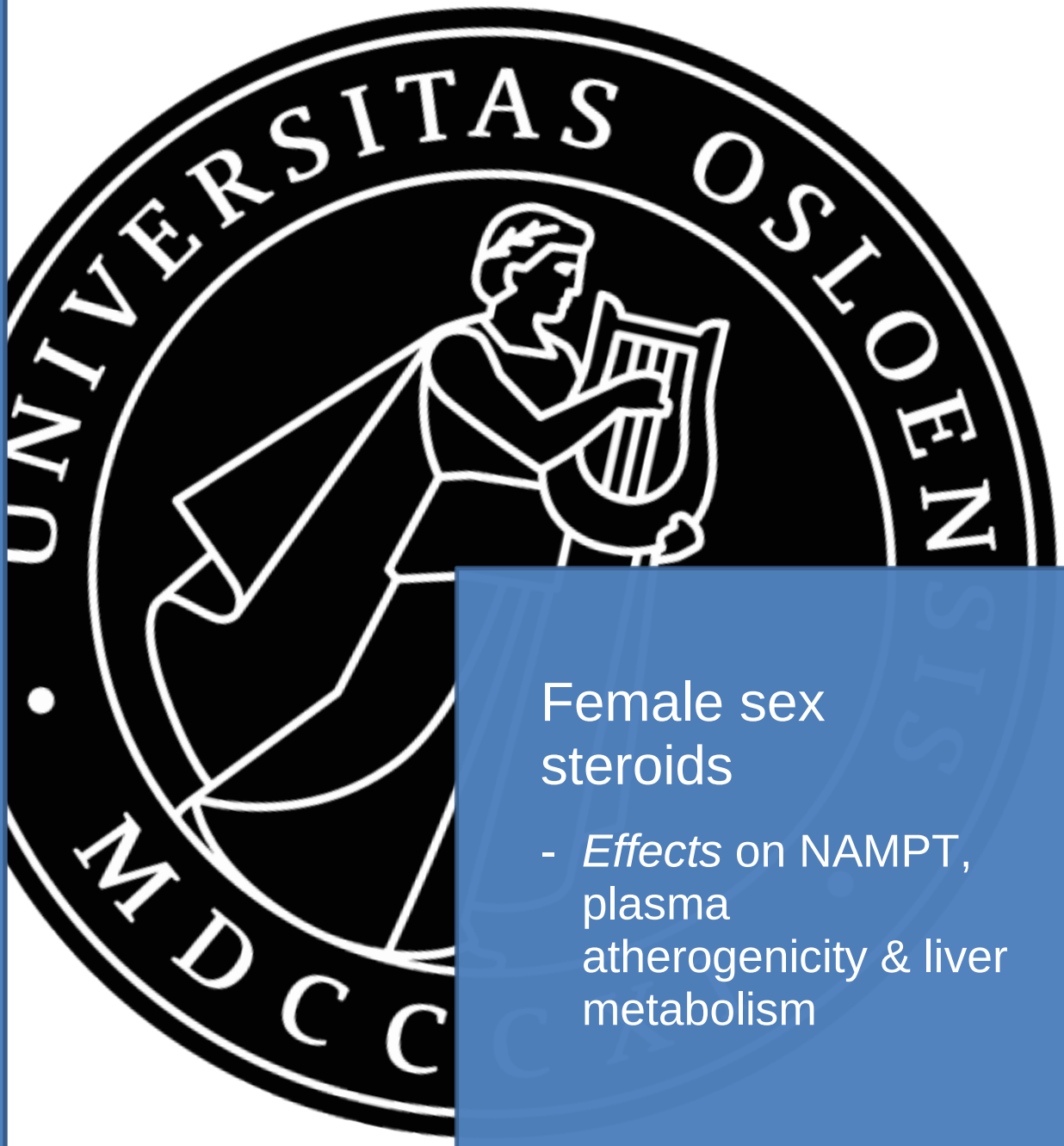

Female sex steroids

- *Effects* on NAMPT, plasma atherogenicity & liver metabolism

Master Thesis by

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May 2015

Female sex steroids

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Oslo, May 2015

Lene Løvdahl

Summary

Background and aims: In menopause, women experience changes in their hormonal milieu. Menopause is associated with cardiovascular complications, proatherogenic changes in lipoprotein pattern and enhanced pathobiology. Hormone replacement therapy (HRT) has shown both favorable and adverse effects on cardiovascular risk. An improved understanding regarding the physiological effects of female sex steroids is crucial. In the present thesis we wanted to study the effect of exogenous female sex steroids on the atherosclerosis associated protein Nicotinamide phosporibosyltransferase **(**NAMPT), plasma atherogenicity, hepatic lipid homeostasis and inflammation.

Methods: Two model systems were utilized for this purpose: A) *in vivo,* studying the effect of oral HRT in postmenopausal women at high risk of venous thromboembolism, randomized to either placebo or 2 mg estradiol and 1 mg norethisterone acetate for 3 months. B) *in vitro*, studying gene expression and cell release in a human hepatocyte cell line (HepG2) after exposure to various doses of 17α-ethinyl estradiol (EE2). The targets of interest for system A) were plasma NAMPT and circulating lipoproteins, apolipoproteins and their ratios and for system B) NAMPT and genes/proteins involved in hepatic lipid homeostasis and inflammation. Gene expression, protein secretion and triglycerides were measured using qRT-PCR, suspension array technology and an enzymatic reaction, respectively.

Results: This thesis is to our knowledge, the first to report the effect of sex steroids on plasma NAMPT and NAMPT cell release. HRT did not have a significant effect on plasma NAMPT, but EE2 exerted a suppressive effect on NAMPT cell release in hepatocytes. We found both favorable and adverse effects on circulating apolipoproteins and lipoproteins, but only favorable effects on lipoprotein ratios. In hepatocytes, some genes involved in the reverse cholesterol transport, bile acid metabolism and *de novo* lipogenesis were induced by EE2. There was no effect on hepatic inflammation or cell release of apolipoproteins.

Conclusions: Although, we found no effect of oral HRT on plasma NAMPT more studies are needed to elucidate this new field of research. Limited to the assessed lipoprotein ratios and the utilized form of HRT, this thesis support an overall favorable effect of HRT on lipid profile in relation to plasma atherogenicity. In addition, it supports an effect of EE2 on hepatic NAMPT secretion and lipid homeostasis. However, further studies are necessary to conclude the effect of estradiol on hepatocytes.

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Abbrevations

% CV	Coefficient of variation
ABCA1	ATP binding cassette transporter A1
ABCG5	ATP binding cassette transporter G5
ABCG8	ATP binding cassette transporter G8
ADRP	Adipose differentiation-related protein
APOA1	Apolipoprotein A-1
APOB	Apolipoprotein B
cDNA	Complementary DNA
CEE	Conjugated equine estrogen
CHD	Coronary heart disease
CRP	C-reactive protein
CVD	Cardiovascular disease
CXCL16	Chemokine (C-X-C motif) ligand 16
CYP7A	Cholesterol 7alpha-hydroxylase
DMEM	Dulbecco`s modified Eagle Medium
dNTP	Deoxynucleotide triphosphate
DVT	Deep venous thrombosis
E2	17β-estradiol
EE2	17α-ethinyl estradiol
ELITE	Early versus late intervention trial with estradiol
eNAMPT	Extracellular NAMPT
ER	Estrogen receptor
ERα	Estrogen receptor alpha
EVTET	Estrogen in venous thromboembolism trial
FAS	Fatty acid synthase
FBS	Fetal bovine serum
HDL	High-density-lipoprotein
HDL-C	High density lipoprotein cholesterol
HRT	Hormone replacement therapy
IL-6	Interleukin 6

iNAMPT	Intracellular NAMPT
KEEPS	Kronos early estrogen prevention study
LDH	Lactate dehydrogenase
LDL	Low-density lipoprotein
LDL-C	LDL-cholesterol
LDLr	Low-density-lipoprotein receptor
L-FABP	Liver-type fatty acid-binding protein
LXRα	Liver x receptor alpha
MCP-1	Monocyte attractant protein-1
mRNA	Messenger ribonucleic acid
NAD	Nicotinamide adenine dinucleotide
NAMPT	Nicotinamide phosphoribosyltransferase
NET-Ac	Norethisterone Acetate
One-way ANOVA	One-way analysis of variance
PARP-1	Poly (ADP-ribose) polymerase-1
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PE	Pulmonary embolism
PMM1	Phosphomannomutase 1
qRT-PCR	Quantitative reverse transcriptase polymerase chain reaction
RCT	Randomized controlled trial
RNA	Ribonucleic acid
RNase	Ribonuclease
SA-PE	Streptavidin-phycoerythin
SIRT	Sirtuin
TC	Total cholesterol
TG	Triglycerides
TNF	Tumor necrosis factor
VLDLr	Very-low-density-lipoprotein receptor
VTE	Venous thromboembolism

1 Introduction

Menopause cause changes in the hormonal milieu of women and is characterized by a loss of circulating estrogen and continuous low progesterone levels. Estrogen deficiency is associated with cardiovascular complications and enhances pathobiology in many body systems. With an aging population in developed countries and increased life expectancy, more women will live in a state of estrogen deficiency and longer in that state than ever before. Consequently more women will be in need of exogenous hormone support for their menopause related disabilities.

1.1 Atherogenicity

Cardiovascular disease (CVD) is the leading cause of death for both genders, worldwide (1) and in Norway (2). It is caused by diseases of the heart and blood vessels and includes coronary heart disease (CHD), cerebrovascular disease, peripheral arterial disease, rheumatic heart disease, congenital heart disease and venous thromboembolism (VTE) comprising deep vein thrombosis (DVT) and pulmonary embolism (PE). These diseases together, accounted for 31 % of all global deaths in 2012, according to the Global status report on noncommunicable diseases 2014 by the World Health Organization. Over three quarters of CVD deaths takes place in low- and middle-income countries (1).

The main contributor to CVD deaths for men and women, both worldwide and in Norway, is CHD (1, 2). CHD comprises several diseases such as stable angina, unstable angina, myocardial infarction, and sudden coronary death (3). Globally, age-standardized mortality rates of CHD have declined significantly since 1980 and particularly in high-income regions (4). In Norway, the lifetime risk of dying from CHD around the year 2000 was 50 % less than in the period 1970-1975 (5).

