction, significant positive associations remained between femoral stiffness $\beta$ and total cholesterol ($r = 0.70$; $P = 0.002$) and BMI ($r = 0.65$; $P = 0.004$) in estrogen users. In postmenopausal nonusers, significant positive associations remained between SBP and carotid $E_p$ ($r = 0.68$; $P = 0.003$) and both femoral stiffness $E_p$ and $\beta$ ($r = 0.72$; $P = 0.001$ and $r = 0.65$; $P = 0.004$, respectively), whereas serum lipids had inverse associations with femoral stiffness $\beta$ (total cholesterol $r = -0.63$; $P = 0.008$; LDL-cholesterol $r = -0.65$; $P = 0.005$). In the women of fertile age, SBP had positive associations with aortic $E_p$ ($r = 0.69$; $P = 0.0007$) and carotid $E_p$ ($r = 0.56$; $P = 0.011$).
Discussion

In the validation study (study I), our findings are sustained by the identification of a three-layered artery wall structure in all arteries with both ultrasonography and histology. The total artery wall thickness was mainly dependent on the media layer, which represented 70% of the total wall on ultrasonography and 76% on histology, while the adventitia and intima layers together represented about 30%. These findings are according with the well-known normal artery wall structure of elastic and muscular arteries (120).

Ultrasonographic estimates of the adventitia layer presented no correlation with histomorphometry and this layer seems to be underestimated by ultrasonography, particularly regarding to the femoral arteries. One reason may be that the external elastic lamina could account for the brightest echoes observed in the adventitia and that the loose tissue conforming the adventitia layer is not able to be imaged with this technique.

Attenuation when the ultrasound wave passes through various tissues becomes more pronounced at higher frequencies (68). Therefore, the resolution is probably better for the structures situated closest to the probe with this method and it can explain the difficulties to visualize the more deeply located femoral artery. This could also account for the poor correlation between ultrasonographic and histomorphometric estimates of intima layer and the apparent overestimation of intima thickness on the ultrasonography. Further, the anatomical structures generating the ultrasonographic image of the intima are small in non-atherosclerotic vessels and may have a size below the resolution limit. Consequently, width of the echo obtained will not accurately correspond to the anatomical structure (68).

The present noninvasive 25 MHz frequency ultrasound method thus gave valid estimates of media thickness and total artery wall thickness on superficial arteries of young, non-atherosclerotic pigs. However, the value of estimating intima thickness by using this method could be further established only by examining arteries with and without developed intima thickening and atherosclerosis. The main findings of study II seem to sustain that value, as separate estimates of intima thickness, as well as media thickness and intima/media ratio could significantly discriminate between subjects with and without atherosclerosis and CVD. The substantial difference in intima/media ratio between subjects with CVD and healthy subjects found in this study clearly results from the known divergent changes in intima and media thicknesses associated with arterial disease. These findings were consistent and similar in women and men, thus suggesting that separate estimates of intima and media thickness and the obtained intima/media ratio might be a valuable tool in the study of pathophysiological changes on the artery wall in both sexes.
In studies II-III, our values for common carotid intima plus media thickness are smaller than IMT obtained by using 7-8 MHz frequency ultrasound. Our values, however, are very similar to those reported in men of similar age (121) and in postmenopausal women (122) by using noninvasive 10 MHz frequency ultrasound. Similar to our data, a significantly thicker IMT have been reported in men when compared to women by using 7-8 MHz frequency ultrasound (123, 124).

The main findings of study III indicate that long-term estrogen therapy, initiated at the time of menopause, preserves a thin intima thickness and maintains media thickness and intima/media ratio at values similar to those of premenopausal women. Estrogen therapy seems to prevent/delay the known increase in intima and decrease in media thickness described with aging (85) and development of atherosclerosis (85, 97). A preserved morphology of the artery wall into high age, including a maintained thin (and presumably healthy) intima may help to explain the well-documented beneficial cardiovascular effects of estrogen therapy initiated early after menopause that have been reported from basic research (54), animal models (39, 45), controlled clinical interventions (45, 79) and in numerous reports from observational studies (12, 125, 126) and meta-analysis (13, 14, 33).

