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**SOY SUPPLEMENTATION AND ROLE OF EQUOL PRODUCTION  
CAPABILITY IN POSTMENOPAUSAL WOMEN USING TIBOLONE:  
EFFECTS ON CARDIOVASCULAR RISK MARKERS**

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**Academic Dissertation**

To be presented and publicly discussed by permission of the Medical Faculty of the University of Helsinki, in the Seth Wichmann Auditorium, Department of Obstetrics and Gynecology, Helsinki University Central Hospital, Haartmaninkatu 2, Helsinki, on November 28<sup>th</sup> at 12 noon.

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ISBN 978-952-92-4730-1 (paperback)  
ISBN 978-952-10-5110-4 (PDF)  
<http://ethesis.helsinki.fi>

Helsinki University Print  
Helsinki 2008

*To my family*

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ORIGINAL PUBLICATIONS

## LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals:

- I Appt S\*, Törmälä R\*, Mikkola TS, Tikkanen MJ, Ylikorkala O, Franke AA, Clarkson TB. Soy-Tibolone Combination – effect on lipids in postmenopausal monkeys and women. *Maturitas* 2008; 60: 216-222.
- II Törmälä R, Appt S, Clarkson TB, Tikkanen MJ, Ylikorkala O, Mikkola TS. Individual differences in equol production capability modulate blood pressure in tibolone treated postmenopausal women; lack of effect of soy supplementation. *Climacteric* 2007; 10: 471-479.
- III Törmälä R, Appt S, Clarkson TB, Groop P-H, Rönnback M, Ylikorkala O, Mikkola TS. Equol production capability is associated with favorable vascular function in postmenopausal women using tibolone; no effect with soy supplementation. *Atherosclerosis* 2008; 198: 174-178.
- IV Törmälä R, Appt S, Clarkson TB, Mueck AO, Seeger H, Mikkola TS, Ylikorkala O. Impact of soy supplementation on sex steroids and vascular inflammation markers in postmenopausal women using tibolone: role of equol production capability. *Climacteric* 2008; 11: 409-415.

\* equal contribution

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## ABBREVIATIONS

AIx	augmentation index
BMI	body mass index
CEE	conjugated equine estrogen
CHD	coronary heart disease
CIMT	carotid artery intima-media thickness
C/L	casein/lactalbumin
CVD	cardiovascular disease
CRP	C-reactive protein
DHEA	dehydroepiandrosterone
DHEAS	dehydroepiandrosterone sulfate
EFI	endothelial function index
EPT	estrogen-progestagen therapy
ER	estrogen receptor
ER $\alpha$	estrogen receptor alpha
ER $\beta$	estrogen receptor beta
E-selectin	endothelial-selectin
ET	estrogen therapy
HDL	high-density lipoprotein
HT	hormone therapy
ICAM-1	intercellular adhesion molecule-1
LDL	low-density lipoprotein
NO	nitric oxide
P-selectin	platelet-selectin
PWA	pulse-wave analysis
SEM	standard error of mean
SERM	selective estrogen receptor modulator
SHBG	sex hormone-binding globulin
VCAM-1	vascular cell adhesion molecule-1
VLDL	very low-density lipoprotein



## ABSTRACT

Tibolone, a synthetic steroid, is effective in the treatment of postmenopausal symptoms. The cardiovascular safety profile of tibolone has been questioned, because it reduces the levels of cardioprotective high-density lipoprotein (HDL) cholesterol. Soy-derived isoflavones may offer health benefits, particularly as regards circulating lipids and also other cardiovascular risk factors. The soy-isoflavone metabolite equol is thought to be the key as regards soy-related beneficial effects. We studied the effects of soy supplementation on various cardiovascular disease (CVD) risk factors in postmenopausal monkeys and postmenopausal women using tibolone. Additionally, the impact of equol production capability was studied.

A total of 18 cynomolgus monkeys were assigned to receive casein/lactalbumin (C/L) (placebo), tibolone, soy (a woman's equivalent dose of 138 mg of isoflavones per day), or soy with tibolone in a random order in such a way that all animals received all four diets. Each treatment phase lasted for 14 weeks, and there was a 4-week washout diet (C/L) before starting the next treatment regimen. Postmenopausal women undergoing long-term tibolone use (N=110) were screened by means of a one-week soy challenge to find 20 women with equol production capability (4-fold elevation from baseline equol level) and 20 control women. Both groups were treated in a randomized placebo-controlled cross-over trial with a soy powder (52 g of soy protein containing 112 mg of isoflavones per day) or placebo for 8 weeks. Before and after the treatments lipids and lipoproteins were assessed in both monkeys and women. In addition, blood pressure, arterial stiffness, endothelial function, sex steroids, sex hormone-binding globulin (SHBG), and vascular inflammation markers were assessed before and after soy and placebo supplementation.

A 14% increase in plasma low-density lipoprotein (LDL) + very low-density lipoprotein (VLDL) cholesterol was observed in tibolone-treated monkeys versus the placebo group (Tibolone vs. C/L  $p=0.004$ ). Soy treatment resulted in a 18% decrease in plasma LDL+VLDL cholesterol (Soy vs. C/L  $p=0.0002$ ), and concomitant supplementation with tibolone did not negate the LDL+VLDL cholesterol-lowering effect of soy (Soy+Tibolone vs. Soy  $p=0.35$ ). A 30% increase in HDL cholesterol (Soy vs. C/L  $p<0.0001$ ) was observed in monkeys fed with soy, whereas HDL cholesterol levels were reduced (~48%) after tibolone (Tibolone vs. C/L  $p<0.0001$ ). Interestingly, Soy+Tibolone diet conserved HDL cholesterol levels (Soy+Tibolone vs. C/L  $p=0.09$ ). Monkeys treated with tibolone alone showed an increase in total cholesterol (TC):HDL cholesterol ratio of 118% compared with placebo (Tibolone vs. C/L  $p<0.0001$ ). Those

treated with Soy or Soy+Tibolone showed reductions of 34% (Soy vs. C/L  $p=0.03$ ) and 2.4% (Soy+Tibolone vs. C/L  $p=0.84$ , Soy+Tibolone vs. Soy  $p=0.05$ ) in the TC:HDL cholesterol ratio.

In postmenopausal women using tibolone, reductions in the levels of total cholesterol ( $p=0.06$ ) and LDL cholesterol ( $p=0.006$ ) were seen after soy supplementation compared with placebo. In contrast to the monkeys, in women soy supplementation had no effect on HDL. In addition, blood pressure, arterial stiffness and endothelial function were unaffected by soy supplementation in women using tibolone. Circulating levels of estrone decreased after soy supplementation, but only in equol producers (12.5% decrease;  $p=0.04$ ), and the levels of testosterone decreased by 15.5% ( $p=0.02$ ) in the entire study population. No changes were seen in the circulating concentrations of androstenedione, dehydroepiandrosterone sulfate (DHEAS), or SHBG. Circulating concentrations of vascular cell adhesion molecule-1 (VCAM-1) increased by 9.2% ( $p=0.06$ ), and platelet-selectin (P-selectin) decreased by 10.3% ( $p=0.002$ ) after soy treatment, whereas C-reactive protein (CRP) and intercellular adhesion molecule-1 (ICAM-1) remained unchanged. At baseline and unrelated to soy treatment, equol producers had lower systolic ( $129.9\pm 2.6$  [SEM] vs.  $138.5\pm 3.1$  mmHg;  $p=0.02$ ), diastolic ( $72.2\pm 1.5$  vs.  $76.6\pm 1.3$  mmHg;  $p=0.01$ ) and mean arterial ( $93.5\pm 1.7$  vs.  $99.9\pm 1.8$  mmHg;  $p=0.007$ ) pressures compared with non-producers. Similarly, baseline arterial stiffness was less ( $25.9\pm 1.1\%$  vs.  $29.6\pm 0.9\%$ ;  $p=0.01$ ), and endothelial function better ( $72.3\pm 5.3\%$  vs.  $55.2\pm 3.8\%$ ;  $p=0.009$ ) in equol producers than non-producers.

To conclude, soy supplementation reversed the tibolone-induced fall in HDL cholesterol levels in postmenopausal monkeys. However, this effect of soy was not seen in postmenopausal women taking tibolone. In these women soy supplementation also failed to modify blood pressure, arterial stiffness and endothelial function, but determined by these CVD risk factors, equol production capability itself was associated with beneficial cardiovascular changes. Our findings imply that there are cardiovascular benefits associated with equol-producing capability, at least in women using tibolone.

## INTRODUCTION

A well established fact is that the risk of cardiovascular diseases (CVDs) increases soon after the onset of menopause (*Rosano et al. 2007*), particularly in women who have entered menopause earlier than normal (*Maxwell 1998, Lobo 2007*). The mechanisms behind this phenomenon are not fully understood, but the decline in the concentrations of estrogens could be a key factor (*Rexrode et al. 2003, Rosano et al. 2007*). This is further supported by data showing that the use of postmenopausal hormone therapy (HT), if initiated early in menopause, may protect against the risk of CVD (*Manson et al. 2007, Mendelsohn and Karas 2007*), although this evidence has been vigorously disputed in recent years (*Barrett-Connor et al. 2005*). However, HT also has unavoidable risks and side-effects, the most feared being the increased risk of breast cancer (*Foidart et al. 2007*). In addition, HT is associated with an increased risk of venous thromboembolic events (*Mendelsohn and Karas 2005*). Alternatives to traditional HT are therefore needed. As CVD continues to be the leading cause of morbidity and mortality among postmenopausal women, the cardiovascular effects of optional menopause treatments need to be evaluated.

Tibolone, a synthetic steroid, is effective in the treatment of climacteric symptoms and in prevention of osteoporosis (*Kenemans and Speroff 2005*). Its use, however, is shadowed by a fall in the circulating concentrations of cardioprotective high-density lipoprotein (HDL) cholesterol (*Mikkola and Clarkson 2002, Notelowitz 2007*), and by increased progression in carotid artery intima-media thickness (CIMT) (*Bots et al. 2006*). Therefore, the cardiovascular safety of tibolone has been questioned.

Postmenopausal women often use various botanical supplements (*Albertazzi and Purdie 2002*), concomitantly with traditional HT or tibolone (*Mahady et al. 2003, Low Dog 2005*). Among alternative treatments are phytoestrogens, plant-derived compounds possessing estrogenic activity (*Murkies et al. 1998*) and with features of Selective Estrogen Receptor Modulators (SERMs) (*Wuttke et al. 2003*). Isoflavones, which represent the main class of the most common phytoestrogens, are abundantly present in soybeans (*Murkies et al. 1998, Valachovicova et al. 2004*). Soy-derived isoflavones have been considered as potential alternatives to traditional HT, although they have little or no effect in the relief of climacteric symptoms (*Nikander et al. 2003a, Tempfer et al. 2007*). Compared with Western countries, the consumption of soy is high in Asia, where the incidences of CVD and breast cancer are low. This may partly be explained by differences in lifestyle and diet (*Tikkanen and Adlercreutz 2000, Valachovicova et al. 2004*), but

the use of soy may be an additional factor. In this regard, equol, an isoflavone metabolite, may be in a key position (*Setchell et al. 2002, Setchell et al. 2005*). It is produced by intestinal bacterial flora from soy-derived daidzein. This characteristic, which is present only in approximately one third of humans, may also affect steroid metabolism (*Duncan et al. 2000, Frankenfeld et al. 2004a*).

Since tibolone and soy are commonly taken together, it is important to study the extent to which soy supplementation modifies the vascular effects of tibolone. Clinical prospective studies would take years, and therefore, before initiating such studies it is wise to assess the effects of soy in women using tibolone with the aid of established vascular surrogate markers. This was the topic of the present study.

## REVIEW OF THE LITERATURE

### Menopause

In Finland, approximately 40 000-50 000 women enter menopause annually, typically at the age of 50-52 years. As the mean life expectancy for a Finnish woman is almost 81 years, women will live approximately 30 years in a postmenopausal state. Menopause may also result from oophorectomy or chemotherapy ending the function of the ovaries. At menopause, ovarian secretion of estrone and 17 $\beta$ -estradiol drastically decreases, but the ovaries continue to produce androgens, such as testosterone, androstenedione and dehydroepiandrosterone (DHEA) (Figure 1). In addition, the adrenal cortex secretes androstenedione, DHEA and its sulfated form (DHEAS). The conversion of these steroids to more active hormones, including estrone, 17 $\beta$ -estradiol and testosterone, takes place primarily in the peripheral adipose tissue, but also in bone, vascular endothelium and brain (Simpson 2002). Indeed, postmenopausal women gain estrogens primarily from androgens through peripheral aromatization, and the most abundant estrogen in postmenopausal women is estrone (Speroff and Fritz 2005).

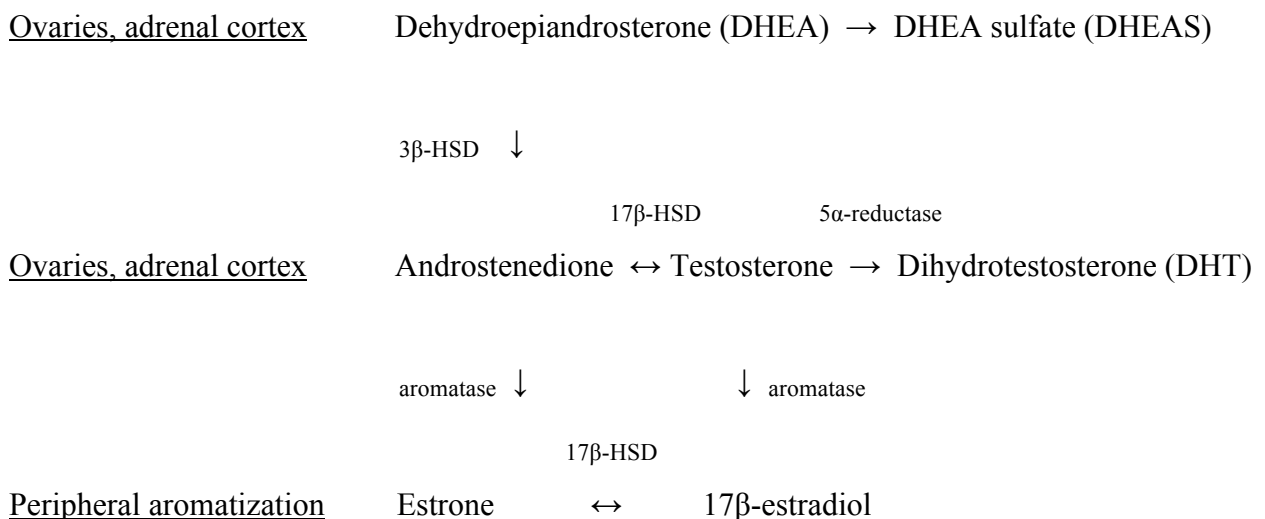
Two nuclear estrogen receptors (ERs), alpha and beta (ER $\alpha$  and ER $\beta$ ), have been discovered. In addition, ERs acting on the cell membrane have been found (Fu and Simoncini 2008, Kampa et al. 2008). Estrogen receptors are present in the reproductive tract, bone, brain, and breast, and also in adipose tissue, liver, intestine, kidney, lungs and cardiovascular tissue (Gruber et al. 2002, Mendelsohn 2002, Mendelsohn and Karas 2005). The ratios of ER $\alpha$  and ER $\beta$  in different tissues may change after menopause (Mendelsohn and Karas 2005). The decline in circulating estrogens appears to be accompanied by health risks in postmenopausal women, particularly in women who have entered menopause earlier than usual (Maxwell 1998, Stearns et al. 2002, Gallagher 2007, Lobo 2007). In the central nervous system estrogen deficiency may result in vasomotor symptoms, such as hot flushes, which are so typical that they can be regarded as diagnostic as regards the onset of menopause (Nelson 2008).