The underlying cause of CHD is atherosclerosis (6). The atherosclerotic process is characterized as an accumulation of lipids and immune cells in the intima of the arterial wall which creates atherosclerotic plaque and a dysfunctional endothelium. The plaque or atheroma may protrude into the arterial lumen, narrowing it and restricting blood flow. This inflammatory process is prone to plaque rupture. Plaque rupture and subsequent development of thrombosis may result in myocardial infarction and stroke (7).

1.1.1 Surrogate markers

Elevated serum cholesterol levels are among the top six most important risk factors for CHD. The others top risk factors are: hypertension, cigarette smoking, diabetes mellitus or elevated glucose levels, and obesity or being overweight (3). Serum cholesterol has been subject to extensive epidemiologic and clinical research and although it has essential functions in the body, elevated levels of serum total cholesterol (TC) and low density lipoprotein cholesterol (LDL-C) levels are recognized as key risk factors of CHD (8, 9). Among the many studies investigating the relationship between CHD and cholesterol, the Framingham study has been of great importance, also in discovering risk factors for CVD in general. The study was initiated in 1947 with an original cohort of 5,209 participants aged 30–62 years. Among other discovered risk factors for CVD, they found elevated serum TC and LDL-C levels (10) to increase the risk of CHD, in addition to low levels of high density lipoprotein cholesterol (HDL-C). Today this knowledge is well established, and LDL-C is the primary target for treatment of hypercholesterolemia (11).

Low density lipoprotein cholesterol

A recent meta-analysis including over 170 000 subjects from randomized trials with statins as intervention, found that every 1 mmol/L reduction in serum LDL-C reduced the annual rate of heart attack, revascularisation, and of ischemic stroke by 20 % (12). LDL-C is essential for transport of cholesterol to peripheral tissues. However, elevated levels are proatherogenic and promote increased uptake by macrophages and retention in intima (13).

Apolipoprotein B

Elevated levels of apolipoprotein B (APOB) is considered a possible risk factor of CVD (14). APOB is directly correlated with plasma LDL-C levels (15). APOB reflects the atherogenic burden in serum as every atherogenic lipoprotein particle contains one molecule of APOB each. The atherogenic lipoprotein particles are: very low density lipoprotein (VLDL), intermediate-density lipoproteins, large buoyant low-density lipoprotein (LDL) and small dense LDL (15).

High density lipoprotein cholesterol

A high serum level of HDL-C is considered cardio protective and is inversely associated with CHD risk (16). However, it is not a target for therapy like LDL-C. Furthermore, it does not have the same support from clinical studies as TC and LDL-C have in terms of a causal relationship with CHD. Instead it serves as a marker of CHD risk (17). High-density-lipoprotein (HDL) is part of the reverse cholesterol transport, and is central in transporting excess cholesterol from peripheral tissue e.g. atherosclerotic plaque to the liver for excretion into the bile. In addition, it wields other anti-atherogenic properties (18).

The variability in LDL and HDL particle size may play a role in their predictive and atherogenic potential (19, 20) . Particularly small dense LDL particles have been found strongly associated with an increased risk of CVD (21-23), regardless of the total level of LDL present (24). This is probably linked to the small LDL particles susceptibility for oxidative modifications which make them more prone to retention in intima. However, there exist inconsistencies on this matter and especially regarding the described inverse association between large HDL particles and CVD risk (22). Large HDL particles may have a greater cholesterol carrying capacity and subsequently more efficient cholesterol removal from tissues.

Apolipoprotein A-1

Apolipoprotein A-1 (APOA1) is a constituent of plasma HDL-C. High levels of serum APOA1 are noted as cardioprotective and it has been suggested as a risk marker for CHD (25, 26).

Lipoprotein (a)

Elevated levels of lipoprotein (a) (Lp [a]) are associated with increased CHD risk. It holds atherogenic properties, is mostly genetically determined and relatively resistant to lipid lowering therapy. Generally, it is not the subject of routine testing, but there exist indications for serum measurements in intermediate to high CVD risk patients (27-30).

Triglycerides

Triglycerides (TG) are transporters of fatty acids and are mainly distributed to tissues packed in VLDL particles. Elevated levels of circulating TG, especially non-fasting, are associated with increased CHD risk (31, 32). However, the association has been debated for 3 decades (33). In 2011 the American Heart Association stated that TG are not directly atherogenic, but rather associated with atherogenic remnant particles and apo CIII. Furthermore, this makes TG important as a biomarker of CVD risk (34).

Lipoprotein ratios

Several lipoprotein ratios have been created in order to optimize the predictive capacity of the lipid profile. The TC/ HDL-C- and the LDL-C/HDL-C ratios, have been suggested to have greater predictive value than isolated lipoproteins used independently, especially with regards to LDL-C (32, 35). The ratio of APOB/APOA1 (36) and the ratio of TC/ HDL-C (37), have been reported to be the strongest predictors of CHD risk.

1.1.2 Sexual dimorphism in lipoprotein metabolism

There exist differences in lipoprotein metabolism and related risk factors for CHD and CVD between men and women. High levels of non-HDL-C and TG seem to be more important risk factors for CVD in women than in men (38). Furthermore, low levels of HDL-C seem to be a better predictor of CHD in women than high levels of LDL-C (39).

Women have a lower CHD risk than age-matched men (40, 41) and a more anti-atherogenic lipid profile before menopausal age. Their lipid profile is characterized with a lower concentration of LDL-C, VLDL cholesterol, VLDL- TG and larger LDL and HDL particle size. The gender differences in lipoprotein metabolism may be the result of a complex network of hormone action and other sex related factors not yet discovered, and this difference may at least partly explain the cardio protective effect of the female sex (42).

1.1.3 Endogenous sex steroids and risk of coronary heart disease

There exists a marked gender difference in respect to when coronary manifestations first occur. Women up to the age of 45-50 years appear to have a relatively low risk of CHD compared with postmenopausal women. From the age of 45-70, women experience a ten year delay in the incidence of CHD compared with age matched men. After the age of 70, this gap declines to a five year difference (40, 41). Interestingly, a recent modelling study of national mortality data from England, Wales, and the United States found acceleration in CHD mortality for men up to the age of 45. After that age, they found a decrease in CHD mortality and hence a more similar risk to CHD as women (43). In addition, the same study reports a lifelong CHD risk among women rather than a sharp increase in risk after menopausal age. However, the causes behind the gender difference in risk of CHD are at present not clear. The change in hormonal milieu that occurs during menopause has been theorized to explain, at least partly, the gender difference in relation to CHD incidence and hence risk. However, the few longitudinal studies that have investigated the association between endogenous sex steroids and CHD risk are not supportive of such an association (44).

Women experience a gradual loss of estrogen, as well as alterations in progesterone secretion during the transition to the menopausal period. The transition phase usually occurs within the age range 40-60 years and the relative estrogen deficiency is associated with cardiovascular complications (45, 46). In postmenopausal women, menopause is associated with modest proatherogenic changes in lipoprotein pattern such as increased TC, LDL-C and APOB (23, 42, 47). However, some data also indicate associations between menopause and small dense LDL particles (48), and yet other data increased circulating levels of TG and Lp (a) (16, 49). Insulin resistance, polycystic ovary syndrome and changes in total body fat and body fat distribution, are also associated with menopause (42), but data may indicate that the change in other CHD risk factors, with the exception of the lipoprotein pattern, may be more due to chronological aging than menopause (47).

1.2 Sex steroids

Sex steroids comprise estrogens, progestogens and androgens. The primary sources of these steroids are the gonads, respectively ovaries for the female and testes for the male. Additionally, sex steroids are also produced locally by peripheral conversion in fat-storing tissues and in the liver. All types of sex steroids exist in both genders, but in different concentrations. The field of sex steroids is quickly evolving. In recent years a well of new information regarding sex steroid targets, their receptors and their different modes of genomic- and non-genomic actions have accumulated. Many of these findings have challenged the classical view on sex steroids in human physiology (50).

1.2.1 Estrogen

The actions of estrogen are essential in both genders and exhibit its functions in many different body systems, such as: The reproductive, cardiovascular, skeletal, central nervous and in the control of energy homeostasis and glucose metabolism (51). Deficiency of estrogen enhances pathobiology and degenerative disorders, affecting the cardiovascular, skeletal and nervous system in addition to predisposing for obesity, the metabolic syndrome, type 2 diabetes and certain types of cancers (50, 52, 53).

Various cellular and animal studies have shown protective effects of estrogen on the vascular endothelium. Among estrogens protective effects are mediation of vasodilatation, promotion of repair and/or regeneration and anti-inflammatory and –oxidative effects. The vascular endothelium is responsive to estrogen through estrogen receptors (ERs). Hence the protective effect exerted by estrogen on the endothelium is dependent on the presence of ERs in the vascular endothelium (54, 55).

17β-estradiol

There exist three types of estrogen: estriol, 17β-estradiol (E2) and estrone. E2 is the predominant form, circulating in healthy premenopausal women. It is produced by the ovaries and act on distant target tissues by functioning as a circulating hormone. Men have low levels of circulating E2, and in postmenopausal women the ovaries fail to produce E2. Consequently, E2 does not function as a circulating hormone in men and postmenopausal

women, but rather exert its function locally. E2 is synthesized in extragonadal sites such as breasts, brain, muscle, bone, and adipose tissue (56). Tissue metabolism and the enzyme tissue estrogen sulfotransferase, control the cellular actions of E2 through degradation and inhibition of ER binding and thereby enhancing urinary excretion of the hormone (57).

1.2.2 Hormone replacement therapy

Hormone replacement therapy (HRT) is a substitution of hormones through medical prescription. HRT can be administered for several reasons, however in this thesis only the replacement of hormones to relieve menopausal symptoms will be discussed. Estrogen can be given as monotherapy, but is often accompanied with a progestin in order to reduce the risk of endometrial carcinoma due to unopposed estrogen (58).