Although the mean age of the postmenopausal women investigated in studies III-IV is similar to that in women of the HERS (18) and WHI (19) studies, our study subjects were on estrogen-only therapy that was initiated at the time of the menopause and before development of severe atherosclerosis. Therefore, our data should only be regarded with respect to the women who started HT at the younger ages in the WHI study and cannot in any respect address the initial increase in cardiovascular events observed in the HERS and WHI studies when women initiated combined HT at a high age (19, 127). However, our data is in agreement with the age-stratified analysis of the WHI study that seem to support beneficial cardiovascular effects when hormone therapy is initiated at early menopausal ages. In the WHI study, the increased risk of CHD events was restricted to women starting HT 20 years or more after menopause (HR = 1.71), whereas women starting HT within 10 years after menopause had a point estimate below unit (HR = 0.89) (22). Similarly, in the estrogen-only treated arm of the WHI study, women starting therapy early after menopause, between 50 and 59 years, had values that might indicate a reduced risk of coronary heart disease (CHD) events, HR = 0.56 (0.30 – 1.03), and a point estimate for total death below unit, HR = 0.73 (0.47 – 1.13) (22).

Our results support the findings in two previous studies, using 22.5 MHz high-frequency ultrasound, indicating preservation of the carotid artery media layer in younger postmenopausal women after short-term estrogen therapy (94, 95). In the present study, however, subjects had a considerably
higher mean age and a longer duration of estrogen treatment (20 years on average), and both an elastic (carotid) and a muscular (femoral) artery were investigated. We also included a group of healthy women of fertile age was included as a premenopausal reference group, to illustrate the thickness of artery wall layers before the development of severe age-related changes.

The general loss of muscle and connective tissue elements with advancing age and their preservation/increase by estrogen therapy (128, 129), might help to explain the observed thinning of the artery media layer with normal aging and its preservation in estrogen users. Estrogen users, compared to premenopausal women, had a significantly lower media thickness in the femoral but not in the carotid artery which might indicate better preservation of the elastic carotid media than of the muscular femoral media.

A reduction of the media thickness of the carotid artery with aging (study III) as well as in subjects with CVD or CHD (study II) may appear inconsistent with the abundant data of an increase in carotid IMT associated with presence of CVD, as assessed by 7-8 MHz frequency probes (70). However, the increase in IMT estimates represents predominantly an increase in intima thickness, as shown in individuals in the seventh and eighth decades of life (84). Moreover, by using high-frequency intravascular ultrasound, Gussenhoven et al. could demonstrate both in vitro (40 MHz) and in vivo (30 MHz) a decrease of the media thickness inversely related to the increase in atherosclerotic lesion thickness (97). Morphometric studies in elderly subjects have further shown inverse correlations between media thickness and degree of stenosis in the aorta, the carotid, coronary, and cerebral arteries (85). The medial thinning observed by high-frequency intravascular ultrasound is inversely related to the extent of intimal thickening and seems to indicate that medial thinning is an essential part of the atherosclerosis process (97).

Medial attenuation in relation to atherosclerotic plaques is poorly understood. By investigating segments of carotid artery and descending aorta, Van der Wal et al. found that increasing severity of intimal lesions was accompanied by a significant increase in medial inflammation and vascularization and by a significant decrease in medial thickness, suggesting that the inflammatory reaction in the media relates to atherosclerosis, has a remodeling effect on medial tissues and may cause medial attenuation (130). Inflammation plays a central role in the pathogenesis of many forms of vascular disease, including atherosclerosis. Atherogenesis begins with endothelial damage, and the damaged endothelium expresses adhesion molecules, chemokines, and proinflammatory cytokines that direct atherosclerotic plaque formation (62). By inducing balloon injury at the carotid artery in ovariectomized rats randomized to estradiol-only, MPA, combined HT or placebo, Xing et al. found that estrogen therapy reduced the granulocyte and monocyte/macrophage populations of injured vessels by approximately 50% (51). These effects were completely blocked by adding MPA. Estradiol may thus
limit the neointimal response to endoluminal vascular injury, at least in part, by limiting leukocyte entry from adventitial/periadventitial tissues into injured vessels early in the injury response (51).