In addition to hot flushes, climacteric symptoms may comprise night sweats, palpitation, mood swings, disturbed night sleep, vaginal dryness and decreased libido (Stearns et al. 2002, Freeman and Sherif 2007). These symptoms, which are present in approximately 80% of postmenopausal women, are disturbingly severe in 20% (Stearns et al. 2002). Hot flushes persist for over 5 years in 30% and for over 15 years in 20% of postmenopausal women (Stearns et al. 2002). After menopause the risk of osteoporosis increases as a result of activated bone resorption

and reduced bone formation, both of which are regulated by estrogen (*Andersen 2007*). Furthermore, estrogen deficiency is associated with an increased risk of CVD (*Mendelsohn and Karas 1999, Mendelsohn and Karas 2007*).

Figure 1.

Principles of estrogen metabolism in postmenopausal women; 3 $\beta$ -HSD: 3-beta-hydroxysteroid dehydrogenase; 17 $\beta$ -HSD: 17-beta-hydroxysteroid dehydrogenase; 5 $\alpha$ -reductase: 5-alpha-reductase.



## Cardiovascular diseases

Approximately 10 000 women in Finland ([www.stat.fi](http://www.stat.fi)) and 8.6 million women worldwide (*The World Health Report 2004, World Health Organization*) die of CVD every year. In premenopausal women the incidence of CVD is lower than in men of the same age group, but it rises markedly at and after the onset of menopause (*Kannel et al. 1976, Maxwell 1998, Collins et al. 2007*).

Cardiovascular disease is manifest in women approximately a decade later than in men (*Collins et al. 2007*). Indeed, among postmenopausal women CVD is the leading cause of morbidity and mortality (*Mendelsohn and Karas 2005*).

There are abundant data on the gender differences that exist in the symptoms, progression, prognosis and management of CVDs (*Collins et al. 2007*). During the past few years, the incidence of coronary heart disease (CHD) in women has been rising perhaps as a result of neglected prevention and management strategies (*Hanratty et al. 2000, Lundberg et al. 2002, Herrington and Klein 2003, Collins et al. 2007*). Moreover, in clinical practice, CHD in a woman is often diagnosed later than in men, and this leads to retarded initiation of treatment.

Postmenopausal women with CVD risk factors, such as diabetes, insulin resistance, hyperlipidemia, hypertension, and overweight – all of which are components of the metabolic syndrome and affected by hormonal changes at the menopausal transition – indicate a higher risk of CVD-associated mortality in women than in men of the same age (*Hu 2003, Collins et al. 2007*). The exact mechanisms behind this phenomenon are not fully understood, but several factors may be implicated as discussed in detail below.

### *Estrogen deficiency*

As mentioned earlier, menopause is associated with an increased risk of CVD (*Mendelsohn and Karas 2005*). One explanation may be hypoestrogenism, because estrogen has several favorable effects on the cardiovascular system (Table 1). These include, for example, the ability to reduce the levels of low-density lipoprotein (LDL) cholesterol and increase those of HDL cholesterol, and to promote vasodilation by altering the production of vasoactive molecules (*Mendelsohn and Karas 1999, Mendelsohn 2002*). Indeed, in a recent study estrogen was associated with reduced subclinical atherosclerosis in postmenopausal women (*Karim et al. 2008*).

### *Polycystic ovary syndrome and hyperandrogenism*

High levels of androgens may jeopardize cardiovascular health through adverse effects on lipids, blood pressure and glucose metabolism (*Rexrode et al. 2003*). Hyperinsulinemia may lead to hyperandrogenism, as insulin stimulates testosterone production in the ovaries. In addition, hyperinsulinemia inhibits the synthesis of sex hormone-binding globulin (SHBG), which at low levels is associated with an increased risk of atherosclerosis (*Reinecke et al. 2002, Salley et al. 2007, Liepa et al. 2008*). An example of hyperandrogenism is in cases of polycystic ovary syndrome, which is associated with an increased risk of CVD (*Martikainen et al. 1998, Liepa et al. 2008*). The effects of androgens and estrogens may differ in women and men; in middle-aged women with CVD levels of androgens have been reported to be lower than in their compared controls (*Khatibi et al. 2007*). In contrast in men, there have also been implications of a beneficial effect of endogenous testosterone on serum lipids (*Mäkinen et al. 2008*). Overall, the role of sex steroids in regard to CVD is not fully established.



Table 1.

Possible mechanisms by which estrogen deficiency may increase the risk of cardiovascular disease (LDL: low-density lipoprotein; HDL: high-density lipoprotein).

Target organ/system	Effect	Reference (reviews or meta-analyses)
Blood pressure	Increase in blood pressure	Mendelsohn and Karas 2005 (review)
Body fat	Android pattern in body fat, increase in visceral fat	Rosano et al. 2007 (review)
Glucose metabolism	Glucose intolerance	Rosano et al. 2007 (review)
Lipid metabolism	Lipid profile becomes more atherogenic; increase in total cholesterol, LDL cholesterol, triglycerides and lipoprotein(a), and decrease in HDL cholesterol	Tikkanen 1996a (review), Godsland 2001 (meta-analysis), Rosano et al. 2007 (review)
Vascular function	Endothelial dysfunction, reduced vasodilation	Mendelsohn and Karas 2005 (review)

### *Lipids*

Elevated levels of serum cholesterol predispose individuals to atherosclerosis, even in the absence of other risk factors (*Schoen 2005*). In the circulation, the major apolipoprotein-B-containing lipoproteins transporting cholesterol are LDL, very low-density lipoprotein (VLDL), HDL and lipoprotein(a) (*Tikkanen 1996b, Stangl et al. 2002*). Before menopause, the levels of total cholesterol and LDL cholesterol are lower and the level of HDL cholesterol is higher in women than in men, whereas after menopause the opposite situation develops. This may contribute to the rise in the risk of CVDs in postmenopausal women (*Tikkanen 1996a*).

Low-density lipoprotein cholesterol is essential in the delivery of cholesterol to peripheral tissues (*Schoen 2005*). In the early stages of atherosclerosis LDL cholesterol is oxidized by free radicals. The oxidized LDL particles are scavenged by macrophages or smooth muscle

cells and become aggregated in the arterial wall (*Moore and Freeman 2006*); accumulation of lipids leads to the formation of foam cells and further to fatty streaks, which may also contain lymphocytes and platelets (*Moore and Freeman 2006, Gossi et al. 2007, Kolodgie et al. 2007*). Fatty streaks develop to atherosclerotic plaques which may eventually rupture and lead to occlusion of the artery (*Moore and Freeman 2006, Kolodgie et al. 2007*).

At low level HDL cholesterol is a major risk factor of CVDs, particularly in women (*Rosano et al. 2007*), whereas at high level HDL cholesterol confers cardioprotection (*Tikkanen and Nikkilä 1987, Collins et al. 2007*). The impact of decreased levels of HDL cholesterol is important, because HDL particles have a role in cholesterol transport from cells to the liver for excretion (*Schoen 2005*). This process called cholesterol efflux is an independent predictor of coronary artery atherosclerosis (*Mikkola et al. 2003*).

In addition to lipoproteins, triglycerides play a significant role in lipid metabolism (*Schoen 2005*). Triglycerides are derived from free fatty acids from the adipose tissue or from ingested foods. In the liver, fatty acids are esterified to triglycerides, which associate with apoproteins. Increased levels of triglycerides have been recognized as an independent risk factor of CVD, again particularly in women (*Stangl et al. 2002, Collins et al. 2007*). Simultaneous elevations in LDL cholesterol and reduction in HDL cholesterol augment the atherogenic risk of hypertriglyceridemia.

On the basis of the results of a large meta-analysis, the ratio of total cholesterol/HDL cholesterol is the strongest predictor of ischemic heart disease (*Prospective Studies Collaboration 2007*). Lowering total cholesterol by 1 mmol/l reduces the risk of dying from ischemic heart disease in both sexes, and in women it is most conspicuous in middle-aged subjects (approximately 30-50% lower mortality). Thus, lowering total cholesterol is beneficial, but improvement of LDL, VLDL and HDL cholesterol and triglycerides status is at least equally important. Furthermore, the impact of non-HDL, apoB and particularly LDL particle concentration is attracting more attention as regards assessment of CHD risk (*Davidson 2008, Hsia et al. 2008*). To sum up, the implications of various lipids and lipoproteins as regards CHD risk require additional study.

### *Hypertension*

Hypertension is the leading CVD risk factor (*Appel et al. 2006, Kshirsagar et al. 2006*). The risk of CVD rises progressively through the range of blood pressure beginning with normotensive values (*Miller and Jehn 2004, Appel et al. 2006, Messerli et al. 2007*). Many apparently healthy

menopausal women have slightly elevated blood pressure (*Appel et al. 2006*). According to the Framingham Study, 55-year-old postmenopausal women have a 90% lifetime probability of developing hypertension (*Vasan et al. 2002*). Individuals with blood pressure ranging from 130-139/85-89 mmHg have a 2-fold risk of CVD compared with normotensive subjects. Moreover, each 20/10 mmHg increase in systolic/diastolic blood pressure doubles the risk of developing CVD (*Miller and Jehn 2004*). The present guidelines are that blood pressure is optimal if <120/<80 mmHg, normal if 120-129/80-84 mmHg, high normal if 130-139/85-89 mmHg, and hypertensive if >140-159/90-99 mmHg (*Consensus Statement of the Finnish Medical Society of Duodecim and the Finnish Association of Hypertension 2006, ESH-ESC 2007 guidelines for the management of arterial hypertension*). These guidelines are infrequently met in clinical practice in Finland, as well as in most other Western countries.

### *Vascular inflammation*

Development of atherosclerosis can also be considered as an inflammatory process, which is initiated with the adherence of leukocytes to damaged endothelium (*Lind 2003*) (Figure 2). Arterial endothelial cells express vascular cell adhesion molecule-1 (VCAM-1), which binds monocytes and T-lymphocytes (*Schoen 2005*). Monocytes adhere to the endothelium, migrate to the intima and are transformed into macrophages, which scavenge lipoproteins and become foam cells. This process is initially protective as potentially harmful lipid particles are removed, but progressive accumulation of lipid-laden macrophages in the arterial wall promotes atherosclerosis. Leukocytes release inflammatory cytokines, which may stimulate the actions of macrophages, vascular endothelial cells and smooth muscle cells. Smooth muscle cells migrate from the arterial media to the intima, proliferate and convert fatty streaks into fibrofatty atheromas and atherosclerotic plaques (*Schoen 2005*). Vascular inflammation also weakens the fibrous cap of the atherosclerotic plaque, leading to its rupture (*Lind 2003*) (Figure 2).

A number of inflammatory biomarkers have been used as risk indicators of CVD (*Blankenberg et al. 2003, Lind 2003*). These include C-reactive protein (CRP), various adhesion molecules, selectins, fibrinogen, leukocytes, cytokines and leukocyte cell surface antigens. Among these the most established are CRP, VCAM-1, intercellular adhesion molecule-1 (ICAM-1), platelet-selectin (P-selectin) and endothelial-selectin (E-selectin) (*Blankenberg et al. 2003, Lind 2003*) (Table 2).

Table 2.

Sources and functions of C-reactive protein (CRP), vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), endothelial-selectin (E-selectin) and platelet-selectin (P-selectin), which are often used as vascular inflammation markers.

Marker	Source	Function	Reference (reviews)
CRP	Liver	Complement activation, counteraction of infections	Lind 2003
VCAM-1	Endothelial cells, smooth muscle cells, tissue macrophages	Signal induction in endothelial cells causing leukocyte migration	Blankenberg et al. 2003, Brevetti et al. 2008
ICAM-1	Endothelial cells, smooth muscle cells, tissue macrophages	Adhesion of leukocytes to the endothelium; leukocyte extravasation	Blankenberg et al. 2003, Brevetti et al. 2008
E-selectin	Endothelial cells, smooth muscle cells, tissue macrophages	Early leukocyte recruitment at the endothelium	Blankenberg et al. 2003, Lind 2003
P-selectin	Platelets, endothelial cells	Early leukocyte recruitment at the endothelium	Blankenberg et al. 2003

### *Arterial stiffness and endothelial dysfunction*

Vascular function can be assessed from the central aortic pulse wave. It is formed from the combination of the forward-moving pressure wave generated by the left ventricle in systole and waves reflected back to the aorta from points of greater impedance along the arterial tree, such as bifurcations and arterioles (*Segers et al. 2001*). Arteries lose their elasticity with advancing age and thus arterial stiffness also increases in an age-related pattern (*Mackenzie et al. 2002, Rönnback et al. 2004*). Hence, young arteries differ from old ones as regards the shape of the arterial pressure waveform and this serves as a measure of arterial stiffness (*Segers et al. 2001*). In young persons the reflected waves reach the ascending aorta in late systole. In the elderly and perhaps hypertensive subjects the magnitude and speed of the waves are increased and add to the forward wave in early systole, thus boosting the systolic pressure (*Segers et al. 2001*). Consequently, pulse

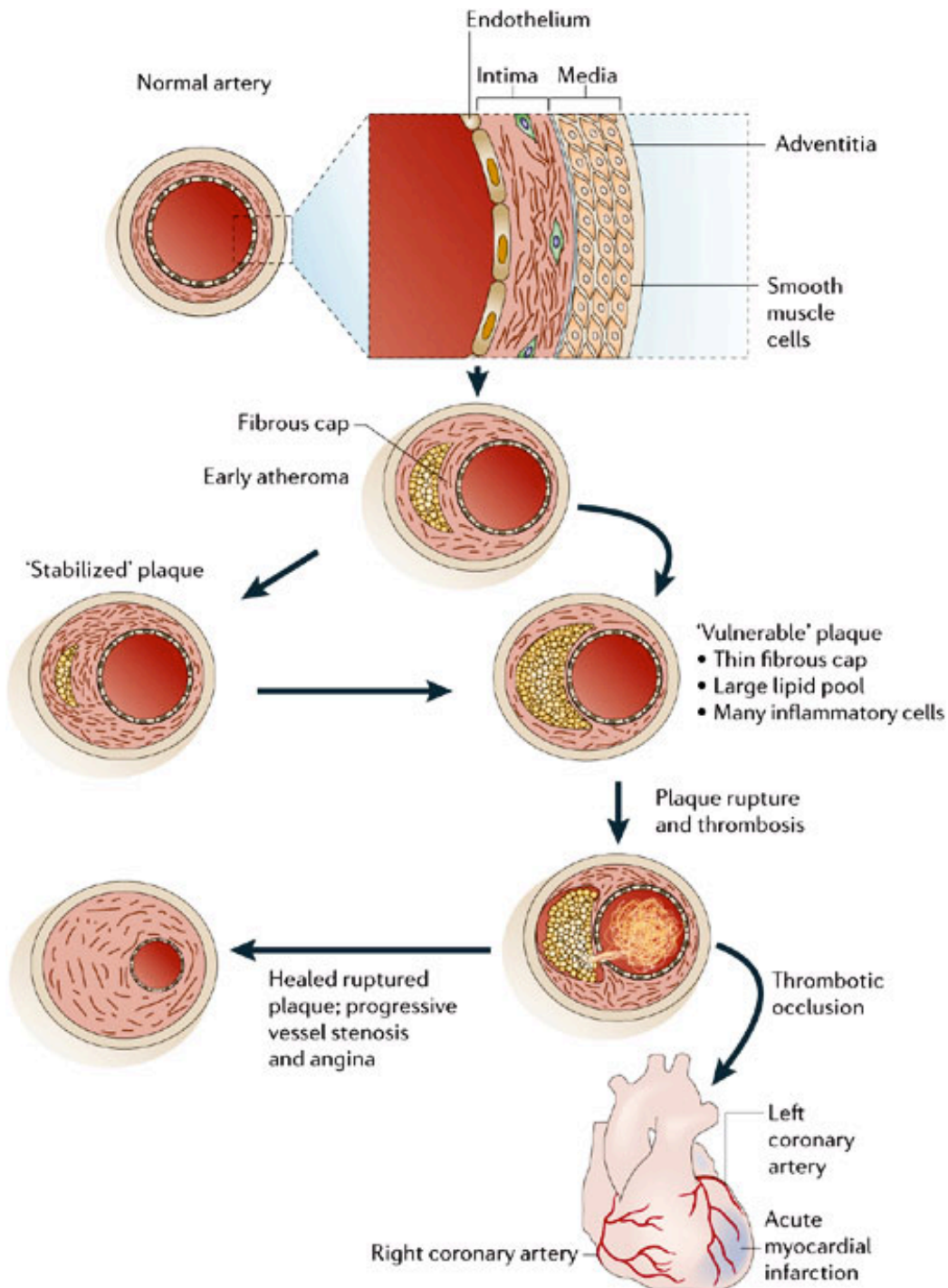
pressure (the difference between systolic and diastolic blood pressure) increases with advancing age and it indicates arterial stiffness (*Mackenzie et al. 2002, Rönnback et al. 2004*).