The "timing hypothesis"

Observational data over several decades consistently demonstrated a decreased risk of CHD with the use of HRT in postmenopausal women (59-61). In contrast to these findings from observational studies and a few randomized clinical trials in support thereafter, the large study called Women's health initiative (WHI), found increased risk of CHD, breast cancer, PE and stroke after HRT use. The WHI was a series of clinical trials investigating primary prevention of CVD and had invasive breast cancer, nonfatal myocardial infarction and CHD death as primary outcomes (62). Many women stopped HRT use after the first publications from the WHI. However, this study stimulated further investigations and after more analysis of the WHI data, it became clear that for women under the age of 60 or within 10 years of menopause, the final findings for all outcomes closely resemble those from observational studies pointing to a protective effect of HRT on CHD risk (63, 64). Interestingly, a study called the Danish Osteoporosis prevention study, initiated around the same time as WHI, found a significant reduction in mortality, heart failure and myocardial infarction in women within 3-24 months of menopause randomized to ten years of HRT, without any apparent increase in risk of cancer, VTE, or stroke (65). A recent meta-analysis (66) of over 100 randomized trials in postmenopausal women involving 39 049 participants in addition to a 2010 scientific statement from the US Endocrine Society (67), came to the same conclusion, that HRT reduced CHD events when administered to younger postmenopausal women. These findings, together with HRT studies in primates and other animal models (46), have led to the

development of the timing hypothesis in which the beneficial effect of HRT is dependent on administration of HRT before the development of advanced atherosclerosis. Estrogen seems to differently affect the endothelial, vascular smooth muscle and inflammatory cells involved in the arteriosclerotic process, depending on the stage of its development (68-72). Early in the atherosclerotic process, estrogen seems to exhibit anti-atherogenic effects whereas later it seems to have proatherogenic effects, as seen in Figure 1.

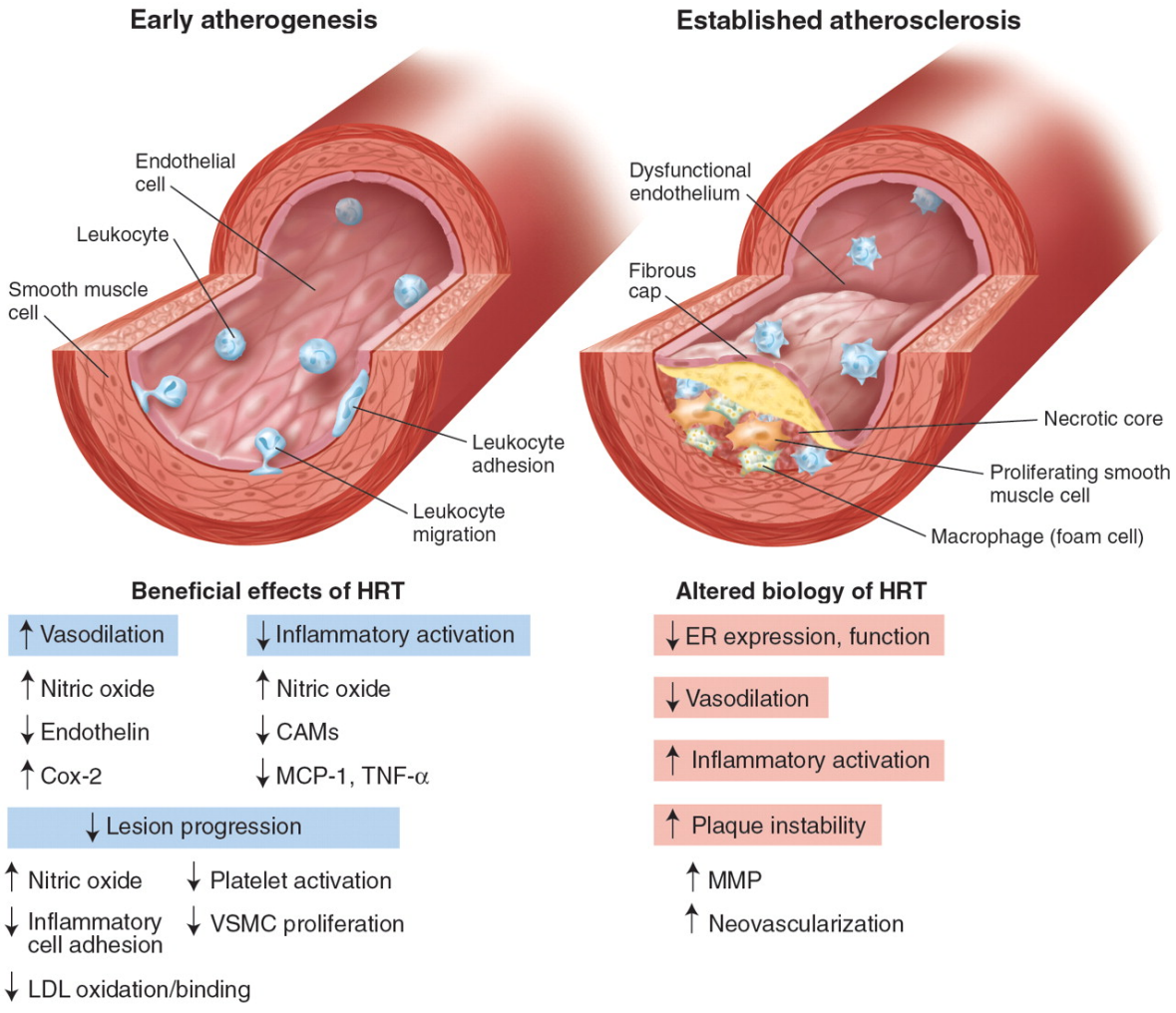

Figure 1. Plausible mechanisms behind the "timing hypothesis". Estrogen differently affects the endothelial-, smooth muscular- and inflammatory cells involved in the arteriosclerotic process, depending on the stage of its development. Atherosclerosis is characterized by a change in vascular biology and ultimately the development of advanced, unstable lesions prone to rupture (7). Early in the atherosclerotic process, estrogen seems to exhibit anti-atherogenic effects whereas later it seems to have proatherogenic effects (68-72). CAMs, cell adhesion molecules; MCP-1, monocyte chemoattractant protein 1; TNF-α, tumor necrosis factor–α; VSMC, vascular smooth muscle cell; MMP, matrix metalloproteinase; COX-2, cyclooxygenase 2. The picture is reprinted with permission from Mendelsohn and Karas (46).

The protective effect of estrogen on the vascular endothelium can be altered by several different factors such as aging, menopause, pregnancy and coexisting pathologies through affecting the ERs and their signaling pathways(54). In support of the timing hypothesis, aging among other factors, dispose for progressive development of atherosclerosis (73). Ultimately this process may lead to loss of vascular endothelium and development of atheroma which is associated with a down regulation of ERs (74, 75). Consequently, there will be a loss of response to estrogen and thereby a loss of its protective effects on the vascular endothelium. Further, findings from experiments in rodents may indicate a loss of estrogen response in estrogen deficient states like menopause (76). Despite accumulated data indicating an effect of aging and menopause on vascular estrogen signaling, the topic is complex and not completely understood (77).

Clinical research on the "timing hypothesis"

Recently, two studies have been specially designed to test the timing hypothesis, the Early versus late intervention trial with estradiol (ELITE)(78) and Kronos early estrogen prevention study (KEEPS) (79). Both studies used surrogate markers for CHD as their end points. In the ELITE trial, a significantly reduced progression rate of carotid artery intima-media thickness was found after 6 years of HRT (oral E2 plus vaginal progesterone) in the early postmenopausal group (less than 6 years) relative to placebo, but not in the late postmenopausal group (more than 10 years). In contrast, the KEEPS study did not find any significant effect on carotid artery intima-media thickness and coronary artery calcium in postmenopausal women (within 3 years of onset of menopause) after 4 years of oral conjugated equine estrogen (CEE) or transdermal estradiol plus progesterone treatment. However, they found significantly reduced insulin resistance and TC in the transdermal group compared to placebo and oral HRT (80).

Clinical relevance of hormone replacement therapy today

During the recent decade our understanding of HRT has increased tremendously and it is interesting to note that the North American menopause society has made a position statement in favor of HRT and the timing hypothesis. Although only as a short term treatment for postmenopausal women in order to treat menopause-related symptoms and to prevent osteoporosis in women at high risk of fracture (81).

Different hormone replacement therapies and routes of administration

In addition to the correct timing of HRT administration, there are other factors that determine the metabolic effect and clinical outcome of this treatment: type of estrogen, addition of a progestin and the type, estrogen dosage and route of administration (45, 82-86).

Several meta analyses have reported that both oral and transdermal estrogen, with or without progestin, may increase lean body mass, reduce abdominal fat, improve insulin resistance, decrease LDL-C/HDL-C ratio, and decrease blood pressure in women without diabetes. Further, that the beneficial effects of HRT on metabolism are dose dependent and reduced by the addition of a progestin. Oral estrogen therapy exerts a stronger beneficial metabolic effect than transdermal HRT (66, 87).

In contrast to transdermal HRT, oral HRT increases serum TG, C-reactive protein (CRP) and has adverse effects on coagulation (66, 82). Furthermore, standard doses of oral HRT may increase the risk of VTE twofold (88) and is associated with an increased risk of stroke and with stroke severity (89). However, orally administrated CEE produce stronger metabolic effects than orally administrated E2 e.g. beneficial effect on LDL-C/HDL-C ratio and adverse effect on serum TG (87). Major randomized control studies like the WHI (90) have used HRT from equine sources (CEE) as intervention and not estradiol from human sources (E2) (23, 91).

The different physiological effects exerted by route of administration of HRT are attributed supra physiological liver exposure and first-pass metabolism (92, 93) . This attribution is based on the fact that the liver is the central regulator of lipoprotein and bile metabolism and producer of coagulation factors and proinflammatory proteins like CRP (94). Hence, oral HRT will affect the hepatic functions differently than transdermal HRT.

1.2.3 Sex steroids and lipid profile

A lot of our knowledge about the effect of sex steroids on plasma lipid homeostasis comes from exogenous sex steroid administration such as HRT in postmenopausal women (42). However, most studies to the best of our knowledge, have assessed isolated constituents of the lipid profile after HRT, in relation to CHD risk (23, 39, 66, 95-98). Thus, the ratios between the constituents remain to be studied. These ratios may add predictive value to evaluation of plasma atherogenicity (32, 35).

1.3 Nicotinamide phosporibosyltransferase

Nicotinamide phosphoribosyltransferase (NAMPT) also known as visfatin or pre-B cell colony-enhancing factor, hereinafter referred to as NAMPT, is a protein which has received considerable attention in recent years. The protein has been shown to be involved in a number of human diseases such as human immunodeficiency virus infection, sepsis, heart failure, metabolic disorders, cancer, neurodegenerative diseases and aging (99). NAMPT is involved in atherosclerosis related diseases in general (19, 99-101) and is not only associated with, but also plays an active role in the development of atherosclerosis (101, 102). The protein has shown both pro-and anti atherogenic effects depending on the biological and pathological processes that it is involved in. More research is needed to elucidate the role of NAMPT in human health and disease to better be able to take advantage of its therapeutic potential (99, 102).