Menopausal HT has been shown to have negative modulatory effects on inflammatory markers, including E-selectin, vascular cell adhesion molecule-1, intercellular adhesion molecule-1, monocyte chemoattractant protein-1, and tumor necrosis factor-alpha, inconsistent effects on interleukin-6, and stimulatory effects on transforming growth factor-beta, a vasoprotective cytokine (62). Oral conjugated estrogens, but not transdermal estradiol, have been shown to increase C-reactive protein, a circulating proinflammatory cytokine produced in both liver and atherosclerotic arteries (131). Although C-reactive protein is clearly linked to increased cardiovascular disease risk in women, the hormone-induced rise in this biomarker may be related to a first-pass effect of C-reactive protein production in the liver after oral estrogen absorption (62).

In study IV, we found no significant differences in arterial stiffness between long-term estrogen users and age-matched nonusers, before or after adjustment for potential confounders and after exclusion of subjects with antihypertensive drugs. Further, the tests of slopes for pair-wise differences in stiffness regressed on age were not significant, supporting that long-term estrogen therapy does not have any substantial effect on the age/menopause-related changes in arterial stiffness of the central elastic and muscular arteries.

Our findings contrast to those reported by Nagai et al. indicating decreased carotid stiffness β index in young postmenopausal women (mean age 55.6 years) receiving ET/HT for at least 6 months (115). However, our results might be in accordance with investigations on regional stiffness estimated by other noninvasive methods. Tanaka et al. found no differences in aortic and carotid stiffness estimated by pulse wave velocity (PWV) and applanation tonometry, respectively, when comparing postmenopausal HT users of about 60 years of age and untreated controls (132), and McGrath et al. reported no significant difference in carotid stiffness estimated as distensibility coefficient (DC) between women on HT (mean age 60) and untreated controls (133). Liang et al. found significantly reduced carotid stiffness in estrogen users (mean age 60 years), compared with age-matched nonusers, with the DC method but not with Young’s modulus (134). The PWV, however, estimates wall mechanics indirectly and not the local elastic properties, while elastic modulus Ep and β are the indices conceptually closest to local arterial stiffness (99). In the present study subjects were older and had been treated with ET for a long time, compared to previous reports. The present and previous reports might indicate that any effects estrogen may have on stiffness are substantially reduced by time and aging.

In the present and previous reports (104, 121), associations between stiffness and age were less steep for femoral artery than for carotid and aorta.
Differences for the femoral artery, between postmenopausal groups and women of fertile age, were smaller than for carotids and aorta, although still significant. Differences in structural and functional characteristics of the different vascular beds might explain why stiffness increases more in the elastic carotids and aorta and less in the muscular femoral artery by age (98, 103).

Stiffness is determined principally by the elastin-to-collagen ratio in the vessel wall (98). Both components are responsible for the non-linear shape of the pressure/diameter curve of the vessel (98) and both change with aging. Estrogens have shown to increase the proportion of the elastic fibers over collagen thus decreasing the stiffness of the vessel wall in rat aorta (135). Arterial stiffness may also be influenced by more distal vascular bed characteristics or may be partly under the functional control of the endothelium (133) as suggested by the significant relationship shown between arterial compliance and total peripheral resistance to indices of endothelial function (136). Gangar et al. found a positive association between pulsatility index (PI) of the internal carotid artery and time since menopause (137). Reduced PI (137) and improved endothelial-dependent vasodilation (138) have also been reported after short-term ET, as well as reduced carotid resistance index (RI) after long-term ET (139). Randomized controlled studies have shown reduction of carotid PI after 6 months and one year of HRT (140, 141) or no significant changes after 6 months of HRT (142). Further, no changes in arterial impedance to flow or endothelial vasomotor function after one and three years of HRT have been reported (143, 144), suggesting a transient effect of HRT on arterial vasodilation.

Adjustment for systolic blood pressure, BMI, and LDL-cholesterol levels, known determinants of arterial stiffness (98, 99), did not change the results. However, it may be inappropriate to adjust for differences in BMI and LDL-cholesterol levels because both are most likely effects of the estrogen therapy by preserved bone mass and probably also muscle mass (145). The cross-sectional design does not exclude a phase earlier in treatment when the treated group did exhibit lower stiffness than the untreated group, that was later outweighed by effects of aging. However, analysis of pair-wise differences regressed on age do not support such a mechanism.