The outer layer of arteries, adventitia, is formed mainly of collagen fibers and fibroblasts. The middle layer, media, contains smooth muscle cells. The inner layer, intima, is formed of an elastic part (interna elastica) and endothelial cells. This endothelium lines the surface of the intima closest to the lumen of the artery (*Schoen 2005*) (Figure 2). Several biologically active mediators regulating vascular tone (such as nitric oxide [NO], prostacyclin, endothelin, angiotensin, and thromboxane), coagulation and fibrinolysis, as well as growth factors and immunological mediators are released by the endothelium (*Brevetti et al. 2008*).

Intact endothelial cells are essential for vascular health, whereas endothelial dysfunction predisposes individuals to vascular disorders (*Schoen 2005*). The specific cause of endothelial dysfunction is not known, but homocysteine, cytokines, viruses and other infectious agents as well as circulating derivatives of cigarette smoke, are potentially involved. The most important determinants of endothelial alterations are, however, thought to be hemodynamic disturbances and adverse effects of hypercholesterolemia, either together or alone (*Schoen 2005*). Endothelial function can be assessed from plasma levels of endothelial cell-generated molecules, such as endothelin-1, intercellular and vascular adhesion molecules and nitrite/nitrate (*Brevetti et al. 2008*). Vascular reactivity may be evaluated by studying flow-mediated dilation, arterial stiffness, or pulse-wave velocity. The ability of the endothelium to release NO is often used as a measure of endothelial function (*Cockcroft 2005*). Endothelial dysfunction is also associated with other risk factors of CVD, for example, hypercholesterolemia, hypertension, age, smoking and diabetes, and in practice it is difficult to delineate which change is primary and which one is secondary (*Sarabi et al. 2001*). Arterial stiffness and endothelial dysfunction are early signs of arteriosclerosis and can be detected before clinically evident vascular disease (*Mackenzie et al. 2002, Wilkinson et al. 2002, Cockcroft 2005*).

Figure 2.

Structure of a normal artery and the formation of atherosclerotic plaque in the arterial wall.  
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“Watkins H, Farrall M. Genetic susceptibility to coronary artery disease: from promise to progress”, copyright 2008.



## Hormone therapy

Estrogen is the most effective treatment for vasomotor symptoms. In women with an intact uterus estrogen is combined with progestagen (estrogen-progestagen therapy; EPT) to avoid endometrial hyperplasia and cancer. Estrogen-only therapy (ET) is used in hysterectomized women. Estrogen is most commonly administered orally or transdermally. Traditional HT effectively relieves climacteric symptoms, prevents osteoporosis, and improves the quality of life (*Nelson 2008*). The various ET and EPT regimens vary in content and dose, and in order to maximize safety, current recommendations are that the lowest dose of HT should be prescribed for the shortest duration necessary to relieve climacteric symptoms (*Nelson 2008, Utian et al. 2008*).

There are several mechanisms by which estrogen may improve vascular health. These include, for example, changes in lipid/lipoprotein metabolism, arterial relaxation by endothelium-dependent and -independent mechanisms, stimulation of endothelial cell proliferation and migration, inhibition of smooth muscle cell growth, contribution to the repair of injured blood vessels and inhibition of intimal thickening (*Mikkola et al. 1995, Ylikorkala et al. 1995, Mendelsohn and Karas 1999*). Data from several studies imply that the use of HT reduces the risk of CVD in previously healthy postmenopausal women by 35-50% (*Mendelsohn and Karas 1999, Grady et al. 2002, Mikkola and Ylikorkala 2005*).

In the past few years, traditional HT has been the subject of major criticism because of reports on the results of randomized clinical trials in which the risks of HT have appeared to outweigh the benefits (*Writing Group for the Women's Health Initiative Investigators 2002, Herrington and Klein 2003, Million Women Study Collaborators 2003*). The adverse outcomes were mainly increases in cardiovascular events (CHD, stroke, venous thromboembolic disease) and breast cancer cases, although there were differences and limitations in the study designs. Overall, the previous assumption that HT would offer protection against CVD regardless of time elapsed since the onset of menopause has been rejected (some time ago) (*Ylikorkala 2000, Mikkola and Ylikorkala 2005, Manson et al. 2007, Clarkson 2008*). Some leading studies and trials are reviewed below.

Of the various observational studies, The Nurses' Health Study (NHS) is the most comprehensive one. It was started in 1976 when 70533 postmenopausal nurses were recruited and followed-up for 20 years. In this large cohort, when adjusted for age and common CVD risk factors, current use of HT was associated with a 39% reduced risk of a major coronary event (*Grodstein et al. 2000*). In postmenopausal nurses with known previous myocardial infarction or atherosclerosis, the recurrence rate was increased by 25% with short-term HT, whereas it was

decreased by 62% with long-term use of HT compared with never-users of HT (*Grodstein et al. 2001, Speroff 2001*).

The Heart and Estrogen/progestin Replacement Study (HERS) involved a total of 2763 postmenopausal women with a history of CHD who were enrolled to receive conjugated equine estrogen (CEE) plus medroxyprogesterone acetate (MPA) or placebo for 4.1 years. This was the first randomized and placebo-controlled trial in which the use of HT in regard to CHD was assessed. The use of CEE+MPA did not reduce the risk of CHD mortality or other CVD outcome compared with placebo (95% CI 0.81-1.22) and therefore the use of EPT was not recommended for secondary prevention of CHD (*Hulley et al. 1998*). It was also noted that the risk of recurrent coronary events was highest in the first year (52% increase), after which the differences between treated and untreated women began to settle and during years 3-5 CEE+MPA was associated with a lower risk (33-13%) of coronary events (*Hulley et al. 1998*), but after 6.8 years the overall risk of CVD was not reduced (*Grady et al. 2002*).

The randomized Women's Health Initiative (WHI) study involving 27347 postmenopausal women treated with CEE+MPA, unopposed CEE in hysterectomized women, or placebo, was aimed at confirming the observational findings that HT is beneficial in primary prevention of CHD. However, the results of the WHI study failed to support this hypothesis, as CEE+MPA was associated with a 22% increased risk of total CVDs and a 29% increased risk of CHD (*Writing Group for the Women's Health Initiative Investigators 2002*). In the WHI sub-study, ET was associated with 45% less coronary artery calcification among 50- to 59-year-old women compared with placebo, although it remained unclear whether this phenomenon would persist if ET was initiated at a younger age (*Manson et al. 2007*). Overall, HT was not recommended for the primary prevention of CVD (*Manson et al. 2003, Manson et al. 2007*). Hormone therapy may, however, be beneficial as regards the risk of CVD if it is initiated soon after the onset of menopause and not several years later (*Dubey et al. 2005, Manson et al. 2007, Mendelsohn and Karas 2007, Rossouw et al. 2007*).

The most common adverse effect associated with HT is the increased risk of breast cancer (*Writing Group for the Women's Health Initiative Investigators 2002, Foidart et al. 2007*), which appears to depend on the duration of HT use, different formulations of ET/EPT, and route of administration (oral vs. transdermal) (*Collins 2006, Lyytinen et al. 2006, Foidart et al. 2007, Opatrny et al. 2008*). Similarly, different lipid and metabolic effects may also be expected depending on the type of HT and particularly on whether it is taken orally or transdermally (*Strandberg et al. 2003*). In addition, some women experience side-effects of HT, such as breast tenderness, uterine bleeding, nausea, headache and weight change (*Vihtamäki et al. 1999, Nelson*



2008). Fortunately, postmenopausal women and their physicians have the possibility to choose from the numerous HT regimens available. Nevertheless, HT can hardly be considered as an ideal treatment. Several guidelines indicate that the use of HT is justified for the relief of climacteric symptoms in appropriate populations. Importantly, an individual risk profile should be evaluated for each woman considering initiation of ET/EPT (*Consensus Statement of the Finnish Medical Society of Duodecim and the Academy of Finland 2004, Grady and Barrett-Connor 2007, Utian et al. 2008*).

### Tibolone

Tibolone, (7 $\alpha$ ,17 $\alpha$ )-17-hydroxy-7 $\alpha$ -methyl-19-nor-17 $\alpha$ -pregn-5(10)-en-20-yn-3-one is a synthetic steroid used as an alternative treatment for climacteric symptoms and the prevention of osteoporosis (*Vos et al. 2002*). Tibolone is given orally at a daily dose of 2.5 mg. It was introduced in 1988 and at present it represents the most popular menopausal treatment in Europe. Tibolone is currently not available in the United States. In Finland, approximately 15 000 women use tibolone (*The Social Insurance Institute of Finland*).

#### *Metabolism and mode of action*

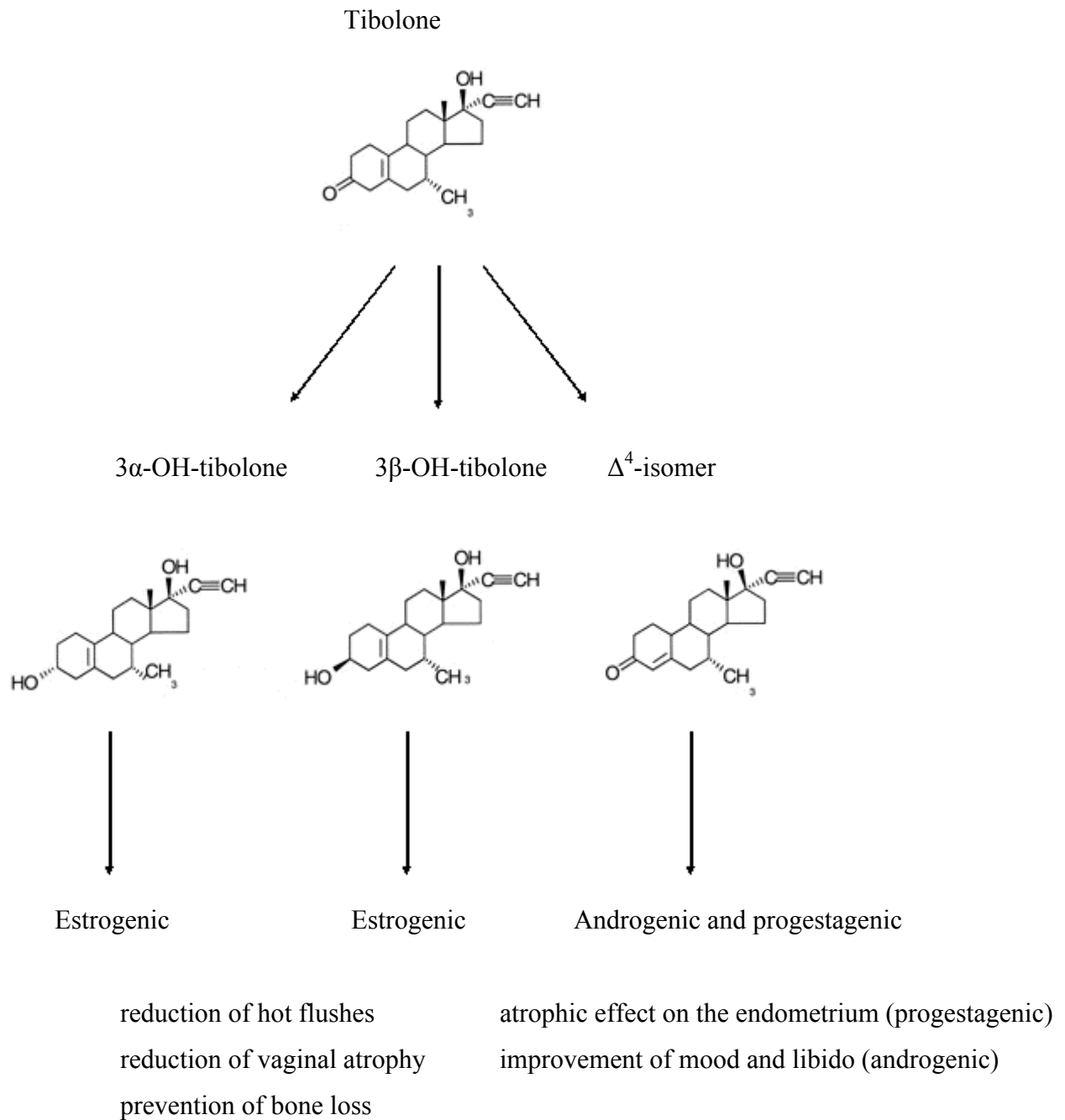
The metabolism of tibolone occurs mainly in the intestine and the liver (*Kloosterboer 2001*). It is metabolized to three biologically active compounds, two of them being the estrogenic metabolites 3 $\alpha$ -hydroxy (OH)-tibolone and 3 $\beta$ -hydroxy (OH)-tibolone, both of which bind to ERs (Figure 3). Because it has estrogenic metabolites, tibolone relieves hot flushes and reduces the risk of osteoporosis and vaginal atrophy (*Kloosterboer 2001, Notelovitz 2007*). The third metabolite is  $\Delta^4$ -isomer which binds to androgen and progesterone receptors (*Kloosterboer 2001, Campisi and Marengo 2007, Notelovitz 2007*) (Figure 3). Due to a locally formed progestin in the endometrium, the use of tibolone does not result in withdrawal bleeding. In addition, tibolone has a positive effect on mood and sexual well-being. This is likely to be the result of the androgenic effects of the  $\Delta^4$ -isomer and/or the reduction in the levels of SHBG and subsequent increase in the levels of free testosterone (*Kloosterboer 2001*). Tibolone has been associated with reduced breast mammographic density in comparison with a conventional form of HT (*Notelovitz 2007*). However, when a total of 3148 women with a history of breast cancer were treated with tibolone (2.5 mg) or placebo for four years, the risk of recurrence/new breast cancer was 48% higher in

women scheduled to tibolone (*Kenemans 2008, Ylikorkala, personal communication*). In contrast, a lower dose of tibolone (1.25 mg) has been associated with a 68% decreased risk of primary breast cancer (*Cummings et al. 2008*). Thus, the dose of tibolone may be critical as regards effects on breast.

Figure 3.

Structure of tibolone and its metabolites. The main target effects are also mentioned.