1.3.1 Functionality

NAMPT is produced in adipose tissue, skeletal muscle, cardiac myocytes, liver, immune cells and in the brain (10). In mammals, NAMPT exists in both intracellular (iNAMPT) and extracellular (eNAMPT) form (101). Whether NAMPT is acting in the same conformation in both environments, and have different or overlapping effects, is not known (99, 102). Both eNAMPT and iNAMPT seem to be involved in the pathophysiology (102).

iNAMPT acts as the speed limiting enzyme during the formation of Nicotinamide adenine dinucleotide (NAD) in the "NAD salvage pathway" (103). NAD^+ and NADH are key molecules in energy metabolism, bio synthesis and the antioxidant defense system. In addition, NAD has several biological functions mediating the regulation of NAD-dependent proteins. Sirtuin's (SIRT) and, especially, SIRT1 and poly (ADP-ribose) polymerase-1 (PARP-1) are two NAD-dependent enzymes that are regulated by the enzymatic activity of iNAMPT. SIRT1 and PARP-1 are involved in cellular processes such as regulation of the cell cycle, in response to DNA damage and apoptosis (104).

The biological effects of eNAMPT are still unclear. Several studies have shown the effect of eNAMPT on cellular functions such as apoptosis, inflammation and matrix degradation, but the basis of these results is debated. Different mechanisms of action have been suggested for eNAMPT, such as enzymatic activity and/or involvement in cytokine signaling. However, the

mechanism of action is still not fully understood and is the subject of intense research. Understanding the mechanism of action is especially important in relation to clarification of the therapeutic potential of eNAMPT (99).

1.3.2 Sex steroids and Nicotinamide phosporibosyltransferase

Circulating NAMPT has been suggested as a biomarker of an atherogenic metabolic profile (19) and an association has been reported between high circulating levels of NAMPT and inflammatory atherothrombotic diseases (105, 106). However, little is known about the factors regulating circulating NAMPT (101). To the best of our knowledge, the effect of sex steroids on circulating levels of NAMPT and NAMPT cell release remains unstudied.

2 Aims of thesis

Improved knowledge regarding the physiological effects of female sex steroids are important to better understand their role in human physiology and to create HRT that only produce the desired physiological effects. In the present thesis, we aimed to study the effect of oral HRT (E2+a progestin), in postmenopausal women and the effect of its synthetic derivative 17α-ethinyl estradiol (EE2) in a human hepatocyte cell line. The study of female sex steroids is limited to the effect of exogenous female sex steroids on parameters related CHD, like NAMPT, lipid metabolism and inflammation.

The aims of the present thesis were:

I) To study HRT`s effect on plasma NAMPT, lipoproteins, apolipoproteins and lipoprotein ratios.

IV) To study estradiol`s effect on gene expression and cell release of NAMPT, proinflammatory cytokines, apolipoproteins, TG and genes involved in reverse cholesterol transport, lipoprotein and bile acid metabolism in the hepatocyte cell line HepG2.

To explore these aims, we employed two model systems:

1. In vivo in humans exposed to HRT in a randomized controlled trial (RCT).

2. In vitro in hepatocytes exposed to estradiol.

3 List of materials

Clinical trial	Distributor	Location
Plasma and data	Per Morten Sandset (principal investigator)	Oslo
Cell culture equipment	**Distributor**	**Location**
Cell line		
HepG2	ATCC	Virginia, USA
Stimuli		
17α-ethinyl estradiol	Sigma Aldrich	Missouri, USA
Fulvestrant	Sigma Aldrich	Missouri, USA
Chemical/compound/equipment	**Manufacturer**	
Biocoat collagen 75 cm^2 vented flask	Corning	The Netherlands
Biocoat collagen 24 well plate with lid	Corning	The Netherlands
Dulbecco`s modified Eagle Medium 1X (DMEM)	Gibco by Life Technologies	California, USA
DMEM w/o L-glutamine and Phenol-red	BioWhittaker by Lonza	Maryland, USA
Gentamicin	Life Technologies	California, USA
Fetal bovine serum	PAA, GE Healthcare	Austria
Penicillin	Sigma-Aldrich	Missouri, USA
Trypsin – EDTA	Life Technologies	California, USA
Phosphate-buffered saline sterile	Life Technologies	California, USA
Chemicals	**Manufacturer**	**Location**
Taqman gene expression master mix	Applied Biosystems	California, USA
Brilliant III ultra-fast SYBR green qPCR master mix	Agilent Technologies	California, USA
Primers	Sigma- Aldrich	Missouri, USA
Primers- LXRα	Invitrogen	California, USA
Primer – Taqman . PMM1	Life Technologies	California, USA
Kits	**Manufacturer**	**Location**
Bio-Plex Pro Diabetes Assay	Bio-Rad	California, USA
Bio-Plex Pro human Chemokine Assay	Bio-Rad	California, USA

Milliplex MAP Kit Human Apolipoprotein Magnetic Bead Panel	Merck Millipore	Germany
Triglyceride Enzymatic PAP 150	BioMerieux	France
Cytotoxicity detection kit (lactate dehydrogenase)	Roche	Germany
High capacity cDNA Reverse Transcription kit	Applied Biosystems	California, USA
PerfectPure RNA Cultured Cell Kit	5 Prime	Germany
Equipment	**Manufacturer**	**Location**
Stratagene Mx3000P 96-well plates	Agilent Technologies	California, USA
Instruments	**Manufacturer**	**Location**
Stratagene Mx 3005P	Agilent Technologies	California, USA
Multigene	Labnet international	New York, USA
Bio-Rad Bioplex 100	Bio-Rad	California, USA
Co_2 incubator MCO-18AIC	Sanyo Electric	Osaka, Japan
Heraesus Megafuge 16 Centrifuge	Thermo Fisher Scientific	Massachusetts, USA
Heraesus Fresco 21 Centrifuge	Thermo Fisher Scientific	Massachusetts, USA
Heraesus Multifuge 3SR+ Centrifuge	Thermo Fisher Scientific	Massachusetts, USA
Motic AE 2000, light microscope with camera	Motic	Xiamen, China
Multiscan Ascent	Thermo Fisher Scientific	Massachusetts, USA
Nano Drop ND-1000 Spectrophometer	Thermo Fisher Scientific	Massachusetts, USA
Software	**Manufacturer**	**Location**
Bio-Plex Manager software 6.0	Bio-Rad	California, USA
EndNote X6	Thomson Reuters	California, USA
Graph Pad Prism 6.0	Graphpad software	California, USA
Microsoft Office 2010	Microsoft	Washington, USA
IBM SPSS statistics 22	IBM	New York, USA
Mx Pro – Mx3005P	Agilent Technologies	California, USA
Ascent software	Thermo Fisher Scientific	Massachusetts, USA
ND-1000	Thermo Fisher Scientific	Massachusetts, USA

4 Sample and Methods

Two model systems were utilized for investigating the effect of HRT and estradiol on NAMPT and selected factors involved in inflammation and lipid metabolism. The effect of HRT was studied *in vivo,* analyzing plasma from postmenopausal women exposed to HRT over a three month period. The other model system was *in vitro* based, using a human hepatocyte cell line to study gene expression and cell release after exposure to various doses of EE2.

4.1 Human trial

For the *in vivo* analysis, frozen plasma samples and data from a study named Estrogen in Venous Thromboembolism Trial (EVTET), were used (84). Prof. Per Morten Sandset, the principal investigator of EVTET, is also co- supervisor for this master thesis.

The aim of the EVTET study was to determinate if HRT would alter the risk of VTE in high risk women who had previously experienced VTE. The primary outcome was VTE either as recurrent DVT or PE. The study was terminated prematurely as other studies were reporting that HRT were likely to increase the risk of VTE during execution of the study.

The study design in EVTET was a randomized, double-blind, and placebo-controlled clinical trial (RCT) with a double triangular sequential design. It included 140 postmenopausal women < 70 years, who were randomized to one tablet (n=71) daily (2 mg estradiol and 1 mg norethisterone acetate) or placebo (n=69). Postmenopausal was defined as not having had natural menstruation for at least one year.

Two studies have previously published data on the EVTET material. One, studied inflammation markers (82) and the other coagulation factors (107). A more detailed description about the methodological aspects of EVTET, can be found in the appendix 3.

Procedures

The EVTET study included several follow-up visits. However, in this investigation only plasma and data from baseline and the first follow-up after three months were used.

The plasma samples were used to quantify circulating NAMPT. These samples had previously been stored in a bio bank at Oslo University hospital HF, Ullevål at -70 ºC until assayed.

To elucidate the effect of HRT on lipid profile, data on file was analyzed statistically. The parameters used from file were: E2, HDL-C, TG, TC, APOA1, APOB (total) and Lp (a). LDL-C was calculated using Friedewald's formula for mmol/L given by: TC-HDL-C-(0.45*TG).

4.1.1 Detection of NAMPT concentration in plasma

Bio-Rad`s Bio-plex® suspension array system was used for detection and quantification of target proteins in plasma. The system is based on xMAP technology and consists of multiplex assays that are able to detect several different analytes in a single well of a 96-well plate. This technology enables the researcher access to more information with less sample volume, costs and time (108). The Bio-plex® suspension array system allow for flexibility with a singleplex or multiplex format.

The Bio-plex® suspension array system is essentially magnetic- bead-based immunoassays. The assays are similar to an ELISA sandwich assay in that they utilize an antibody sandwich for detection (Figure 1). However, ELISA does not have the ability to multiplex different analytes in a single 96-well plate.

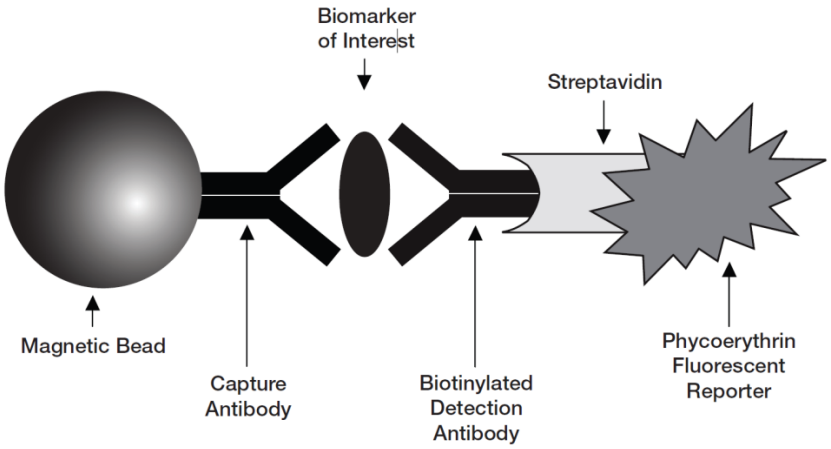

Figure 1. Bio-Plex sandwich immunoassay. Detection complex with magnetic bead, capture antibody, protein target/biomarker, detection antibody and fluorescent reporter conjugate of streptavidin-phycoerythin. The picture is reprinted with permission from the Bio-Plex Pro™ human diabetes immunoassay kit instruction manual (109).