The small sample size in our study, in combination with no significant differences between estrogen users and nonusers may raise the possibility of a type-II error. However, the mean pair-wise differences were numerically very small and the P values far from statistical significance. Further, according to post hoc power calculation, mean differences of about 1/3 of the standard deviation of stiffness indices could be detected, differences that are considerably smaller than those assumed to be of clinical importance. Hirai et al. reported a difference of about 4 units for carotid β index and 13 units for aorta β index between patients with myocardial infarction and healthy subjects of about 60 years of age (100) and Gatzka et al. reported a differ-
ence of 7 units in aorta $\beta$ index between patients with newly diagnosed coronary artery disease (mean age 63 years) and healthy age-matched subjects (146). Those differences were statistically significant and of apparent clinical relevance.

With regard to the validity, our estimates of stiffness indices and the standard deviations were numerically very similar to those previously reported in the literature for women of similar ages (105, 113). Moreover, we found highly significant differences (4 to 6 units) for stiffness $\beta$ indices between premenopausal women and either of the postmenopausal groups, Figure 4. The coefficients of variation of about 5% in our study were also low for this type of estimates.

**Study limitations:** The CVD categories presented in study II, i.e., CHD, MI, stroke and hypertension, are overlapping and may include other CVD. Due to the relative small sample of 100 subjects, further analysis by excluding subjects with combined CVD diagnoses were not performed. The inclusion of individuals of the same age only, makes it impossible to estimate the effect of aging on the thickness of the artery wall layers within the study group. The cross-sectional design in studies III-IV may raise the possibility that selection bias (presence of differences between the groups before the start of estrogen treatment) may have affected our results. However, any bias at the time of the menopause would probably have only a limited impact on artery thickness and stiffness 20 years later, compared with presumed effects of aging and long-term estrogen therapy (on average 20 years). The present studies address effects of estrogen-only therapy and not estrogen/progestogen combined therapy. Further, we did not monitor dietary variables that might have modified the rate of atherosclerotic development.
General conclusions

The following conclusions can be drawn from the results of the performed investigations:

Noninvasive 25 MHz high-frequency ultrasound could image superficial arteries in vivo. The method gave valid estimates of total artery wall and media thickness in the pig. Adventitia thickness was underestimated and intima thickness overestimated when non-atherosclerotic vessels were imaged.

Separate estimates of carotid intima and media thickness and the obtained intima/media ratio assessed by 25 MHz high-frequency ultrasound differed significantly between subjects with and without CVD. This method might be a useful tool in monitoring changes in artery wall morphology associated with aging and development of atherosclerosis.

Long-term estrogen therapy initiated at the time of menopause may counteract the increase in intima and decrease in media thickness associated with aging and development of atherosclerosis. The maintenance of a thin intima layer with preservation of the intima/media ratio, might be a mechanism for the documented cardioprotective effects of estrogen when therapy is initiated at or early after menopause.

Long-term estrogen therapy has no substantial effect on the age-related changes in arterial stiffness estimated at the central elastic and muscular arteries. This suggests that any long-term protective effect that ET has on cardiovascular disease is unlikely to be mediated by an impact on arterial stiffness.
Figures

Figure 1. Ultrasonographic B-scan (top) and A-scan (bottom) images of the near common carotid artery wall obtained in a 40 year-old woman by using noninvasive 25 MHz frequency ultrasound. The highest peaks in the A-scan correspond to the hyperechogenic layers adventitia and intima imaged in the B-scan. The total artery wall thickness measured here from the leading edge of the adventitia to the far edge of the intima was 0.86 mm.
Figure 2. Ultrasonic B-image of the common carotid artery in a 34 year-old woman with the echo-tracking cursors locked to the intima-lumen interface at the near and far wall of the vessel (top). The mean diameter curve obtained from ten consecutive cardiac cycles is also shown (bottom).
*Spearman Rank Correlation Test

Figure 3. Correlations between ultrasonographic and histomorphometric estimates of total wall (top) and media thickness (bottom) for cutaneous arteries
Figure 4. Stiffness index $\beta$ at the carotid, femoral and aorta in the three study groups. Scale is in arbitrary units (stiffness units)
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42
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