OH: hydroxy;  $\Delta^4$ : delta-four. For comparison, see Figure 4 for the structure of  $17\beta$ -estradiol.



## *Cardiovascular safety*

Many studies have been concerned with assessment of the effects of tibolone on several established CVD risk factors (Table 3). Tibolone does not affect blood pressure (*Lloyd et al. 2000, Cagnacci et al. 2004*) and it may reduce arterial stiffness and improve endothelial function (*Bruce et al. 2005, Castelo-Branco et al. 2005, Somunkiran et al. 2006*). Furthermore, tibolone has been shown to increase plasma levels of NO in postmenopausal women, implying a possible cardioprotective effect (*Cicinelli et al. 2002*). Moreover, it reduces the circulating levels of cell adhesion molecules, such as VCAM-1, ICAM-1 (*Egarter et al. 2003, Cicinelli et al. 2006*) and selectins (*Cicinelli et al. 2006, Sator et al. 2006*), or leaves them unchanged (*Vitale et al. 2005*). Yet, tibolone may increase the level of CRP (*Barnes et al. 2005*) suggesting an increased CVD risk, but this finding has not been seen in all studies (*Campisi and Marengo 2007*).

Tibolone lowers the levels of total cholesterol, LDL cholesterol and lipoprotein(a). In addition, it reduces the levels of HDL cholesterol by 20-30% (*Mikkola and Clarkson 2002, Notelovitz 2007*), which has been a cause of concern regarding its cardiovascular safety. The decrease in HDL cholesterol may be compensated, at least in part, by the ability of tibolone to reduce the levels of triglycerides and thus also the triglyceride/HDL-ratio, which is a predictor of CHD risk (*Koh et al. 2003, Campisi and Marengo 2007*).

The cardiovascular safety profile of tibolone has been questioned, as conflicting data have emerged from clinical trials. In one study the use of tibolone (2.5 mg/day) was associated with progression of carotid artery intima-media thickness (CIMT) (*Bots et al. 2006*). However, this effect appeared only in European, but not in American women, a phenomenon the cause of which remained unexplained (*Clarkson 2006*). In contrast, CIMT has also been shown to reduce (*Erenus et al. 2003*), or remain unaltered (*Morris et al. 1999, Somunkiran et al. 2006*) during tibolone therapy. Recently, a smaller dose of tibolone (1.25 mg/day) for 3 years was associated with an increased risk of stroke (*Cummings et al. 2008*). In this trial, however, the mean age of the women was as high as 68 years (range 60-85 years). To conclude, with HDL cholesterol excluded, the effects of tibolone on the above-mentioned CVD surrogate markers closely resemble those of traditional HT (*Campisi and Marengo 2007, Cummings et al. 2008*).

Table 3.

Effects of tibolone on various vascular surrogate markers in postmenopausal women. LDL: low-density lipoprotein; HDL: high-density lipoprotein; CRP: C-reactive protein; VCAM-1: vascular cell adhesion molecule-1; ICAM-1: intercellular adhesion molecule-1; P-selectin: platelet-selectin.

Marker	Effect and reference		
	Decrease	No effect	Increase
Total cholesterol	Prelevic et al. 2002, Koh et al. 2003, Christodoulakos et al. 2006	Cagnacci et al. 1997	
LDL cholesterol	Campisi and Marengo 2007, Koh et al. 2003	Christodoulakos et al. 2006	
HDL cholesterol	Prelevic et al. 2002, Koh et al. 2003, Christodoulakos et al. 2006	Cagnacci et al. 1997	
Triglycerides	Cagnacci et al. 1997, Prelevic et al. 2002, Koh et al. 2003, Christodoulakos et al. 2006		
CRP		Koh et al. 2003, Vitale et al. 2005, Campisi and Marengo 2007	Prelevic et al. 2002, Barnes et al. 2005, Eilertsen et al. 2008
VCAM-1	Simoncini and Genazzani 2000, Egarter et al. 2003, Cicinelli et al. 2006	Vitale et al. 2005	
ICAM-1	Egarter et al. 2003, Cicinelli et al. 2006	Simoncini and Genazzani 2000, Vitale et al. 2005	
P-selectin	Eilertsen et al. 2008		

## Soy-derived isoflavones

The three main classes of phytoestrogens are isoflavones, lignans and coumestans, among which isoflavones are the most important due to their wide range of biological activities (*Murkies et al. 1998, Valachovikova et al. 2004, Finné Nielsen and Williamson 2007*). Isoflavones are abundant in soybeans, but they are also found in various fruits, vegetables and other plants. The primary isoflavones are genistein and daidzein (*Murkies et al. 1998, Valachovikova et al. 2004*).

Isoflavones undergo metabolism in the intestine and the liver, where they are subject to enterohepatic circulation and excretion into the bile. Isoflavones in soy protein are bound to sugars called glucosides. Deglycosylation by  $\beta$ -glucosidase to the biologically active aglycones takes place in the gut microflora, where they are quickly absorbed; genistein and daidzein aglycones attain maximum serum concentrations at 5-6 hours after ingestion (*Setchell et al. 2003b, Finné Nielsen and Williamson 2007*). The free aglycones are transported to the liver, where they are hydroxylated and reconstituted to more water-soluble metabolites and eventually excreted in the urine (*Murkies et al. 1998, Yuan et al. 2007*).

The consumption of isoflavones is substantially higher in Asia (20-80 mg/day) than in Europe and the USA (0.5-3 mg/day) (*Tham et al. 1998, Keinan-Boker et al. 2002*). The low incidence of CVD and cancers of the breast, endometrium and prostate in Asian countries has raised the question of whether or not isoflavones have a protective role against these diseases (*Tikkanen and Adlercreutz 2000, Valachovikova et al. 2004, Sacks et al. 2006*).

Data on the efficacy of isoflavones in the treatment of hot flushes and vaginal dryness are not uniform (*Adlercreutz and Mazur 1997, Albertazzi and Purdie 2002*); hot flushes have been found to be reduced in some (*Messina and Hughes 2003, Nahas et al. 2007*), but not all studies (*Dalais et al. 1998, Nikander et al. 2003a*). Overall, it seems that the effect of isoflavones on climacteric symptoms is absent or minimal (*Royal College of Obstetricians and Gynaecologists 2006, Albertazzi 2007, Lethaby et al. 2007, Reed et al. 2008*). Additionally, isoflavones appear to be ineffective in the prevention of osteoporosis (*Sacks et al. 2006, Brink et al. 2008*).

### *Mechanism of action*

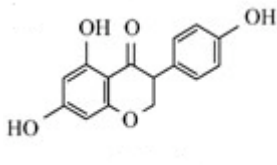
Isoflavones are structurally similar to  $17\beta$ -estradiol (Figure 4) and thus they bind to ERs with higher affinity to ER $\beta$  than to ER $\alpha$  (*Valachovikova et al. 2004*). Isoflavones can behave as both estrogen agonists and antagonists (*Tempfer et al. 2007*) and thus they are sometimes called natural Selective Estrogen Receptor Modulators (SERMs) (*Mikkola and Clarkson 2002, Yuan et al. 2007*).

In addition, isoflavones bind to progesterone and androgen receptors (*Beck et al. 2003*). Isoflavones have also been shown to downregulate androgen receptors (*Lund et al. 2004a, Hamilton-Reeves et al. 2007*). If this is the case in vivo, it could potentially reduce the risk of prostate cancer. However, the efficacy and safety of isoflavones in the prevention of breast, endometrial and prostate cancer are not established, and adverse effects may also be possible (*Sacks et al. 2006*).

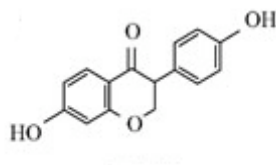
Figure 4.

Chemical structure of genistein and daidzein, and that of 17 $\beta$ -estradiol for comparison.

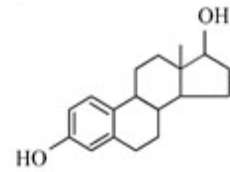
Genistein



Daidzein



17 $\beta$ -estradiol



*Equol*

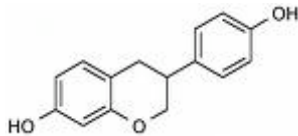
Equol, 7-hydroxy-3-(4'-hydroxyphenyl)-chroman, was first discovered in the urine of a pregnant mare and thus the equine-based nomenclature was created (*Setchell et al. 2002*). Equol has been characterized as a nonsteroidal estrogen (Figure 5) which binds to ER $\alpha$  and ER $\beta$  (*Setchell et al. 2002*). Equol has two isomers: the S-isomer, which is found only in humans, binds to ER $\beta$  with a similar affinity as 17 $\beta$ -estradiol; the R-isomer, which is not found in humans, shows less affinity to ER $\beta$  (*Setchell et al. 2005*).



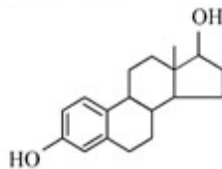
Figure 5.

Chemical structure of equol, and that of 17 $\beta$ -estradiol for comparison.

Equol



17 $\beta$ -estradiol



Equol is produced by the intestinal bacterial flora from daidzein in approximately 30% of humans. This is thought to be a stable characteristic that is best revealed after a soy challenge (*Setchell et al. 2002, Atkinson et al. 2004, Frankenfeld et al. 2004b, Frankenfeld et al. 2005*). The definition of an equol producer and non-producer varies in the literature. Often a cut-off-point for non-producers has been a plasma equol level <40 nmol/l (10  $\mu$ g/l), and for equol producers >83 nmol/l (20  $\mu$ g/l) or a urinary equol level >1000 nmol/l (250  $\mu$ g/l) (*Setchell et al. 2002*). The main intestinal bacteria responsible for equol production are not known, but at least *Bifidobacterium*, *Escherichia coli*, *Ruminococcus productus*, *Streptococcus intermedius*, *Bacteroides ovatus* (*Setchell et al. 2002, Atkinson et al. 2005*) and the recently discovered novel bacterium *Adlercreutzia equolifaciens* (*Maruo et al. 2008*), are involved. Equol production capacity may be transitorily affected by the use of antibiotics or by dietary factors; consumption of fat results in low equol production, whereas non-starch polysaccharides stimulate bacterial fermentation and increase equol formation (*Rowland et al. 2000, Setchell et al. 2002*). Induction of equol production could occur also with probiotics. The most commonly used probiotics are *Lactobacilli* and *Bifidobacteria* (*Fooks and Gibson 2002*).

Equol has a half-life of 8.8 hours (*Setchell et al. 2002*). In the circulation, 50% of equol circulates non-bound, whereas 18.7% of daidzein appears to be free (*Setchell et al. 2002*). Equol binds to SHBG and competitively inhibits the binding of 17 $\beta$ -estradiol and testosterone to SHBG in a dose-dependent manner (*Martin et al. 1996*). Thus, equol increases the availability of biologically active fractions of 17 $\beta$ -estradiol and testosterone. On the other hand, equol production has been associated with reduced concentrations of estrogens, androgens and cortisol but elevated

concentrations of SHBG and progesterone in women of fertile age (*Duncan et al. 2000*). Equol production capacity has been related to a lowered risk of breast and prostate cancer, although the data are not uniform (*Yuan et al. 2007*). Nevertheless, it seems that equol production capacity may alter steroid production or metabolism, perhaps through specific colonic bacteria (*Duncan et al. 2000, Frankenfeld et al. 2004a*). There are several biological effects in association with equol/equol production capacity (Table 4), but the definitive role of this isoflavone metabolite in health and disease remains unsettled.

Table 4.

Biological effects of equol production capacity.

Effect	Reference
Inhibition of lipid peroxidation	Setchell et al. 2002
Release of nitric oxide from the endothelium	Hwang et al. 2003, Joy et al. 2006
Altered steroid metabolism	Duncan et al. 2000, Frankenfild et al. 2004a
Antioxidant activity: scavenging of free radicals	Setchell et al. 2002
Lowered risk of breast cancer?	Duncan et al. 2000, Atkinson et al. 2005
Lowered risk of prostate cancer?	Lund et al. 2004b, Atkinson et al. 2005, Yuan et al. 2007

*Effects on cardiovascular risk factors*

The assumed vascular benefits of soy were previously thought to be mainly based on its ability to lower the concentrations of total cholesterol, LDL cholesterol and triglycerides, and modestly to increase the levels of HDL cholesterol (*Anderson et al. 1995*). The mechanisms behind this action are not fully understood, but they may include alterations in cholesterol metabolism and LDL-receptor activity (*Anderson et al. 1995, Clarkson 2002, Wagner et al. 2003*). In 1999, the American Heart Association stated that 25 g of soy protein daily is protective against CHD (*Erdman Jr 2000*). This recommendation was, however, reversed in 2006 and the cardiovascular benefit of soy was regarded as being unlikely (*Sacks et al. 2006*). Overall, the effect of soy alone on lipids and perhaps also on CVDs is nowadays considered minimal (*Clarkson 2002, Kreijkamp-Kaspers et al. 2004, Hall et al. 2005, van der Schouw et al. 2005, Sacks et al. 2006*).

Blood pressure has been shown to decrease during soy supplementation in some (*Rivas et al. 2002, Welty et al. 2007*), but not all studies (*Jenkins et al. 2002*). Similarly, endothelial function (*Colacurci et al. 2005*) and arterial compliance (*Nestel et al. 1997*) has either improved or remained unaltered (*Hale et al. 2002*) during treatment with soy or isolated

isoflavones. Soy or isolated isoflavones have decreased (*Hall et al. 2005*) or caused no changes in CRP levels (*Nikander et al. 2003b, Hanson et al. 2006, Matthan et al. 2007*). Furthermore, soy or isolated isoflavones have not affected the levels of VCAM-1, ICAM-1 or E-selectin (*Blum et al. 2003, Hall et al. 2005, Greany et al. 2007*) (Table 5). The discrepancies between the data accumulated from different studies may be explained by differences in type, dose and duration of soy supplementation (*Clarkson 2002, Dewell et al. 2006*). In addition, the presence of both soy protein and isoflavones appears to be essential for biological effect (*Greaves et al. 1999, Clarkson 2002*). This is important, because different investigators may have used different preparations, including intact soy protein, soy protein with isoflavones extracted, or isolated isoflavones (*Anderson et al. 1995, Erdman Jr 2000, Dewell et al. 2006*). Research evidence appears to support a role for soy protein in improving lipid status (*Sacks et al. 2006*).

In postmenopausal monkeys soy supplementation increases the levels of HDL cholesterol (*Clarkson et al. 2001a*) and decreases the levels of LDL+VLDL cholesterol (*Greaves et al. 1999, Clarkson et al. 2001a*) and this results in inhibited progression of atherosclerosis (*Honoré et al. 1997, Clarkson et al. 2001a, Wagner et al. 2003*). Reduction in the levels of VCAM-1 has been observed in atherosclerotic monkeys on a soy diet (*Register et al. 2005*). It should be noted that female monkeys are all equal producers and this may explain different effects of soy in monkeys and women (*Gu et al. 2006*).

Table 5.

Effects of soy or isolated isoflavones on various vascular surrogate markers in postmenopausal women. LDL: low-density lipoprotein; HDL: high-density lipoprotein; CRP: C-reactive protein; VCAM-1: vascular cell adhesion molecule-1; ICAM-1: intercellular adhesion molecule-1; P-selectin: platelet-selectin.