The test process starts with a magnetic bead with a specific spectral address that is covalently coupled to a monoclonal antibody which is specific for the protein target of interest (Figure 2). Several washings steps are needed throughout the whole process to remove unbound protein and ensure that only the magnetic bead complexes are left in the wells. Coupled beads then attach to the protein target in the sample. Next, a sandwich complex is created by adding a biotinylated detection antibody that attach to the protein target. A conjugate consisting of streptavidin-phycoerythin (SA-PE) is added as a final step. This conjugate attaches to the detection antibody and makes up the final detection complex. Phycoerythin generates a fluorescent reporter signal when excited by a laser at 532 nm which allows for quantification of the protein target. The use of a detectable bead with a specific spectral address enables the detection and quantification of different analytes simultaneously in the same sample. A flowcytometer with a classification laser and a reporter laser is used to identify and quantify the protein target or targets. The classification laser detects and identifies the magnetic beads and the reporter laser detects and quantifies the fluorescent signal given off by Phycoerythin when excited by the laser at 532 nm.

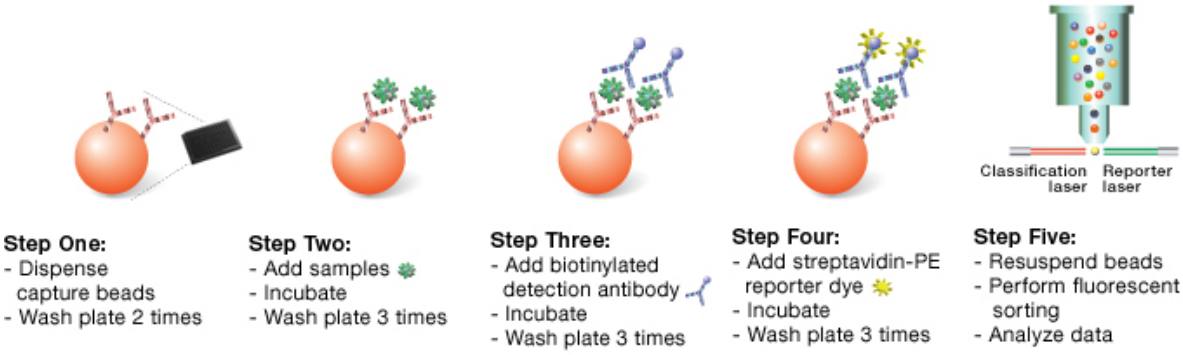

Figure 2. Schematic representation of Bio-Plex sandwich immunoassay workflow. There are 5 steps in which the protein target is detected. Step number 1-4 starts with a magnetic bead with a specific spectral address that is covalently coupled to a monoclonal antibody which is specific for the protein target of interest. The detection complex is further made in to a sandwich complex with bonding of capture and detection antibody to protein target/biomarker and a fluorescent reporter conjugate of streptavidin-phycoerythin binding to the detection antibody. Several washings steps are needed throughout the whole process to remove unbound protein. In step number 5, beads and fluorescent signals are detected and quantified with a flowcytometer. The use of a detectable bead with a specific spectral address enables the detection and quantification of different analytes simultaneously in the same sample. The picture is reprinted with permission from Wei Geng et al. (110).

NAMPT was detected in plasma in a singleplex format with the Bio-Plex Pro™ human diabetes immunoassay kit from Bio-Rad. The work flow was conducted according to the instruction manual dispatched with the kit (109).

For the initial preparation, plasma samples were thawed on ice. Meanwhile the Bio-Plex system was primed and calibrated and a single vial of the standards was reconstituted in Bio-Plex standard diluent and used to make an 8 point standard diluent series in addition to blank. Thawed samples were diluted in Bio-Plex sample diluent. Next, coupled beads were mixed with assay buffer and protected from light until reaching room temperature of both samples and beads.

Briefly, beads were added to an assay plate and the plate was washed in a magnetic Bio-Plex Pro wash station. Samples, standards, blank and controls were added to the assay plate and incubated in darkness while on a plate shaker. The plate was washed again before incubated with detection antibody in the dark while shaking. Meanwhile SA-PE was prepared in assay buffer. After an additional washing step, washing away any unbound biotinylated antibodies, the beads were incubated in the dark while shaking with the reporter conjugate SA-PE. After an additional washing step, the beads were re-suspended in assay buffer, put on a plate shaker and placed in the flowcytometer Bio-Plex 100 from Bio-Rad for detection and quantification of NAMPT. Data was processed by the Bio-Plex Manager software 6.0.

In order to estimate the precision of the immunoassays, an intra- and inter-assay assessment was performed to calculate coefficient of variation (% CV) (Appendix 1). Intra-assay % CV is a measure of the variation in the same sample replicated many times in one analysis. Whereas inter-assay % CV is a measure of the variation in the same sample analyzed several times. The inter-assay % CV reflects plate-to-plate consistency and standardized procedures and storage conditions are essential for the outcome. The % CV is given as mean of standard deviation, divided by mean of sample and multiplied by 100 (111).

4.1.2 Data analysis

Statistics

All data were tested parametric or non-parametric according to their distribution. When possible, log transformation was carried out on non-parametric data. All statistical testing were conducted with the software IBM SPSS statistics 22 and a probability value of $\leq .05$ was set as significant. Data is presented as mean or median with 95% confidence interval.

Independent samples test (t-test or Mann Whitney U) was used for testing for differences in change score between placebo and treatment group.

4.2 Cell culture experiment

HepG-2 cells, a human hepatocyte cell line, was used in the cell experiments to study the cellular release and gene expression of NAMPT and selected factors involved in inflammation and lipid homeostasis after stimulation with EE2. EE2 is a synthetic derivative of E2 and is the most potent naturally occurring type of estrogen in humans (112).
The experiment was set up as a dose-response assessment with various doses of 0.1-100 nM EE2 and two time-points, 6 and 24 hrs. Fulvestrant, an estrogen receptor alpha (Erα) inhibitor was included as a control (1 μM+ 100 nM EE2) for the ERα signal transduction pathway (113, 114).
The utilized type of cell or tissue and the quality of the work done with the cells prior to the analysis of cell release and gene expression, determines the outcome and the reliability of the results. The essential work done before analysis is: handling of the culture prior to and during experiments, seeding, stimulation, incubation and harvest of cells. In addition, each analysis holds its own potential pitfalls for errors. Strict adherence to the appropriate procedure is of outmost importance for the results of the experiment.

4.2.1 Treatment

Cell viability and growth

A decreased viability and growth may indicate a bacterial infection or increased death among cells. This may affect the quality of the culture, the number of cells per sample, alter gene expression and the release from cells and thus, affect the experimental results. It is therefore important to monitor cell viability and growth to ensure reliable results.

Microscopic evaluation

The viability and growth of the HepG2 cells was monitored under a Motic AE2000 microscope every third day throughout sub culturing and during experiments.

Measure of cell viability

The cytotoxicity kit (lactate dehydrogenase) from Roche was used for the quantification of dead and damaged cells at both time points (6 and 24 hrs.) The cytotoxicity kit used is based on calorimetric detection of lactate dehydrogenase (LDH) in cell-free culture supernatant.

LDH is stably expressed in all cells and is rapidly released into the cell culture medium upon damage of the cell membrane. An increase in the amount of dead or plasma membrane-damaged cells results in an increase of the LDH enzyme activity. The LDH activity is determined in an enzymatic test where tetrazolium salt INT is reduced to formazan salt catalyzed by LDH. The amount of LDH directly correlates to the amount of formazan formed. Formazan salt is a water-soluble dye with maximum absorption around 500 nm. The tetrazolium salt INT shows no absorption around that wave length (115).

Conditioned medium from three experiments at two time points (6 and 24 hrs.), were incubated in duplicates with the reaction mixture from the cytoxicity kit for 20 minutes at room temperature. Next, the absorbance was read at 492 nm with the spectrophometric microplate reader, Multiscan Ascent from Thermo Fisher Scientific. The assay medium was used as background control and subtracted from the results.

Cell line

The HepG2 cell line originates from a liver biopsy of a 15 year old Caucasian male with a well differentiated hepatocellular carcinoma. The cells had been grown in large scale cultivation systems prior to distribution. The HepG2 cells used in this experiment was obtained from ATCC and cryopreserved in complete growth medium supplemented with 5% (v/v) DMSO.

Culture

In order to culture the cells, the cells were first thawed in a 37 °C water bath. Next, DMSO was removed and the cells resolved in complete growth medium. The complete growth medium was made with Dulbecco`s modified Eagle Medium 1X (DMEM) with 1 g/L D-Glucose, 25 mM HEPES, L-Glutamine and Pyruvate supplemented with 1 % penicillin, 10% fetal bovine serum (FBS) and 0.1 % Gentamicin. The cells were cultured for two weeks prior to the first experiment and kept incubated at 37 °C with 5% CO2. Sub culturing was maintained for 3.5 months.

The complete growth medium was changed every third day throughout the sub culturing process and the cells were kept in 75 cm^2 collagen coated plastic containers from Corning. The cells were passaged at a subconfluency of 70-80 % to maintain growth in monolayer.

The passage was done with Trypsin/EDTA. Hepatocytes grow in monolayer at subconfluency attached to the bio coat. Trypsin is a proteolytic enzyme which breaks down the proteins that enables the cells to adhere to the bio coat. After adding Trypsin/EDTA, the cells were incubated in 37 °C with 5% CO2 for a few minutes. To ensure a dispersed cell layer, the cells were observed under an inverted microscope before trypsin/EDTA inactivation with complete growth medium. Cells were normally split 1:8 and transferred to a new 75 cm^2 container with 13 ml complete growth medium.

Experimental set-up

The experiments were set up in triplicates with six conditions and two time-points for harvest, 6 and 24 hours. The 6 hour time-point was repeated three times and the 24 four hour time-point, four times (Figure 3).