Marker	Effect and reference		
	Decrease	No effect	Increase
Total cholesterol	Allen et al. 2007, Taku et al. 2007	Nikander et al. 2004 Matthan et al. 2007	
LDL cholesterol	Allen et al. 2007, Taku et al. 2007	Nikander et al. 2004, Colacurci et al. 2005	
HDL cholesterol		Nikander et al. 2004, Colacurci et al. 2005, Taku et al. 2007	Baum et al. 1998, Goodman-Gruen and Kritz-Silverstein 2001
Triglycerides	Lichtenstein et al. 2002, Meinertz et al. 2002	Colacurci et al. 2005, Matthan et al. 2007, Taku et al. 2007	
CRP	Hall et al. 2005	Nikander et al. 2003b, Hanson et al. 2006, Matthan et al. 2007	
VCAM-1	Colacurci et al. 2005, Register et al. 2005	Blum et al. 2003, Hall et al. 2005, Greany et al. 2007	
ICAM-1	Colacurci et al. 2005	Blum et al. 2003, Hall et al. 2005, Greany et al. 2007	
P-selectin		Blum et al. 2003, Colacurci et al. 2005	

## AIMS OF THE STUDY

The purpose of the present study was to investigate the effects of soy supplementation on predominant markers of cardiovascular risk in non-human primates and postmenopausal women using tibolone. Additionally, the impact of equol producing capacity was studied. The specific targets were

- the circulating levels of lipids
- blood pressure, arterial stiffness and endothelial function
- the circulating levels of estrone,  $17\beta$ -estradiol, testosterone, androstenedione, DHEAS and SHBG
- the circulating levels of CRP, VCAM-1, ICAM-1 and P-selectin

## SUBJECTS, MATERIALS AND METHODS

### Subjects and design

#### *Monkeys*

All primate studies were carried out on 18 cynomolgus monkeys (*Macaca fascicularis*). These animals had been made postmenopausal by means of bilateral oophorectomy 4-6 years prior to the study. The monkeys were  $19 \pm 0.7$  (SEM) years of age (range 13-25 years), corresponding to middle-aged women.

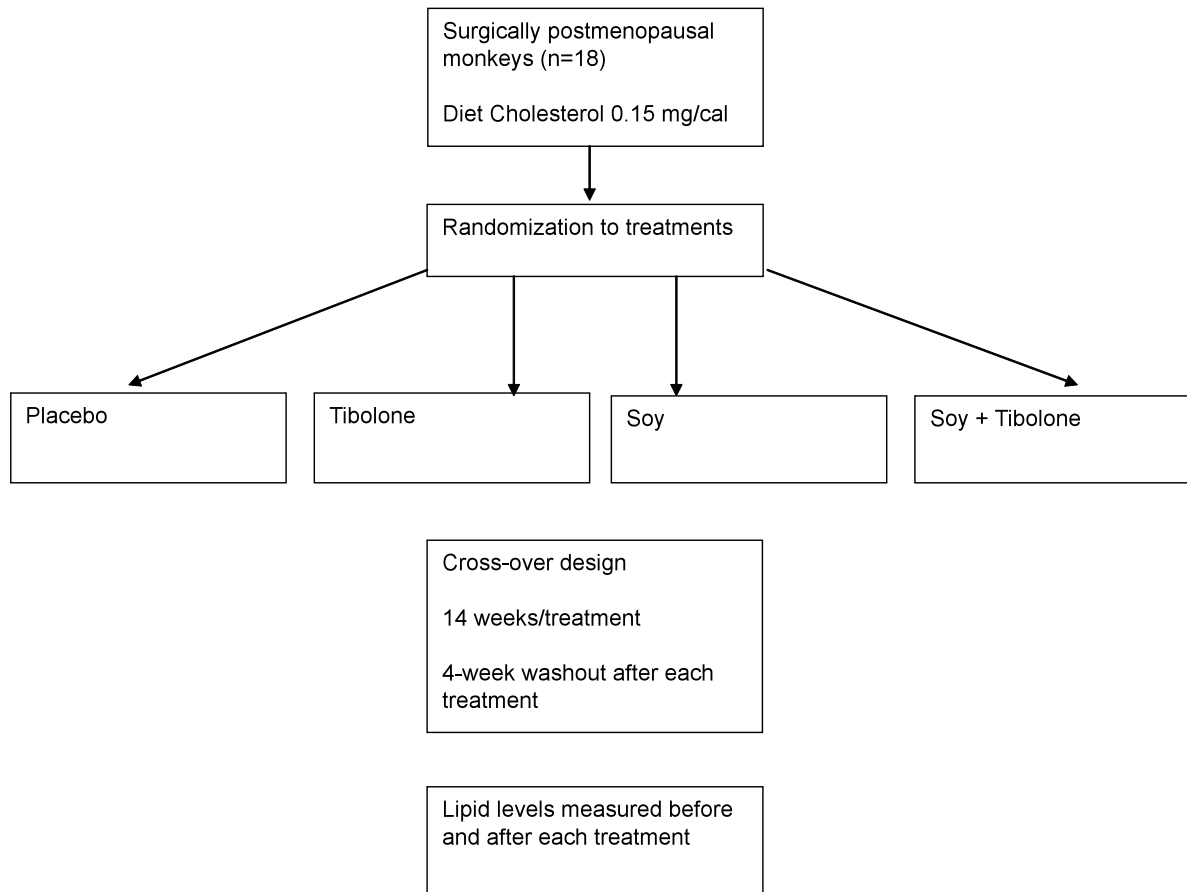
The monkeys were studied in compliance with state and federal laws, standards of the US Department of Health and Human Services, and guidelines established by the Wake Forest University Animal Care and Use Committee.

The monkeys were assigned to receive one of the four dietary regimens in a random order in such a way that all animals received all four diets. The diets contained either casein/lactalbumin (C/L) (placebo), tibolone, soy, or soy with tibolone (Figure 6). All diets were prepared in the diet laboratory of the Comparative Medicine Research Center (Wake Forest University School of Medicine, Winston-Salem, NC, USA) and were formulated to be equivalent in cholesterol and macronutrient content (protein, fat, carbohydrate). An essential amino acid, DL-methionine, was added to the soy-containing diets to make the content of sulfur-containing amino acids comparable to that in C/L diets. Detailed composition of the diets is presented in Table 1 of Study I.

The soy diet provided a woman's equivalent dose of 138 mg of isoflavones (phytoestrogens – genistein, daidzein, glycitein) per day. The soy protein (SUPRO 670-HG, Solae) contained, on average, 1.03 mg of genistein, 0.72 mg of daidzein and 0.13 mg of glycitein per gram of soy protein isolate (expressed in aglycone units). The tibolone dose was equivalent to a woman's daily dose of 1.25 mg. This dose was adjusted for metabolic differences between monkeys and equated to approximately 0.4 mg/monkey/day. Each treatment phase lasted for 14 weeks. All the monkeys were fed a washout diet (C/L) for 4 weeks before starting the next treatment regimen. Blood samples for determination of plasma lipid concentrations were obtained twice during each treatment phase.

Figure 6.

Design of the study on monkeys.





## Women

A total of 110 women were studied with the approval of the Ethics Committee of the Department of Obstetrics and Gynecology, University of Helsinki, Finland. The study was conducted during the years 2003-2007 and all of the women were recruited and studied in Helsinki. All the study subjects received written information on the trial and signed a consent document before participation. The investigation conforms with the principles outlined in the declaration of Helsinki.

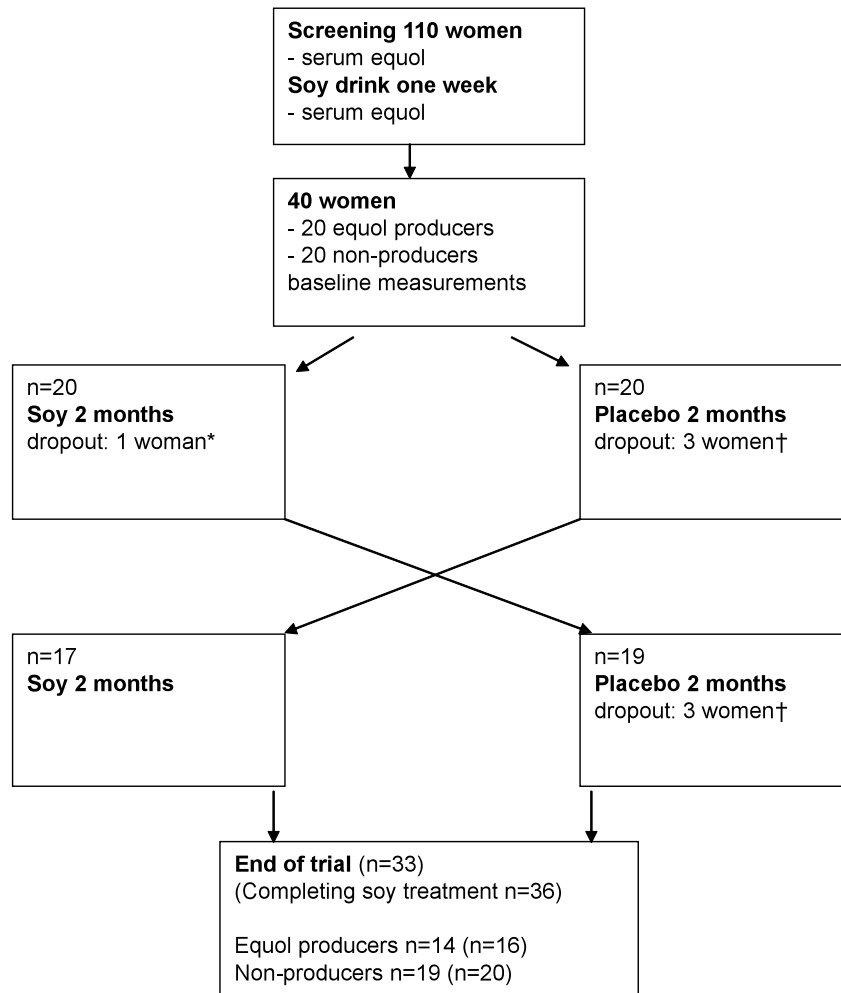
The volunteers were healthy non-smoking postmenopausal women who had used tibolone (Livial® 2.5 mg) for  $\geq 3$  months. They consumed a typical Western diet, and findings in general and pelvic examination were normal. Further inclusion criteria were body mass index (BMI)  $< 33 \text{ kg/m}^2$ , no use of antihypertensive medication and no use of antibiotics within 3 months prior to or during the study.

The women were screened for equol production capacity by means of a challenge test; soy powder (52 g of soy protein containing 112 mg of isoflavones; 63 mg of genistein, 43 mg of daidzein and 6 mg of glycitein expressed as aglycones) (Solae Co. St. Louis, Missouri) mixed with food or beverages once a day for one week (*Setchell et al. 1984, Setchell et al. 2003a*). In our study, a 4-fold rise from baseline serum equol concentration was the criterion for equol production capability. Twenty women of the 110 screened (18%) fulfilled this criterion. Twenty subsequent women without a rise in equol concentrations were studied as non-producers (controls). There was a minimum of 4 weeks between the soy challenge and initiation of the actual trial.

In the final trial, the two groups of women, 20 equol producers and 20 non-producers, were treated in a randomized order for 8 weeks either with a soy powder (52 g of soy protein containing 112 mg of isoflavones expressed as aglycones) or with a similar-looking placebo powder (containing 52 g of milk protein) (Solae Co. St. Louis, Missouri). The regimens could be mixed with food or beverages and taken as a single dose or divided into two daily doses. The first treatment period was followed by a washout period of 4 weeks, and then the treatments were crossed over for another 8 weeks (Figure 7). The women were encouraged to lead normal lives without changes in normal dietary habits, but they were strictly to avoid consuming additional soy-containing food or supplements. The women were seen at the research center before and at the end of each treatment period and blood samples were collected after an overnight fast. Compliance was evaluated by counting the used/unused study portions and by analyzing serum levels of genistein.

Figure 7.

Design of the study on women on long-term tibolone use.



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## Samples

To obtain serum, blood was allowed to clot and serum was separated by centrifugation. Aliquots of serum were stored at -80 °C until analyzed.

To obtain plasma, blood was drawn to heparin-containing tubes. Plasma was prepared by centrifugation and stored at -80 °C until analyzed.

## Assays

### *Isoflavones – Monkeys*

Serum concentrations of soy isoflavones (genistein, daidzein, equol) were determined by liquid chromatographic-photodiode array-electrospray ionization-mass spectrometric analysis at the Cancer Research Center of Hawaii, using techniques described previously (*Franke et al. 2002*). The reagents were obtained from Dr. Adrian Franke, Hawaii, USA. Measurements were carried out on serum samples collected 4 hours after feeding. Intra-assay coefficients of variation were <14% and interassay coefficients of variation ranged between 3-22% (8-22% at levels below 20 nmol/l, 7-14% at levels of 20-100 nmol/l and 3-12% at levels over 100 nmol/l) for genistein, daidzein and equol. The detection limits were 10 nmol/l for genistein and daidzein and 15 nmol/l for equol.

### *Isoflavones – Women*

Equol and genistein concentrations were assessed by time-resolved fluoroimmunoassays using europium chelate as label and coupling the isoflavone compounds to bovine serum albumin (Table 6). The final classification for equol producers and non-producers was based on data in the 2 month soy treatment and we used an equol elevation 4 times over baseline level as a discriminating factor.

Table 6.

Characteristics of the assays used for the measurement of genistein and equol in the women on long-term tibolone use. TR-FIA: time-resolved fluoroimmunoassay.

Analyte	Source of reagents	Principle of assay	Coefficient variation		Detection limit
			Intra-assay	Interassay	
Genistein	Labmaster Ltd.	TR-FIA	2.9-	5.9-	1.5
	Turku, Finland		4.7%	6.7%	
Equol	Labmaster Ltd.	TR-FIA	5.5%	6.0%	0.25
	Turku, Finland				

### *Lipids – Monkeys*

Blood samples for determination of plasma lipid concentrations were obtained twice during each treatment phase and collected after food had been withheld for 18 hours. Plasma total cholesterol and HDL cholesterol concentrations were measured in the laboratory of the Wake Forest University Primate Center using enzymatic methods, and a COBAS FARA II analyzer (Roche Diagnostics, Inc., Montclair, New Jersey). The protocols and reagents were supplied by Boehringer Mannheim. Plasma HDL cholesterol concentrations were determined using the heparin-manganese precipitation procedure (*Burstein and Samaille 1960, Manual of Laboratory Operations, Lipid Research Clinics Program 1974*) (Table 7). Plasma LDL cholesterol plus VLDL cholesterol was calculated as the difference between total cholesterol and HDL cholesterol.

Table 7.

Characteristics of the assays used for the measurement of total cholesterol and high-density lipoprotein (HDL) cholesterol in the monkeys.

Analyte	Source of reagents	Principle of assay	Coefficient variation		Detection limit
			Intra-assay	Interassay	
Total cholesterol	Boehringer Mannheim	Enzymatic methods on the COBAS FARA II analyzer	<5%	<5%	*
HDL cholesterol	Boehringer Mannheim	Heparin-manganese precipitation procedure	<5%	<5%	*

\* no detection limits; detection possible to extend as far as possible by diluting samples

### *Lipids – Women*

Concentrations of plasma total cholesterol, LDL cholesterol, HDL cholesterol and triglycerides were measured by means of standard methods: plasma total cholesterol and triglycerides concentrations were assessed by enzymatic colorimetric methods. Plasma lipoproteins were isolated by sequential ultracentrifugation (*Havel et al. 1955*) using a Beckman optima LE-80K ultracentrifuge and a Ti 50.4 rotor; LDL cholesterol was isolated at density range 1.019-1.063 g/ml and HDL at density range 1.063-1.21 g/ml. Ultracentrifugally isolated LDL and HDL were further purified by gel filtration on Sephadex G25 with phosphate-buffered saline (pH 7.4) as eluting solvent. The reagents were supplied by ABX Diagnostics Cholesterol for total cholesterol, LDL cholesterol and HDL cholesterol, and by ABX Diagnostics Triglycerides for triglycerides. Intra-assay coefficients of variation ranged between 0.42-1.26% for total cholesterol, LDL cholesterol and HDL cholesterol, and 1.46-1.91% for triglycerides. Interassay coefficients of variation ranged between 1.48-2.25% for total cholesterol, LDL cholesterol and HDL cholesterol, and 1.85-3.68% for triglycerides. The detection limits were 0.04 mmol/l for total cholesterol, LDL cholesterol and HDL cholesterol, and 0.08 mmol/l for triglycerides.