6 hours incubation

→ Harvesting

	EE2	EE2	EE2	EE2	EE2 + Fulvestrant
Control	0.1	1.0	10	100	100 + 1µM
Control	0.1	1.0	10	100	100 + 1µM
Control	0.1	1.0	10	100	100 + 1µM

n=3

24 hours incubation

→ Harvesting

	EE2	EE2	EE2	EE2	EE2 + Fulvestrant
Control	0.1	1.0	10	100	100 + 1µM
Control	0.1	1.0	10	100	100 + 1µM
Control	0.1	1.0	10	100	100 + 1µM

n=4

Figure 3. Set up for cell experiments. The experiments were set up in triplicates with six conditions and two time-points for harvest, 6 and 24 hours. The conditions were 0.1-100 nM EE2, 100 nM EE2+ 1 µM Fulvestrant and unstimulated cells. The 6 hour time-point was repeated three times and the 24 four hour time-point, four times.

As preparation for the experiments, cells were first seeded in bio coat collagen 24 well plates with lid. The procedure started with a passage of the cells by trypsinisation at subconfluency and split 1:2. Next, the cells were centrifuged at 200 g for 3 minutes at room temperature, the supernatant was discarded and the cell pellets re-suspended in 24 ml complete growth medium DMEM. 500 µl of the cell suspension was added to each well in two plates, one plate for each time-point. The plates were incubated at 37 °C with 5% CO2 until reaching subconfluency and adequate cell adherence to the bio coat.

The stimulation part of the experiment, started with two washes with sterile Phosphate-buffered saline (PBS) in order to remove any traces of complete growth medium. Next, 500 µl research medium with DMEM w/o L-glutamine and Phenol-red and supplemented with the respective concentration of EE2 (0, 0.1, 1, 10 and 100 nM), was added to its corresponding wells. The Fulvestrant conditioned wells were washed and incubated with 500 µl research

medium and 1 µl Fulvestrant at 37 °C with 5% CO2, for two hours prior to the PBS wash of the other wells (Figure 4).

Condition	[EE2] + 500 µl DMEM	[EE2]: C1 x V1=C2 x V2	V1 (µl)	V1x3x10%	(V1x3x10%) x 2 plates
1	0	100 nM x V1= 0 nM x 500 µl	0	0	0
2	0.1 nM	100 nM x V1= 0.1 nM x 500 µl	0.5	1.65	3.3
3	1 nM	100 nM x V1= 1 nM x 500 µl	5	16.5	33
4	10 nM	10 000 nM x V1= 10 nM x 500 µl	0.5	1.65	3.3
5	100 nM	10 000 nM x V1= 100 nM x 500 µl	5	16.5	33
6	0 nM, 1 µM Fulvestrant (F)	10 µM F x V1= 1 µM F x 500µl	50	165	330
6	After 2 hours preincubation, 100 nM	10 000 nM x V1= 100 nM x 500 µl	5		

Figure 4. Calculation overview of stimulants and experimental medium used in the cell experiments. 500 µl research medium with DMEM w/o L-glutamine and Phenol-red and supplemented with the respective concentration of EE2 (0, 0.1, 1, 10, 100 nM), was prepared for its corresponding wells. The Fulvestrant conditioned wells were washed and incubated with 500 µl research medium and 1 µM Fulvestrant at 37 °C with 5% CO2, for two hours prior to stimuli of the other wells.

After cell stimulation, the cell plates were incubated at 37 °C with 5% CO2 for 6 or 24 hours before being harvested on ice. The supernatants were quickly extracted and added into pre-marked Eppendorf tubes. The cell wells were washed two times with PBS before adding lysis solution. The supernatants were centrifuged and transferred to new tubes to ensure a cell-free supernatant. Cell plates and conditioned medium were frozen down at – 80 °C.

4.2.2 Analysis of gene expression

For reliable results from gene expression analysis, it is important to keep the sample intact. Directly after harvesting, changes in the gene expression pattern occur due to specific and nonspecific Ribonucleic acid (RNA) degradation as well as to transcriptional induction. To prevent this immediate stabilization of RNA is necessary (116).

Isolation and purification of total RNA

To attain total RNA from the harvested cells RNA has to be isolated from the cells and purified. The PerfectPure RNA Cultured Cell Kit from 5 Prime was used for this process. The procedure was done according to the instruction manual from the manufacturer (117). The isolation and purification procedure is based on column technology which is a combination of columns containing a silica based membrane with specific binding properties, and high speed centrifugation. The kit from 5 prime is most efficient at isolating RNA species greater than 150 bases in length. Highly structured RNA molecules such as transfer RNA which is less than 150 bases in length are selectively excluded. Most of transfer RNAs are involved in protein synthesis (118).

To ensure intact samples, it is important with a Ribonuclease (RNase) free environment as RNases degrade RNA. As for initial preparation, special care to create an RNase free environment was facilitated for instance with RNase decontamination of pipettes, the use of certified RNase-free reagents and equipment and extra meticulous hand washing before putting on gloves with more. The whole process was done on the lab bench in room temperature without the need for a fume hood as no hazardous chemicals were used in the process.

The frozen cells with lysis buffer from 4.2.2 was thawed on ice and homogenized to completely disrupt the cell membranes. This released RNA and the cell content in general, into the lysis solution. Next, the samples were added to a purification column and centrifuged through the column. In this process the RNA was bound to the column. The first wash of several washes was applied to the columns to effectively eliminate RNase activity and wash away DNA, protein and residual lysate. Next, any residual DNA was degraded by an on-column DNase treatment. To remove the DNA fragments and residual salts, several washing steps were performed throughout the purification process.

At the end of the process, purified RNA was eluted from the column into DEPC-treated water. To ensure a high RNA concentration in the samples, 50 µl of elution solution was used. This is the minimum recommended amount to completely saturate the column. Purified RNA samples were placed on ice immediately after elution and stored at –80ºC.

Quantification of RNA

To ensure standardized conditions between the samples it is necessary to measure both RNA concentration in each sample and the RNA quality. It is important that equal amounts of RNA from each sample and RNA of the same quality are used to make complementary DNA in order to receive correct information about gene expression.

Measurement of RNA concentration and quality was done simultaneously by using the Nano-Drop ND-1000 Spectrophometer. This instrument is a full spectrum spectrophometer that has the ability to measure a wide range of biological molecules, including the concentration and purity of nucleic acids (119). The system is based on a combination of surface tension and fiber optic technology to hold the sample in place between two optical surfaces while measuring the sample.

RNA concentrations were measured by the system by penetrating the sample with ultra violet light with a wave length of 260 nm. According to Beer-Lambert law the absorbed light can be related to the concentration of molecules, and one absorbance unit is equal to 40 µg/ml RNA.

The measurement of the RNA concentration in each sample was started by thawing the samples and cleaning the two optical surfaces on the Nano-Drop pedestal with RNase free water. Next, 2 µL of the elution solution from the RNA purification process was set as blank to adjust for background absorbance. The samples were first mixed and then measured in 2 µL loads. Between each sample measurement the two optical surfaces was wiped clean with RNase free water.

Nucleic acids can be contaminated with other molecules like proteins and other organic compounds. The absorption ratio between 260 and 280 nm was calculated by the Nano-Drop system to assess the purity of the nucleic acids.

Quantitative reverse transcriptase polymerase chain reaction

The Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) involves two steps. The first step copies all RNA transcripts present in the sample into complementary DNA strands (cDNA). The second step amplifies a selected transcript of interest for further quantification (Figure 5).

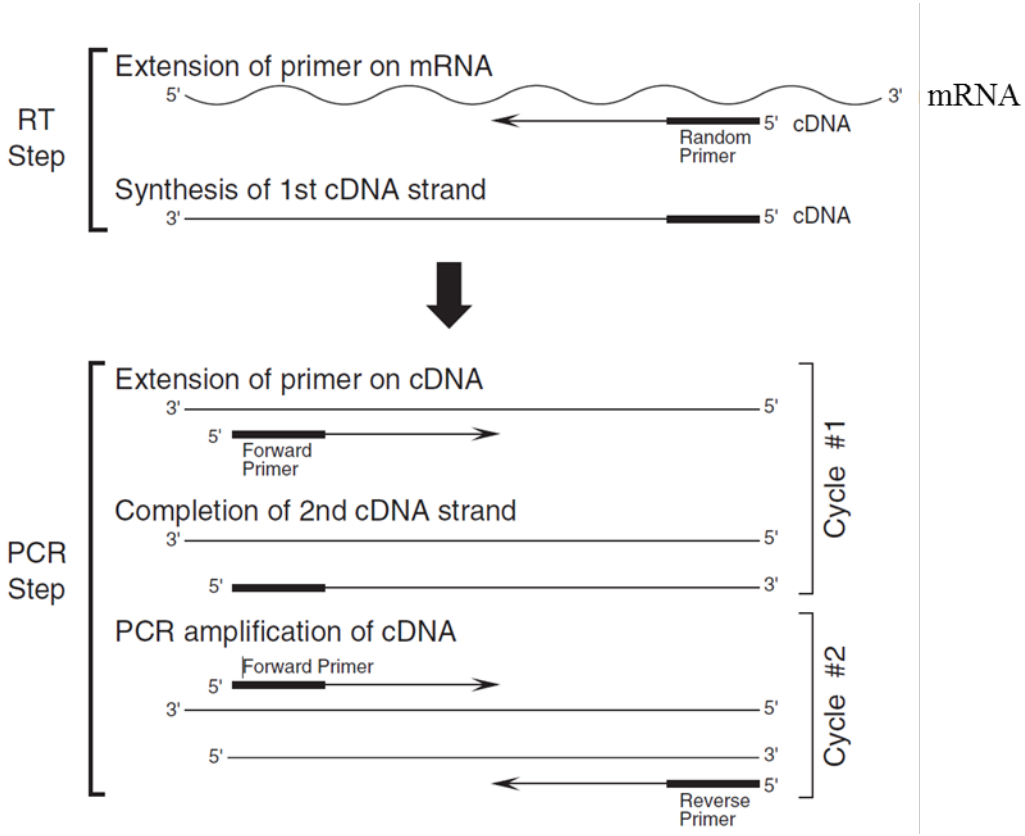

Figure 5. Overview of the qRT-PCR reaction. The Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) reaction involves two steps. In the first step, reverse transcriptase copies all RNA transcripts present in the sample into complementary DNA strands (cDNA). The second step amplifies a selected transcript of interest. The number of cycles needed to reach a certain number of copies is used to calculate the relative amount of target mRNA in the sample. The picture is reprinted with permission from the TaqMan Gene Expression Master Mix Protocol from Applied Biosystems (120).