### *Sex steroids and sex hormone-binding globulin*

Serum samples from the women were assayed by radioimmunoassay for total estrone (DSL Beckmann Coulter, Sinsheim, Germany), by chemiluminescence immunoassay for total 17 $\beta$ -estradiol, total testosterone (Ortho Clinical Diagnostics, Neckergemünd, Germany), androstenedione and DHEAS (DPC Biermann Siemens Medical Solutions, Bad Nauheim, Germany). Serum samples were assayed by chemiluminescence immunoenzymetric assay for SHBG (DPC Biermann Siemens Medical Solutions, Bad Nauheim, Germany) (Table 8).

The free estradiol index was calculated as the ratio: 17 $\beta$ -estradiol (nmol/l) x 100 / SHBG (nmol/l) and the free androgen index as the ratio: total testosterone (nmol/l) x 100 / SHBG (nmol/l).

Table 8.

Characteristics of the assays used for the measurement of estrone, 17 $\beta$ -estradiol, total testosterone, androstenedione, dehydroepiandrosterone sulfate (DHEAS) and sex hormone-binding globulin (SHBG) in the women on long-term tibolone use.

Analyte	Source of reagents	Principle of assay	Coefficient variation		Detection limit
			Intra-assay	Interassay	
Estrone	DSL Beckmann Coulter Sinsheim, Germany	Radio-Immunoassay	9.0%	14.0%	3.7 pmol/l
17 $\beta$ -Estradiol	Ortho Clinical Diagnostics, Neckergemünd Germany	Chemiluminescence-Immunoassay	7.8%	11.8%	9.9 pmol/l
Testosterone	Ortho Clinical Diagnostics, Neckergemünd Germany	Chemiluminescence-Immunoassay	5.9%	7.9%	0.17 nmol/l
Androstenedione	DPC Biermann Siemens Medical Solutions, Bad Nauheim Germany	Chemiluminescence-Immunoassay	6.7%	11.3%	1.1 nmol/l
DHEAS	DPC Biermann Siemens Medical Solutions, Bad Nauheim Germany	Chemiluminescence-Immunoassay	8.0%	9.8%	0.1 $\mu$ mol/l
SHBG	DPC Biermann Siemens Medical Solutions, Bad Nauheim Germany	Chemiluminescence Immunoenzymetric Assay	2.3%	2.5%	0.02 nmol/l

### *Vascular inflammation markers*

Levels of CRP in the women were assayed by chemiluminiscence immunoenzymetric assay (DPC Biermann Siemens Medical Solutions, Bad Nauheim, Germany). Levels of VCAM-1, ICAM-1 and P-selectin in the women were assayed by enzymeimmuno-assay (R&D Systems) (Table 9).

Table 9.

Characteristics of the assays used for the measurement of C-reactive protein (CRP), vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1) and platelet-selectin (P-selectin) in the women on long-term tibolone use.

Analyte	Source of reagents	Principle of assay	Coefficient variation		Detection limit
			Intra-assay	Interassay	
CRP	DPC Biermann Siemens Medical Solutions, Bad Nauheim Germany	Chemiluminiscence Immunoenzy- metric Assay	4.7%	9.7%	0.1 mg/l
VCAM-1	R&D Systems	Enzymeimmuno- assay	5.4%	7.9%	0.8 ng/ml
ICAM-1	R&D Systems	Enzymeimmuno- assay	7.3%	9.3%	0.3 ng/ml
P-selectin	R&D Systems	Enzymeimmuno- assay	6.9%	8.5%	0.35 ng/ml



### *Estrogenic metabolites of tibolone*

At the end of the one-week soy challenge, additional serum samples from 20 women (equol producers n=11, non-producers n=9) were taken 1-2 hours after tibolone administration to measure the estrogenic metabolites of tibolone; 3 $\alpha$ -OH-tibolone (Org 4094) and 3 $\beta$ -OH-tibolone (Org 30126) (NV Organon, Oss, the Netherlands), as described before (*Verheul et al. 2007*). The analytes and internal standards were extracted from plasma by solid phase extraction using 100 mg, 3 ml Isolute C18 (EC) columns (Sopachem, Wageningen, the Netherlands) and eluted with acetonitrile. The analytes were quantified by derivatization under alkaline conditions at room temperature using validated gas-chromatography mass-spectrometry (Agilent 6890/5973 GC-MSD) operated in the positive mode. Coefficients of intra-assay and interassay variation were <20% and the detection limits were 0.1 ng/ml for both metabolites.

### Blood pressure

Blood pressure of the women was measured before and after each study period between 3-7 pm. After a minimum of 10 minutes rest in a supine position in a quiet room, blood pressure was assessed from the right arm using a validated oscillometric technique (Omron M4-I Intellisense, Omron Corporation, Japan) with medium cuff-size. Duplicate measures were taken in each case and the mean of the two recordings was used for data analysis. Mean arterial pressure was calculated by means of the equation  $([2 \times \text{diastolic blood pressure}] + \text{systolic blood pressure}) / 3$  (*Franklin et al. 1997*).

### Pulse-wave analysis

Arterial stiffness of the women was assessed by means of pulse-wave analysis (PWA), a validated and repeatable method (*Wilkinson et al. 2002*), using SphygmoCor® equipment (AtCor Medical, Sydney, Australia). The pulse-wave was recorded from the right radial artery using a hand-held micromanometer-tipped probe.

### *Augmentation Index*

As the pulse-wave was registered, a generalized transfer function was used to generate a waveform to correspond the ascending aortic waveform (central waveform). The aortic augmentation index (AIx) was calculated as the augmentation of aortic systolic blood pressure by the reflected pulse-wave, expressed as a percentage of the aortic pulse pressure. Since AIx is influenced by heart rate, AIx adjusted to a heart rate of 75 beats/min, as calculated by the software, was used in the analyses (*Wilkinson et al. 2002*).

### *Endothelial Function Index*

Endothelial function was assessed by using a nitroglycerin and salbutamol challenge. Nitroglycerin causes maximal vasodilation independently of the endothelium, whereas salbutamol triggers the release of NO from the endothelium and causes maximal endothelium-dependent vasodilation. After the baseline PWA recordings, 0.5 mg of nitroglycerin (Nitro resoriblet, Orion Pharma, Espoo, Finland) was administered sublingually and AIx was measured 3, 5, 10, 15 and 20 minutes later. After an hour had passed from the nitroglycerin intake, 2x200 µg salbutamol (Ventoline Evohaler, GlaxoSmithKline, Ware, UK) was inhaled using a Volumatic-spacer device) and AIx was measured at 5, 10, 15 and 20 minutes. All the PWA measures were carried out in triplicate and the mean of the triplicate baseline measurements, and the lowest AIx value after nitroglycerin- or salbutamol-induced vasodilation were included in the analyses. An endothelial function index (EFI), which expresses endothelium-dependent vasodilation as a percentage of endothelium-independent vasodilation, was calculated (*Lind et al. 1999, Sarabi et al. 2001, Rönnback et al. 2007*).

### Statistics

The monkey data were analyzed using Statistical Analysis Software (Version 9.1; SAS Institute, Inc., Cary, NC). All variables were first evaluated for their distribution and equality of variances between groups using univariate analysis and Levene's test for homogeneity of variance. A mixed general linear model with repeated measures was used to determine means and to test for main effects of treatment. All variables were screened in the initial model for phase-by-treatment

interactions and none were found. All results are expressed as mean $\pm$ SEM (standard error of mean) and a p-value  $<0.05$  was considered statistically significant.

The human data were analyzed using NCSS software (version 2001, Kaysville, Utah). Differences between baseline values and those during treatment were analyzed by the paired *t*-test. Comparison of two treatments (soy versus placebo) and comparison between equol producers and non-producers was carried out by the two-sample *t*-test. Since we used a crossover design, the possibility of a period effect was tested by means of the paired *t*-test, where we compared the differences between the periods in the two groups of subjects (those beginning with soy and those beginning with placebo); no carryover effect was detected. Thus, the women using soy or placebo were analyzed as single groups regardless of the treatment order. The Mann-Whitney *U*-test, the Wilcoxon Rank Sum Test or the Kolmogorov-Smirnov test was used in case of non-normal data distribution. Associations between different variables were evaluated by using linear regression analysis. All results are expressed as mean $\pm$ SEM (standard error of mean) and a p-value  $<0.05$  was considered statistically significant.

## RESULTS

Detailed results are given in the original publications and therefore only the main results are presented here.

### Monkeys

#### *Isoflavones*

Total isoflavone concentrations in monkeys fed either the Soy or Soy+Tibolone diet were within the range previously reported in other studies (*Clarkson et al. 2001a*) and were not significantly different from each other ( $p=0.99$ ). The proportion of isoflavone metabolites, equol, daidzein and genistein, did not differ between the two diet groups ( $p>0.15$  for all metabolites). Isoflavone levels detected in monkeys fed with isoflavone-free diets were below or at the detection level for the assay.

#### *Lipids*

An approximately 14% increase in plasma LDL+VLDL cholesterol was observed in tibolone-treated monkeys compared with those fed with the control diet (Tibolone vs. C/L  $p=0.004$ ). Soy treatment resulted in an approximately 18% reduction in plasma LDL+VLDL cholesterol (Soy vs. C/L  $p=0.0002$ ) and concomitant supplementation with tibolone did not negate the LDL+VLDL cholesterol-lowering effect of soy (Soy+Tibolone vs. Soy  $p=0.35$ ) (Table 10).

A 30% increase in HDL cholesterol (Soy vs. C/L  $p<0.0001$ ) was observed in monkeys fed with soy. Monkeys treated with tibolone showed large reductions in plasma HDL cholesterol (~48%, Tibolone vs. C/L  $p<0.0001$ ). Soy+Tibolone diet resulted in conservation of plasma HDL cholesterol concentrations, in such a way that monkeys fed with this diet did not differ from controls (Soy+Tibolone vs. C/L  $p=0.09$ ) (Table 10).

Monkeys treated with tibolone alone showed an increase in the total cholesterol (TC):HDL cholesterol ratio of 118% compared with controls (Tibolone vs. C/L  $p<0.0001$ ). Those treated with Soy or Soy+Tibolone showed decreases in the ratio of TC:HDL cholesterol of 34% (Soy vs. C/L  $p=0.03$ ) and 2.4% (Soy+Tibolone vs. C/L  $p=0.84$  [Soy+Tibolone vs. Soy  $p=0.05$ ]) (Table 10).

Table 10.

Plasma lipid concentrations (mmol/l) of low-density lipoprotein + very low-density lipoprotein cholesterol (LDL+VLDL cholesterol), high-density lipoprotein (HDL) cholesterol, and the ratio of total cholesterol (TC) to HDL cholesterol (TC:HDL-ratio) in the monkeys fed either with Soy, Tibolone, Soy+Tibolone, or Casein/Lactalbumin (C/L), measured at the end of each diet. Values of p denote the differences between the diets. Data are presented as mean±SEM.

Analyte	Diet			
	Soy	Tibolone	Soy+Tibolone	C/L
LDL+VLDL	5.55±0.55 * °	7.67±0.55 **	5.26±0.55 ** °	6.76±0.55 *
HDL	1.57±0.1 ***	0.63±0.1 †	1.09±0.1 °	1.21±0.1 *** † °
TC:HDL-ratio	5.38±1.5 †† °°	18±1.5 ***	8.0±1.5 ° °°	8.2±1.5 *** †† °

\* p=0.0002

\*\* p=0.004

\*\*\* p<0.0001

† p<0.0001

†† p=0.03

° p=NS

°° p=0.05

### Women

Of the 40 women, 33 (82.5%; equol producers n=14, non-producers n=19) completed the study. Seven women discontinued the trial; six for personal reasons and one because of a desire to quit using tibolone. Three of these (equol producers n=2 and non-producers n=1) completed the 2-month soy treatment period adequately before dropping out of the trial.

At baseline, no differences in age, weight, BMI, parity, time since onset of menopause or in duration of tibolone use between the equol producers and non-producers were found (Table 11). Concentrations of the estrogenic 3 $\alpha$ -OH- and 3 $\beta$ -OH-metabolites of tibolone showed no differences between the equol producers and non-producers.

Table 11.

Clinical characteristics of the women on long-term tibolone use at baseline. Values are expressed as mean±SEM (range). BMI: body mass index; y: years; m: months.

Clinical characteristics	All n = 40	Equol producers n = 20	Non-producers n = 20
Age (y)	57.7±0.8 (47-68)	57.9±1.1 (47-65)	57.4±1.3 (47-68)
Time in menopause	7.8±0.8 (1-21)	8.6±1.2 (1-21)	7.1±1.1 (1-16)
Weight (kg)	66.4±1.5 (48-85.2)	67.4±2.3 (49.8-85.2)	65.5±1.8 (48-80.4)
BMI (kg/m <sup>2</sup> )	24.6±0.5 (19.4-32.1)	24.4±0.8 (19.4-32.1)	24.8±0.7 (19.8-31.4)
Parity			
nulliparous	n=12 (30%)	n=6 (30%)	n=6 (30%)
parous	n=28 (70%)	n=14 (70%)	n=14 (70%)
Tibolone in use (m)	21.2±2.4 (3-60)	21.3±3.8 (3-60)	21±3.1 (5-54)

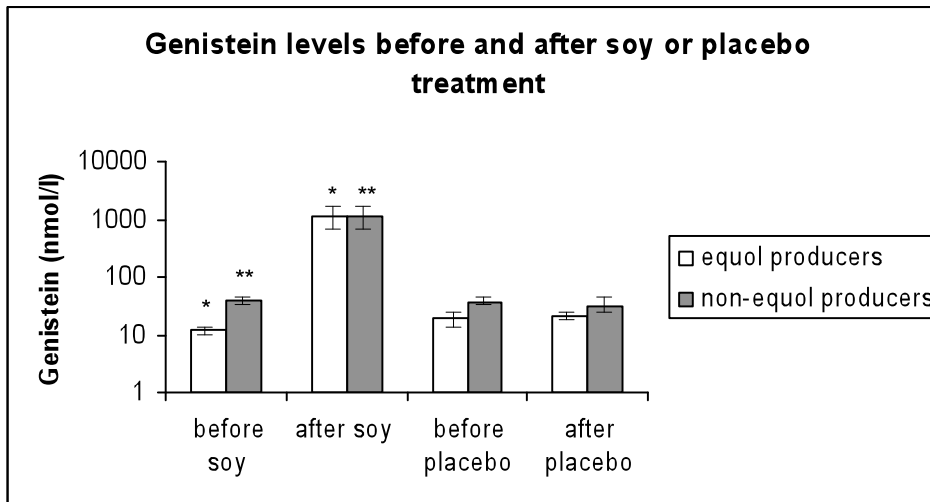
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### *Isoflavones*

The use of soy was confirmed by 40-fold rises in genistein concentrations (from 28±9.8 up to 1120±170 nmol/l, p<0.0001) in the entire study group. In the equol producers the genistein rise was 92-fold (from 12.6±3.8 up to 1160±282 nmol/l, p=0.0004) and 27-fold in the non-producers (from 40.3±17.1 up to 1092±212 nmol/l, p<0.0001) (Figure 8). No changes were seen during placebo use.