Complementary DNA synthesis

RNA is a much less stable polymer than DNA. Conversion of RNA into cDNA stabilizes and preserves the RNA expression at the time of harvest which is important to ensure reliable results. Production of cDNA from total RNA was carried out using the reverse transcription reaction (RT). The enzyme reverse transcriptase synthesizes cDNA by the using primers and four deoxynucleotide triphosphates (dNTP). Random primers were used in the experiments.

The RT reaction consists of three steps. In the first step, denaturation takes place, as secondary structures are removed under heat exposure at 85-95 ºC. The second step is the annealing phase where a lowering of the temperature to 37 ºC allows for the attachment of random primers. In the last step, reverse transcriptase elongates the strand from the primer attachment site (121).

The high-capacity cDNA Reverse Transcription kit from Applied Biosystems was used for the cDNA synthesis. The procedure was in accordance with the protocol from the manufacturer (122). In each experiment, a set amount of RNA (ng) from each sample was mixed with buffer, dNTP mix, random primers, multiscribe and nuclease-free H_2O in 8 strips tubes on ice to a final volume of 20 µl (Table 1). As the samples had different concentrations of RNA, the amount to extract in micro liter from each sample varied. The amount to be extracted was calculated from the RNA concentration measured with the nano-drop in the previous step. The volume of both RNA and nuclease-free H_2O was to be 14.2 µl in total and nuclease-free H_2O was used to adjust the volume. The cDNA synthesis was carried out in a thermal cycler machine called Multigene with a thermal program of 10 minutes at 95 ºC, 120 minutes at 37 ºC and 5 minutes at 85 ºC. Next, the cDNA was kept at 4 ºC until diluted 5X with RNase free water. Diluting of the cDNA enables more accurate pipetting of cDNA for the amplification step. The cDNA was stored at -20 ºC.

Table 1. Reaction mix for cDNA synthesis. For each sample 5.8 µl master mix and a set amount of RNA (ng) and nuclease-free H2O with a total volume of 14.2 µl were mixed and centrifuged prior to initiation of the RT-PCR.

Component	**Reaction volume per sample (µl)**
10X RT Buffer	2.0
25X dNTP Mix (100 mM)	0.8
10X RT Random Primers	2.0
Multiscribe Reverse Transcriptase	1.0
Sum Mastermix	5.8
+	
RNA and nuclease-free H_2O	14.2
Total reaction mix	20

Amplification of selected transcript

The next step in the qRT-PCR is the amplification of the selected transcript of interest. The selected transcript is amplified by running cDNA and a reagent mixture through a polymerase chain reaction (PCR) thermal cycler. The reagent mixture consists of two transcript specific primers (forward and reverse), nuclease free water and a master mix with a type of fluorescent technology, DNA polymerase enzyme, the four nucleotide bases and optimized buffer components.

The PCR consists of several steps. As with the cDNA synthesis, the time interval and temperature varies between the different steps. In the first step the DNA polymerase enzyme is activated. For the second step, the DNA fragment is opened and ready for the primers to bind. In step three, hydrogen bonds are formed between the primers and the DNA-templates, and DNA synthesized by the DNA polymerase enzyme. In the fourth step, the hydrogen bonds between the DNA strands are broken and ready for the DNA polymerase to bind again.

In the first PCR reaction, the cDNA strands give rise to a new cDNA strand and generate a double stranded DNA. After the first step, amplification of the target sequence proceeds at an exponential rate and doubles for each cycle (Figure 5). The amplification rate leaves the exponential phase and enters a more linear phase after 10 cycles or more due to that primers and reagents are no longer in excess. At additional cycles, the amplification rate will approach zero and a plateau, where only a negligible amount of product is made.

A threshold value is set sufficiently above background and in the exponential phase. A fluorescent signal is reflecting the amount of target sequence being produced in the sample. The number of cycles that the target needs to produce a strong enough fluorescent signal to reach above the set threshold value gives a C_t value. Genes that are expressed at high levels reach the threshold levels early, whereas genes that are expressed at low levels need additional cycles and consequently have a higher C_t value (Figure 6) (123).

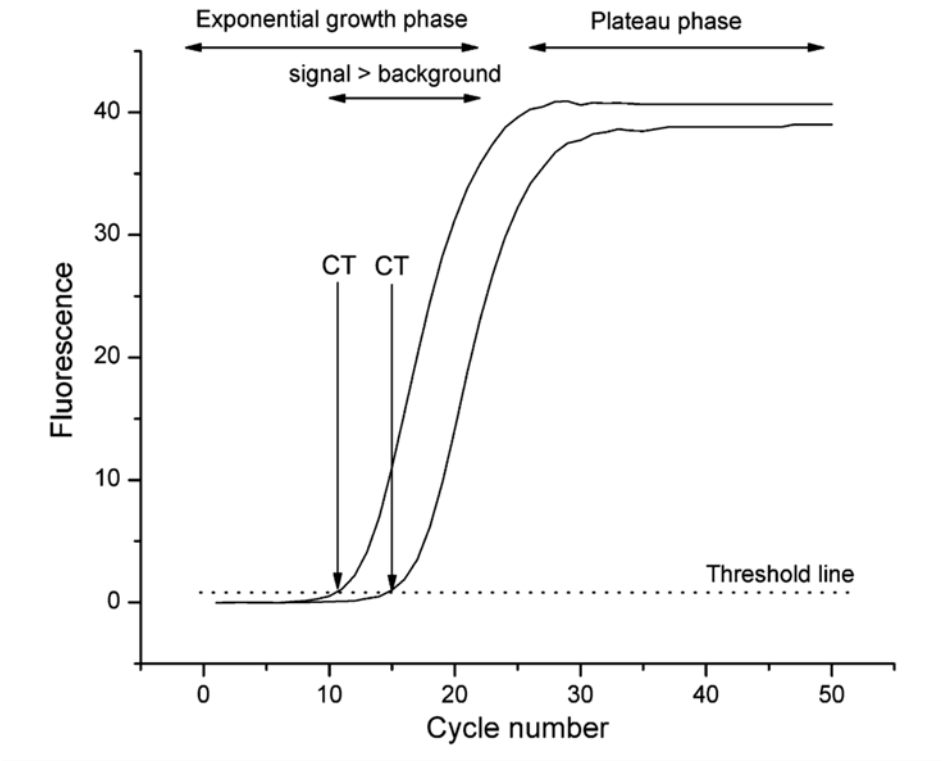

Figure 6. Real-time PCR response curves. A threshold level is set sufficiently above background and at the exponential phase. The amount of target sequence being produced in the sample is reflected by a fluorescent signal. The number of cycles needed for a target gene to produce a fluorescent signal above the set threshold value gives a Ct value. The Ct value is used for further calculations of the relative amount of target mRNA in the sample. The picture is reprinted with permission from Kubista et al. (123).

Fluorescent technologies for detection of amplified target sequence

Two different technologies were used in this experiment to detect the amplified target sequence, the SYBR green dyes and the TaqMan probe system (Figure 7).

The TaqMan probe system was used for detecting the housekeeper gene. In this system, three primers bind to the target sequence. Two amplify the target sequence, and the third which is called a probe, binds in the region between the two primers. The probe contains a fluorescent reporter dye attached to the 5'end and a quencher attached to the 3' end. The quencher absorbs the emission from the reporter dye when they are in close proximity. The DNA polymerase exhibit endonuclease activity which will hydrolyze the probe into single nucleotides as it reaches the probe. As a result the reporter will release from the quencher. The quencher will no longer be able to absorb the fluorescence emitted from the reporter, and the fluorescence signal can therefore be measured (120).

The SYBR green dyes bind to any double stranded DNA and emits strong fluorescence upon binding. The emission is a measure of the total mass of double stranded DNA, and not only the number of target sequences amplified. Amplification of primer-dimers and non-target sequences may also give rise to double stranded DNA. Primer dimers consist of primers that have attached to each other because of complementary bases in the primers. Unspecific primers might bind to non-target sequences in addition to target sequence. DNA contamination might also contribute to the total pool of double stranded DNA.

In order to detect amplification of primer-dimers, non-target sequences and DNA contamination, a dissociation curve was run at the end of each PCR with SYBR green dyes. A melting point where 50 % of the DNA is dissociated creates a top on the dissociation curve. Each amplified product has a unique melting point and dissociation curve thus several melting points might indicate that non-target sequences and/or primer-dimers have been amplified.

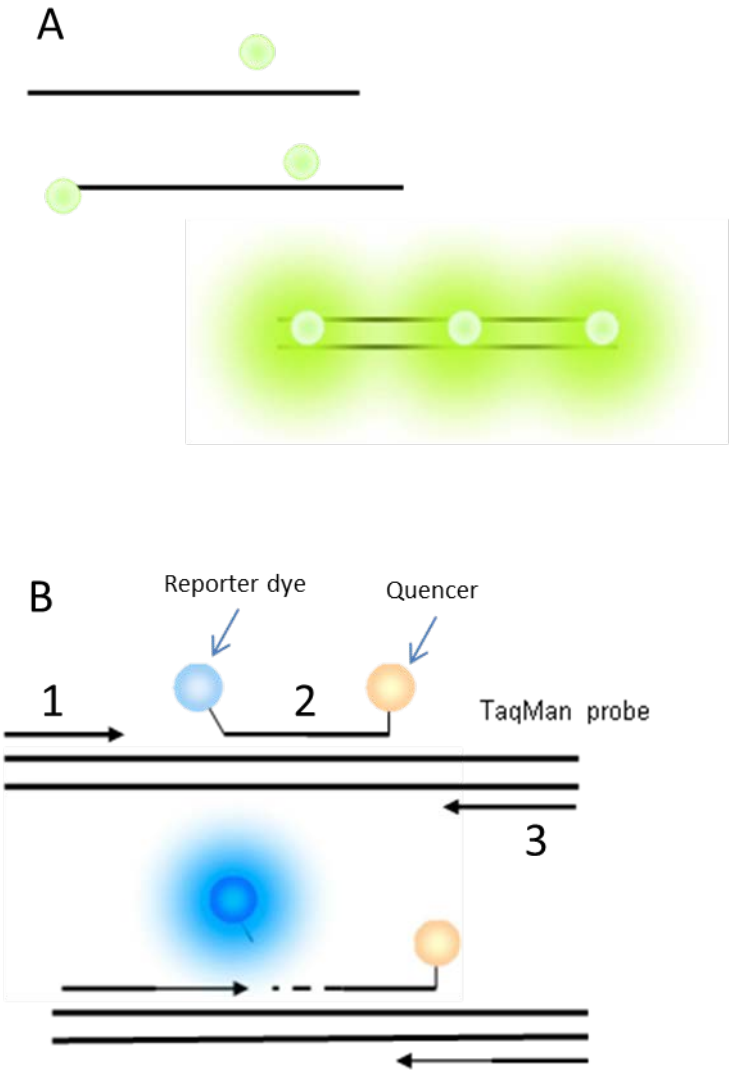

Figure 7. Visual presentation of two fluorescent technologies for detection of amplified target sequence. SYBR green dyes (A) emits weak fluorescence in solution. The dyes bind to any double stranded DNA and emits strong fluorescence upon binding. The TaqMan probe system (B) consists of three primers that bind to the target sequence. Two amplify the target sequence, and the third which is called a probe, binds in the region between the two primers. The probe contains a fluorescent reporter dye attached to the 5'end and a quencher attached to the 3' end. The quencher absorbs the emission from the reporter dye when they are in close proximity. The DNA polymerase exhibit endonuclease activity which will hydrolyze the probe into single nucleotides as it reaches the probe. As a result the reporter will release from the quencher. The quencher will no longer be able to absorb the fluorescence emitted from the reporter, and the fluorescence signal can therefore be measured. The picture is modified and reprinted from a lab protocol at the Department of Nutrition UIO (124).