**Figure 8.**

Changes in levels of genistein after soy and placebo treatment in the women on long-term tibolone use; data for equol producers (n=16) and non-producers (n=20) are presented separately. The p-values denote the difference between baseline and soy treatment. Values are expressed as mean±SEM.

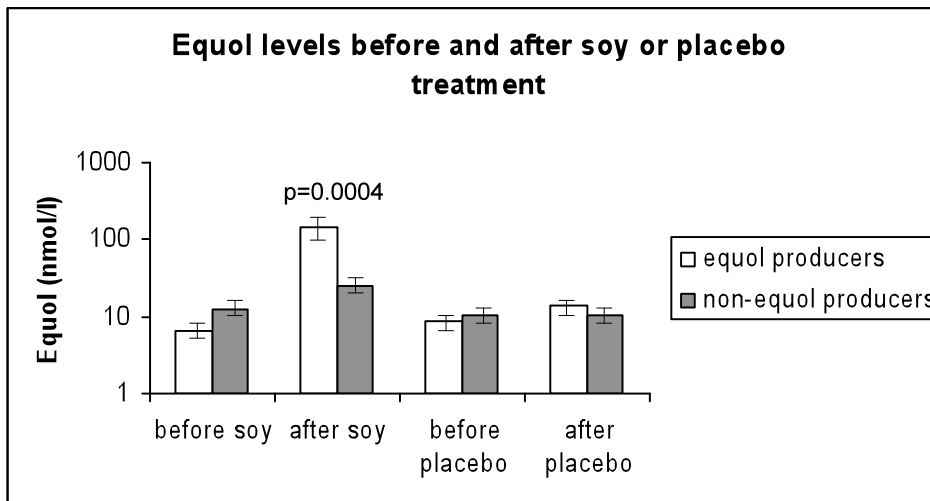


\* p=0.0004  
\*\* p<0.0001

The concentrations of equol rose 22-fold in the equol producers (from 6.6±1.2 to 144±33.9 nmol/l, p=0.0004) and 1.9-fold in the non-producers (from 12.7±1.0 to 24.4±2.8 nmol/l). No changes were seen during placebo use (Figure 9).

**Figure 9.**

Changes in levels of equol after soy and placebo treatment in the women on long-term tibolone use; data for equol producers (n=16) and non-producers (n=20) are presented separately. The p-value denotes the difference between baseline and soy treatment in the equol producers. Values are expressed as mean±SEM.



### *Lipids*

Soy supplementation for 2 months resulted in a significant decrease (p=0.006 for difference between soy and placebo) in LDL cholesterol compared with placebo treatment. A similar trend (p=0.06 for difference between soy and placebo) was observed in total cholesterol concentrations, but no effect on HDL cholesterol or triglycerides was seen (Table 12). When the equol producers and non-producers were evaluated separately, the reduction in LDL cholesterol levels was significant only in the equol producers. There were no differences in lipid concentrations between equol producers and non-producers at baseline.



Table 12.

Changes in total cholesterol (TC), low-density lipoprotein cholesterol (LDL), high-density lipoprotein cholesterol (HDL) and triglycerides (TG) (all mmol/l) from baseline after soy and placebo treatment in the women on long-term tibolone use. Values of p denote the difference between baseline and after treatment, or soy vs. placebo. Data are presented as mean±SEM for the entire study population (A), and equol producers (B) and non-producers of equol (C) separately.

A. All (n=32)

Analyte	Baseline	Soy	p	Placebo	p	Soy vs. Placebo
TC	5.25±0.1	4.89±0.2	0.001	5.1±0.1	0.09	p=0.06
LDL	3.75±0.1	3.37±0.2	0.0003	3.61±0.1	0.07	p=0.006
HDL	1.38±0.1	1.36±0.1	0.7	1.33±0.1	0.3	p=0.3
TG	0.97±0.1	0.93±0.1	0.4	0.92±0.1	0.3	p=0.8

B. Equol producers (n=14)

Analyte	Baseline	Soy	p	Placebo	p	Soy vs. Placebo
TC	5.35±0.2	4.86±0.2	0.0009	5.09±0.3	0.02	p=0.2
LDL	3.88±0.3	3.39±0.2	0.0002	3.63±0.2	0.05	p=0.04
HDL	1.33±0.1	1.34±0.1	0.9	1.27±0.1	0.5	p=0.3
TG	0.95±0.1	0.94±0.1	0.5	1±0.1	0.5	p=0.5

C. Non-producers of equol (n=18)

Analyte	Baseline	Soy	p	Placebo	p	Soy vs. Placebo
TC	5.18±0.1	4.92±0.3	0.1	5.13±0.2	0.7	p=0.2
LDL	3.65±0.1	3.35±0.2	0.04	3.59±0.2	0.6	p=0.07
HDL	1.42±0.1	1.38±0.1	0.5	1.38±0.1	0.5	p=0.9
TG	0.99±0.1	0.92±0.1	0.4	0.86±0.1	0.09	p=0.3

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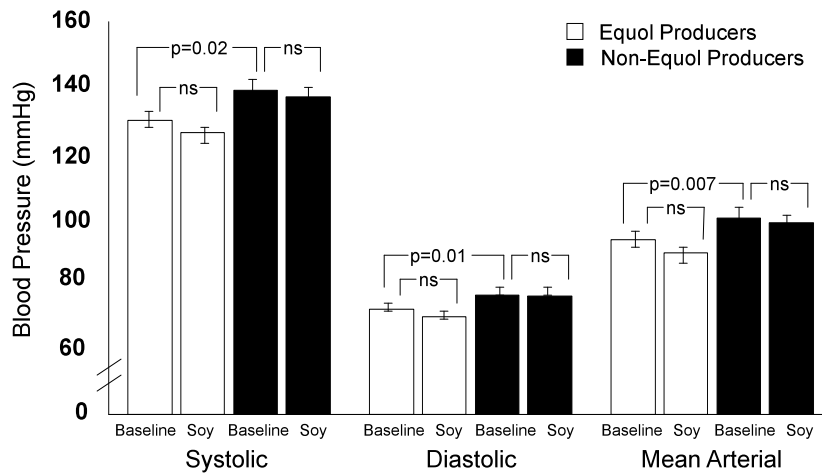
Neither soy nor placebo supplementation resulted in a change in body weight. No changes in the levels of any lipid correlated with individual changes in serum equol or genistein levels, nor with any baseline characteristics.

### *Blood pressure*

The equol producers compared to the non-producers had significantly lower baseline systolic ( $129.9 \pm 2.6$  vs.  $138.5 \pm 3.1$  mmHg,  $p=0.02$ ), diastolic ( $72.2 \pm 1.5$  vs.  $76.6 \pm 1.3$  mmHg,  $p=0.01$ ) and mean arterial ( $93.5 \pm 1.7$  vs.  $99.9 \pm 1.8$  mmHg,  $p=0.007$ ) blood pressure. Soy supplementation had no effect on blood pressure, but the differences between the equol producers and non-producers persisted as regards the systolic, diastolic and mean arterial blood pressure during soy intake (Figure 10).

**Figure 10.**

Difference in blood pressure among long-term tibolone users producing (n=14) and not producing (n=19) equol at baseline and after soy supplementation. Values of p denote differences between equol producers and non-producers. NS: non-significant difference. Data are presented as mean±SEM.



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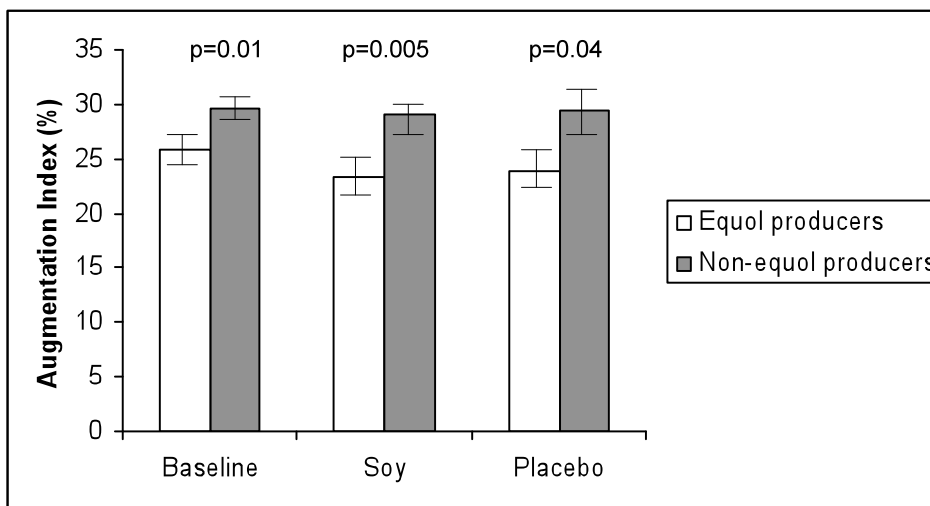
### *Arterial stiffness and endothelial function*

At baseline AIX was lower (p=0.01) in the equol producers (25.9±1.1%) than in the non-producers (29.6±0.9%). Soy or placebo supplementation had no effect on these levels (Figure 11).

Augmentation index was not associated with age, BMI or individual equol concentrations before soy intake.

Figure 11.

Differences in arterial stiffness (expressed as augmentation index) between equol producers (n=16) and non-producers (n=20), before and during soy and placebo supplementation among long-term tibolone users. Two baseline recordings from each woman are included. Values of p denote differences between the groups (equol producers vs. non-producers). Values are expressed as mean%±SEM.

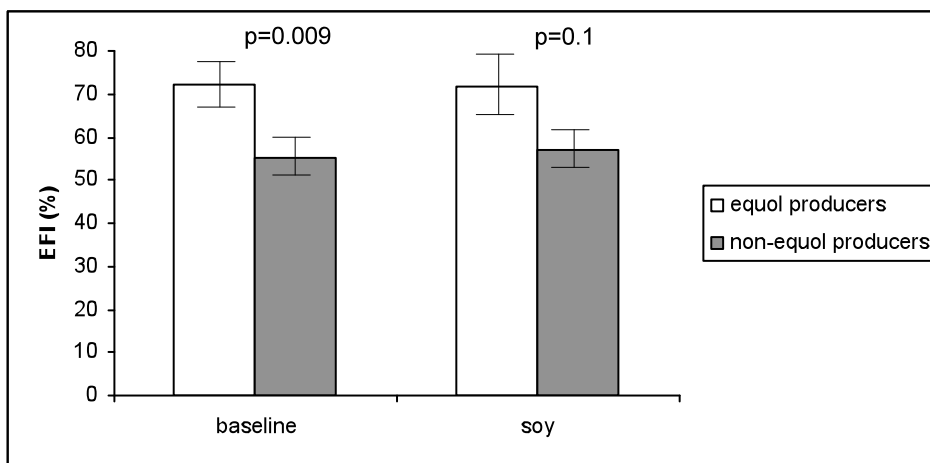


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Similarly, EFI was significantly better (p=0.009) in the equol producers (72.3±5.3%) than in the non-producers (55.2±3.8%) at baseline. Soy intake had no significant effect on EFI in either group, although a similar trend was observed after soy treatment compared with baseline (Figure 12). No correlations were seen between AIX and EFI, or between genistein levels and AIX or EFI.

Figure 12.

Differences in endothelial function (expressed as EFI levels) between equol producers (n=16) and non-producers (n=20) at baseline and after soy supplementation among long-term tibolone users. Two baseline recordings from each woman are included. Values of p denote differences between the groups (equol producers vs. non-producers). Values are expressed as mean%±SEM. EFI: endothelial function index.



### *Sex steroids and sex hormone-binding globulin*

At baseline, there were no differences in the levels of sex steroids or SHBG between the equol producers and non-producers. Moreover, these levels showed no association with the baseline levels of equol or genistein. Parity, age, time since menopause and the duration of tibolone use were not determinants of the baseline levels of sex steroids, genistein or equol.

In the entire study population soy supplementation was accompanied by a 7.1% reduction in estrone levels, whereas placebo had no such effect. The reduction was significant only in equol producers (12.5%; p=0.04). The level of testosterone decreased by 15.5% (p=0.02) after soy, and this reduction was also seen only in equol producers (22.1%; p=0.03) rather than non-producers of equol (10%; NS). Free estradiol and androgen indexes showed no changes during soy intake (Table 13).

Table 13.

Baseline and post-treatment levels of estrone, 17 $\beta$ -estradiol, total testosterone, free estradiol index, free androgen index, androstenedione, dehydroepiandrosterone sulfate (DHEAS) and sex hormone-binding globulin (SHBG) in the entire study population (n=36) of long-term tibolone users. Data are separately presented for equol producers and non-producers if significant differences emerged. Differences denoted by asterisks and daggers refer to differences between baseline and post-soy measurements. Values are expressed as mean $\pm$ SEM.

Analyte	Baseline	After soy	After placebo
Estrone (pmol/l)	69.6 $\pm$ 5.6	64.7 $\pm$ 4.0	70.8 $\pm$ 9.2
Equol producers (n=16)	80.2 $\pm$ 10.8 *	70.3 $\pm$ 7 *	67.6 $\pm$ 7.4
Non-producers (n=20)	61.1 $\pm$ 4.6	60.3 $\pm$ 4.6	73.2 $\pm$ 15.2
17 $\beta$ -estradiol (pmol/l)	64.9 $\pm$ 4.8	67.5 $\pm$ 4.8	68.4 $\pm$ 7.4
Free estradiol index	0.43 $\pm$ 0.1	0.5 $\pm$ 0.1	0.6 $\pm$ 0.1
Testosterone (pmol/l)	586 $\pm$ 62.6 **	495 $\pm$ 50.1 **	549 $\pm$ 68.6
Equol producers (n=16)	595 $\pm$ 111 ***	464 $\pm$ 82.1 ***	632 $\pm$ 133
Non-producers (n=20)	578 $\pm$ 72.4	520 $\pm$ 63.3	489 $\pm$ 67.9
Free androgen index	3.7 $\pm$ 0.5	4.0 $\pm$ 0.5	4.3 $\pm$ 0.6
Androstenedione (nmol/l)	4.7 $\pm$ 0.3	4.4 $\pm$ 0.3	4.4 $\pm$ 0.3
DHEAS (umol/l)	3.1 $\pm$ 0.3	3.3 $\pm$ 0.3	3.1 $\pm$ 0.3
SHBG (nmol/l)	19.3 $\pm$ 1.5 † ††	15.6 $\pm$ 1.2 †	15.0 $\pm$ 1.2 ††

\* p=0.04    \*\* p=0.02    \*\*\* p=0.03    † p=0.0003    †† p=0.03

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### *Vascular inflammation markers*

At baseline, there were no differences in the levels of CRP or adhesion molecules between the equol producers and non-producers. Parity, age, time since menopause or the duration of tibolone use were not determinants of the baseline levels of inflammation markers, genistein or equol.

The levels of CRP and ICAM-1 showed no changes after the use of soy, whereas the levels of VCAM-1 increased by 9.2% (p=0.06) and those of P-selectin decreased by 10.3% (p=0.002). The reduction in P-selectin was larger the longer tibolone had been used (r=-0.63; p=0.009). Equol producers showed a larger reduction in P-selectin than non-producers of equol (13.5% vs. 7.7%), whereas it was not a factor as regards changes in VCAM-1 (Table 14).

Table 14.

Baseline and post-treatment levels of C-reactive protein (CRP), vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1) and platelet-selectin (P-selectin) in the entire study population (n=36) of long-term tibolone users. Data are separately presented for equol producers and non-producers if significant differences emerged. Differences denoted by asterisks refer to differences between baseline and post-soy measurements. Values are expressed as mean±SEM.

Analyte	Baseline	After soy	After placebo
CRP (mg/l)	2.4±0.4	2.46±0.4	2.3±0.3
VCAM-1 (ng/ml)	512±17.5 *	559±22.8 *	511±19.8
ICAM-1 (ng/ml)	211±10.1	210±9.6	198±8.3
P-selectin (ng/ml)	184±9.1 **	165±11 **	182±14.3
Equol producers (n=16)	186±10.9 ***	161±10.9 ***	211±29.1
Non-producers (n=20)	182±14.2	168±18.0	161±11.0

\* p=0.06

\*\* p=0.002

\*\*\* p=0.007

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Levels of equol showed no relationship to changes in the levels of steroids or inflammation markers, except a significant relationship between concentrations of equol and DHEAS in equol producers (r=0.65, p=0.007) at baseline and after soy supplementation (r=0.51, p=0.04). The soy-induced changes in estrone and testosterone concentrations were related to each other (r=0.51; p=0.002).

## DISCUSSION

Tibolone is an effective alternative for the treatment of menopausal symptoms (*Speroff and Clarkson 2003, Kenemans and Speroff 2005, Notelovitz 2007*) and approximately 15 000 women used it in Finland in 2007 (*The Social Insurance Institute of Finland*). It does not cause withdrawal bleeding and may improve sexual well-being (*Kloosterboer 2001, Reed and Kloosterboer 2004*). A major concern as regards the use of tibolone has been the reduction in the levels of cardioprotective HDL cholesterol (*Notelovitz 2007*). Although tibolone decreases the levels of triglycerides at the same time, which may counteract the negative effect of falling concentrations of HDL cholesterol, the cardiovascular safety of tibolone has been questioned (*Bots et al. 2006*). Indeed, CIMT progression rates have been found to be higher with tibolone compared with placebo. Interestingly, separating the women into Europeans and Americans an increase in mean CIMT was found in European women after tibolone compared with placebo, whereas no effect was seen in American women (*Bots et al. 2006, Clarkson 2006*). So far there exists no explanation for this alarming phenomenon, but perhaps dietary factors could be involved. This brings out the use of soy, which shows national differences (*Keinan-Boker et al. 2002, Consumer attitudes about nutrition 2007*).

The cynomolgus macaque study model has been used for more than three decades and is suitable for evaluating the effects of a given regimen on atherosclerosis progression, especially in women (*Clarkson et al. 2001a-b*). First, cynomolgus macaques resemble humans in that there is 94% DNA homology (*Clarkson et al. 2004*). Second, female macaques have a similar menstrual cycle and menopause profile as in women. Again, similarly to women, premenopausal female macaques have higher circulating HDL cholesterol levels and less coronary artery atherosclerosis than male monkeys (*Clarkson 2007*). In a previous study with monkeys, tibolone did not worsen coronary artery atherosclerosis despite a reduction in HDL cholesterol levels (*Clarkson et al. 2004*). In the present study in postmenopausal monkeys, the tibolone-induced HDL cholesterol reduction (-48%) was reversed with a soy diet; the ratio of total cholesterol to HDL cholesterol was also reduced (*Study I*).

The results from the monkey study led us to explore whether soy supplementation would have similar effects on circulating lipids in postmenopausal women using tibolone. Additionally, other predominant markers of CVD risk (blood pressure, arterial stiffness, endothelial function) were included. Furthermore, of the various circulating surrogate markers of CVD (*Mueck and Seeger 2006*), we chose to study estrogens, testosterone, androstenedione,



DHEAS, SHBG and markers of vascular inflammation and adhesion, since changes in these are connected to CVD risk, particularly in women (*Davison and Davis 2003, Mueck and Seeger 2006, Clarkson 2008*). In our study, the female volunteers were healthy long-time users of tibolone. Based on previous studies, it was judged that both the exposure time and dosage of the soy regimen we used in our study should be adequate for triggering any effects of soy if such existed (*Tham et al. 1998, Dalais et al. 2003, Nikander et al. 2004*). Consistent elevations in serum genistein levels in our study subjects confirmed that the women had used soy as instructed. We used a minimum of a 4-fold elevation from baseline serum equol level as a sign of equol producing capacity, and this is a more strict criterion than employed in many previous studies (*Frankenfeld et al. 2004a-b, Grace et al. 2004, Kreijkamp-Kaspers et al. 2005*). Therefore, only 18% of our study subjects fulfilled this criterion, but we may assume that all these subjects were truly equol producers.

We observed decreases in the levels of total and LDL cholesterol during soy supplementation in postmenopausal women using tibolone, but no effect on HDL cholesterol. The longer soy supplementation period (14 weeks) in the monkeys compared with the women (8 weeks) may account for differences in LDL cholesterol reductions and the lack of effect on HDL cholesterol. In addition, in monkeys, cholesterol in the blood is mainly derived from freely absorbed cholesterol, whereas in women the majority of circulating cholesterol originates from the liver. Finally, all monkeys produce equol (*Gu et al. 2006*), whereas only 18% of women did so in our study.

Soy supplementation showed no effect on blood pressure in tibolone users in our study. Previously, soy has been found to decrease (*Rivas et al. 2002, Welty et al. 2007*) or has not affected (*Jenkins et al. 2002*) blood pressure. However, in these studies the postmenopausal women were not using tibolone and therefore direct comparison is difficult, as no previous data exist on the concomitant use of tibolone and soy. Interestingly, in our study blood pressure was already lower in equol producers before the beginning of soy supplementation, and this feature persisted during soy treatment. Equol itself is unlikely to reduce blood pressure, since the baseline equol concentrations did not differ between equol producers and non-producers. Our findings are not likely to have resulted from equol-related changes in the metabolism of tibolone, because levels of the estrogenic metabolites of tibolone showed no difference between equol producers and non-producers. More likely, equol production capability may just be a new marker in the regulation of blood pressure. Equol may increase renal sodium output (*Giménez et al. 1997*), which in theory should decrease blood pressure. Furthermore, equol has shown vasorelaxant activity in the cerebral arteries of normotensive rats, similarly to daidzein, whereas in hypertensive

rats this was the case only with equol (*Jackman et al. 2007*). Of course it is also possible that equol may have direct vascular effects (*Chin-Dusting et al. 2001*), but the exact mechanism and whether it is endothelium-dependent or -independent, is unknown.

Soy supplementation did not affect arterial stiffness or endothelial function in tibolone users in our study. Previous data have shown improved endothelial function during soy supplementation in postmenopausal women not using tibolone (*Cuevas et al. 2003, Steinberg et al. 2003, Colacurci et al. 2005*), although this finding has not been seen in all studies (*Hale et al. 2002, Kreijkamp-Kaspers et al. 2005, Evans et al. 2007*). Soy may, however, have different effects in tibolone users and non-users, particularly if the women are equol producers. In our study, equol producers at baseline had less arterial stiffness and better endothelial function than non-producers of equol. The lower augmentation index and higher endothelial function index also persisted after soy supplementation. Thus, tibolone users producing equol may have more compliant arteries than non-producers, even in the absence of soy intake. This finding may also imply a direct vascular effect of equol.

The levels of estrone and testosterone decreased during soy treatment in tibolone users. Similar reductions during the use of soy have been reported earlier in premenopausal women (*Duncan et al. 2000*), and also in monkeys not exposed to tibolone (*Wood et al. 2007*). Soy may have acted via various pathways, perhaps by increasing the activity of 5 $\alpha$ -reductase, which converts testosterone to 5 $\alpha$ -dihydrotestosterone. It also increases the activity of sulfotransferase, which results in increased elimination of estrone. The latter is, however, unlikely in view of a previous study where soy, and equol in particular, were potent inhibitors of this enzyme (*Harris et al. 2004*) and therefore other explanations seem more likely. According to Atkinson et al., the fecal bacteria of equol producers are capable of metabolizing more estrone to 17 $\beta$ -estradiol, and 16 $\alpha$ -hydroxyestrone to estriol (*Atkinson et al. 2006*). This may also have been the case in our study, as changes in the levels of testosterone and estrone were more pronounced in equol producers. No significant changes occurred in the free estradiol index, the free androgen index, or levels of SHBG, androstenedione, or DHEAS. The significant relationship observed between the circulating levels of DHEAS and equol in the equol producers may imply that equol is involved in the regulation of androgens of the adrenal cortex in women using tibolone, and in principle a similar finding has been reported in men (*Lewis et al. 2005*).

Supplementation with soy or isolated isoflavones has decreased (*Hall et al. 2005*) or brought about no changes in the levels of CRP (*Nikander et al. 2003b, Hanson et al. 2006, Matthan et al. 2007*), and brought about no changes in the levels of VCAM-1, ICAM-1 or E-selectin (*Hall et al. 2005*). In our tibolone users, soy supplementation did not affect the levels of

CRP or ICAM-1, whereas the levels of VCAM-1 increased; the latter can be taken as a sign of potential endothelial dysfunction (*Blankenberg et al. 2003*). In contrast, at the same time, the levels of P-selectin fell, which in theory is a favorable effect (*Blankenberg et al. 2003*). The reduction in P-selectin was more pronounced in the equol producers and hence equol might participate in platelet aggregation. Taken together, soy supplementation brought about such small changes in the levels of vascular inflammation and adhesion markers in postmenopausal women using tibolone that their clinical significance is likely to be modest.

To conclude, soy supplementation reversed the tibolone-induced fall in HDL cholesterol levels in postmenopausal monkeys. However, this effect of soy was not seen in postmenopausal women using tibolone. Soy supplementation in tibolone users failed to modify blood pressure, arterial stiffness or endothelial function, but equol production capability itself was associated with beneficial cardiovascular changes in postmenopausal women using tibolone. Our data do not justify recommending assessment of equol production status in postmenopausal women in clinical practice in general. More data are needed in women using and not using tibolone to evaluate whether equol production capacity truly has an impact on cardiovascular health.

## CONCLUSIONS

In postmenopausal monkeys, co-administration of soy and tibolone improved the lipid profile by maintaining the level of HDL cholesterol (compared with a 48% decrease with tibolone alone), by lowering the level of LDL+VLDL cholesterol in a similar manner (22%) as soy treatment alone (18%), and by maintaining the ratio of total cholesterol to HDL cholesterol. These effects may offer cardiovascular protection.

In postmenopausal women, the administration of soy in long-term tibolone users modestly improved the lipid profile by way of reductions in the levels of total cholesterol and LDL cholesterol, whereas HDL cholesterol was not affected.

Postmenopausal women using tibolone and producing equol had lower blood pressure, more compliant arteries and better endothelial function than non-producers of equol. These findings may imply cardiovascular benefits in postmenopausal tibolone users producing equol.

The decreases in the levels of estrone and testosterone brought about by soy supplementation or equol production capacity may imply differences in steroid metabolism between equol producers and non-producers, but the clinical significance of this finding is unknown.

The effect of soy supplementation or equol production capacity on vascular inflammation and adhesion markers in postmenopausal women using tibolone were only minor and the changes are unlikely to be of clinical significance.

## ACKNOWLEDGMENTS

This study was carried out at Helsinki University Women's Hospital and at the Research Laboratory of the Department of Obstetrics and Gynecology, Helsinki University Central Hospital, during the years 2003-2007.

My deepest gratitude goes to my supervisors, professor Olavi Ylikorkala and docent Tomi Mikkola, for the excellent guidance throughout this project. Professor Ylikorkala is an outstanding teacher and scientist on whom I could always count to offer solutions, share his knowledge and guide me when times were difficult. He made me work hard, but for that I am grateful and more competent today. During these years my appreciation for docent Mikkola's talent has constantly grown. He is an enthusiastic and hard-working scientist, utterly critical and always capable of staying positive and seeing the essential. Keeping up with the pace of these two gentlemen was a great challenge, but the journey was well worthwhile.

Docent Antti Perheentupa and docent Tapio Ranta, the official reviewers, are warmly thanked for their careful revision and valuable comments, which markedly improved this thesis. Doctor Nick Bolton is warmly thanked for his expert revision of the language of this thesis.

I am deeply indebted to professor Thomas B. Clarkson and doctor Susan Appt, our sympathetic collaborators over the Atlantic; without their preliminary monkey studies, belief, and professional support, this study would not have been possible. The important contribution of professor Adrian A. Franke is equally acknowledged.

The scientific expertise of professor Matti J. Tikkanen, his generous collaboration with our research group and interest in my work during these years is gratefully acknowledged – not forgetting his excellent laboratory personnel.

I warmly thank professor Per-Henrik Groop for offering us the equipment we used for the pulse-wave analysis studies, and the young doctor from his research group, Mats Rönnback, for the many days and hours he spent patiently instructing us in the method.

Doctor Alfred O. Mueck and doctor Harald Seeger are warmly thanked for their collaboration in this project. Doctor Helenius J. Kloosterboer/Organon is warmly thanked for measuring the serum estrogenic metabolites of tibolone.

My warmest appreciation goes to doctor Eini Nikander for giving me the opportunity to participate in her project, to professor Aila Tiitinen for her contribution in my first publication, and to doctor Mervi Väisänen-Tommiska for support and guidance with statistics and the whole process of writing my thesis.

We all depended on the expert staff of the lab, especially Eira Halenius, who made my life so much easier patiently teaching and encouraging me, as well as Kirsti Räsänen and Tomi Silvennoinen, who offered me guidance, support and company in the lab. The important work of Pirkko Timonen with our subjects cannot have been an easy task, but she managed it wonderfully and for that I am grateful and appreciate her part in this study a great deal. This study, like many others, would not have been possible without our subjects and I am extremely grateful to these volunteers.

Laila Selkinen and I go way back to 1983. I am deeply grateful for her role in initiating the actions that led me to join the research group and for always supporting and defending me. Leena Vaara is warmly thanked for her technical advice in several situations and for always organizing, knowing and taking care of everything. Maaria Puupponen is warmly thanked for her help with the final steps in preparing this thesis.

Fellow researchers Maija and Robert Badeau, Hanna Hautamäki, Päivi Rahkola, Hanna Savolainen-Peltonen and Pauliina Tuomikoski are warmly thanked for their support, smiles and understanding the ups and downs of a Ph.D student. Doctor Minna Tikkanen is warmly thanked for her valuable advice in practical matters, encouragement and friendship.

Friends and colleagues from Hyvinkää Hospital and Helsinki University Central Hospital, Department of Obstetrics and Gynecology, are warmly thanked for their interest, support and enjoyable company; particularly Jenni, who understands so many things in so many fields. Doctor Minna Kauko, a wonderful person and a pioneer of our time, will always be my spiritual leader in the field of gynecology and responsible for the beginning of my clinical career. I consider the period of working under her guidance a tremendous honor.

Friends and colleagues outside the research world are deeply thanked for being there, for clearing my mind, and for reminding me of the important things in life.

Finally, and most importantly, my heartfelt thanks go to my family; my loved-ones, my everything. My parents, my brother, my sisters; you are always on my mind. Mikko, huge thanks for everlasting technical assistance. Laura, huge thanks for keeping high spirits. Jemppa and Justus, you represent love, happiness, harmony and all that is good in life.

This study was financially supported by grants from the Finnish Menopause Society, the Yrjö Jahnsson Foundation, the Päivikki and Sakari Sohlberg Foundation, the Juho Vainio Foundation, Finnish State Funds (TKK 6695, TKK 7695) and grants from the research funds of Helsinki University Central Hospital. Solae Company kindly provided the soy and the placebo products.

Helsinki, October 2008

Riina Jernman

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