Quantification of the amount of target in the sample

The comparative Ct or comparative threshold method was used to calculate the relative amount of target messenger ribonucleic acid (mRNA) in each sample. The mRNA was calculated and the Ct values for each sample was normalized against an endogenous housekeeping gene, with the formula $2^{-\Delta\Delta Ct}$. The amount of target mRNA was compared with unstimulated controls. Phosphomannomutase 1 (PMM1) was used as housekeeping gene. PMM1 was in our experiments (Appendix 2) and is also known to be, unregulated by estradiol treatment (125).

Procedure

Gene expression from 21 genes were analyzed with cDNA from the first experiment. These genes were PMM1, Bactin, ATP binding cassette transporter A1 (ABCA1), ATP binding cassette transporter G8 (ABCG8), ATP binding cassette transporter G5 (ABCG5), Sortilin, Cholesterol 7alpha-hydroxylase (Cyp7a), NAMPT, Tissue factor pathway inhibitor, nicotinamide nucleotide adenylyltransferase 1-3, PARP1, Chemokine (C-X-C motif) ligand 16 (CXCL16), Adipose differentiation-related protein (ADRP), Very-low-density-lipoprotein receptor (VLDLr), Low-density-lipoprotein receptor (LDLr), Scavenger receptor class B member 1, Liver-type fatty acid-binding protein (L-FABP), Fatty acid synthase (FAS), Liver x receptor alpha (LXRα). In the second experiment fewer genes were analyzed and in the third, only eight genes were analyzed. Only information about the eight genes that were analyzed in all experiments is presented in this thesis.

Taqman gene expression master mix or Brilliant III ultra-fast SYBR green qPCR master mix with target sequence specific primers was added to Stratagene Mx3000P 96-well plates, 35 µl in total. Next, 5 µl cDNA was added and the solution gently mixed with a pipette before duplicate wells were made with 50 % of the volume (20 µl). The qPCR reaction was carried out on a Stratagene Mx 3005P thermal cycler with the accompanying software Mx Pro – Mx3005P according to the manufacturer`s protocol (120, 126). Primer sequences used with the SYBR green dye are listed in Table 2.

Table 2. Primers sequences used with the SYBR green dye in the qPCR procedure.

Human gene	**Forward primer**	**Reverse primer**
ABCA1	5`ACATGAATGCCATTTTCCAA`3	5`GTAACGGAAACAGGGGTTGT`3
ABCG8	5`AAAATGCCTCTCGGGAACCT`3	5`GGTACGAGTCCAGCTCCATGA`3
ABCG5	5`GCATGCTGAACGCTGTGAAT`3	5`TGGTAGAGGCCGTCCTGACT`3
Cyp7a	5`ACGGGTGAACCACCTCTAGAGA`3	5`CTCAAGAGGATTGGCACCAAA`3
NAMPT	5`CTTCTGGTAACTTAGATGGTCTGGAA`3	5`GCTCCTATGCCAGCAGTCTCTT`3
CXCL16	5`TATACTACACGAGGTTCCAGCTCCTT`3	5`CAAGACAGCTCATCAATTCCTGAA`3
ADRP	5`GAATCAGCCATCAACTCAGATTGT`3	5`AGTAGTCGTCACAGCATCTTTTGC`3
VLDLr	5`AGAAAAGCCAAATGTGAACCCT`3	5`CACTGCCGTCAACACAGTCT`3
LDLr	5`TGGACCGGAGCGAGTACAC`3	5`GACGCCGTGGGCTCTGT`3
L-FABP	5`TTCTCCGGCAAGTACCAACTG`3	5`CAGACCGATTGCCTTCATGA`3
FAS	5`GCAAATTCGACCTTTCTCAGAA`3	5`GCACCCCGTGGAATGTCA`3
LXRα	5`CGCACTACATCTGCCACAGT`3	5`TCTTCAGGCGGATCTGTTCT`3

4.2.3 Detection of protein- and lipid secretion in cell supernatant

xMAP technology, magnetic bead-based immunoassays and multiplex assays were used for detection and quantification of NAMPT, Tumor necrosis factor (TNF), Monocyte attractant protein-1 (MCP-11), Interleukin 6 (IL-6), CXCL16, APOA1 and APOB100 in cell supernatants. The methods are described in detail in section 4.1.1.

NAMPT was detected in a singleplex format with the Bio-Plex Pro™ human diabetes immunoassay kit and TNF, MCP-1, IL-6 and CXCL16 in a multiplex format with Bio-Plex Pro™ human Chemokine Assay. Both kits are from Bio-Rad. APOA1 and APOB100 were multiplexed with the kit Milliplex MAP Kit Human Apolipoprotein Magnetic Bead Panel from Merck Millipore.

Detection of TG was determined enzymatically with the triglyceride Enzymatic PAP 150 kit from BioMerieux. The kit facilitates an enzymatic reaction with TG which can be detected spectrophotometrically. The amount of color created during the enzymatic reaction is proportional with the amount of TG present in the sample and thus used for quantification. The absorbance was read at 505nm with the BioTek Synergy H1 Hybrid Multi-Mode microplate Reader. All assays were conducted according to the instruction manual from the manufacturer (109, 127-129).

4.2.4 Data analysis

Ct values from the qPCR process obtained during analysis of gene expression were imported to Microsoft Excel 2007 for calculation of $2^{-\Delta\Delta Ct}$ and normalization of data to controls. Raw data on cell release was exported from the Bio-Plex Data Pro™ software as pg/ml and also normalized to controls in Microsoft Excel 2007. All statistical processing of data was done in Graph Pad prism 6.0.

During experimentation, the 6 hour time-point was evaluated as the relevant time-point for mRNA expression study and the 24 hour time-point for cell release. Consequently, data from the 6 hour time-point for mRNA expression and 24 hour time-point for cell release are presented herein. The reason was that we were interested in primary effects of estradiol and not secondary or tertiary, as we were expecting from the 24 hour time-point. A linear increase in cell release over time was observed for all proteins. This occurrence is in accordance with general cell biology knowledge were protein translation happens after mRNA transcription and thus takes longer time to occur (55). In addition, the cell needs to transport the proteins to the plasma membrane to release them.

The cytotoxicity test (LDH) as described in section 4.2.1, was used to study EE2`s effect on cell death in HepG2 cells. As seen in Figure 8, there was a significant increase in cell death at the 6 hour time point after EE2 exposure relative to controls. Cells exposed to 100 nM EE2 were interpreted as the conclusive factor. Consequently, data from the 100 nM EE2 and 100 nM EE2 with 1µM Fulvestrant were interpreted as toxic and omitted at both time-points (6 and 24 hrs.). Thus, data from 0-10 nM EE2 exposure at the 6 and 24 hrs time-points are presented in this thesis.

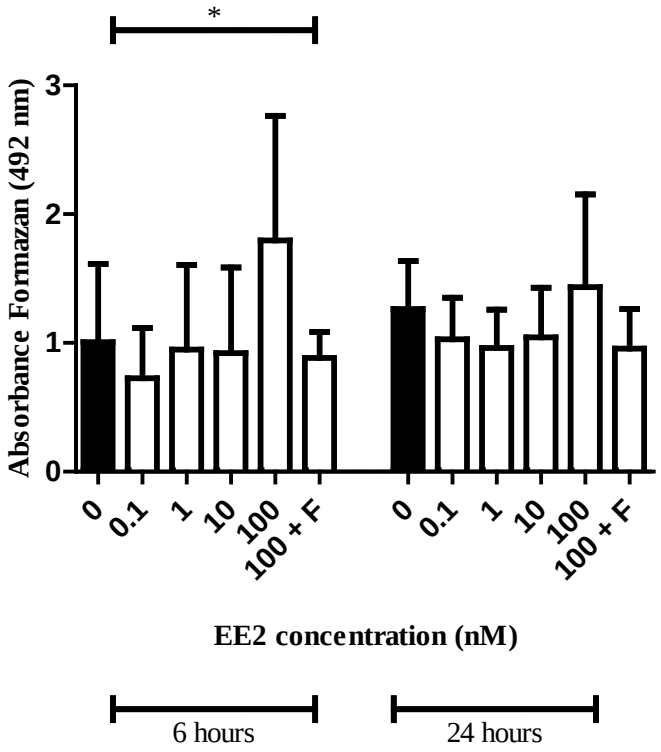

Figure 8. Difference in cell death in EE2 treated HepG2 cells after 6 hours. Cell supernatant was analyzed with a LDH based cytotoxity detection kit and the absorbance was read in a spectrophotomer. The quantity of Formazan is directly proportional to cell death and cell lysis at the time points of cell harvest (6- and 24 hours). HepG2 cells were treated with 0.1-100 nM EE2 or 100 nM EE+ 1µM Fulvestrant for 6- and 24 hours. Controls were given DMEM. The bars represent the relative mean ±SD at the respective concentrations. *=$p < 0.05$ relative to unstimulated cells, one-way ANOVA with Bonferroni correction. F= 1 µM Fulvestrant (EE2 antagonist).

Statistics

One-way analysis of variance (one-way ANOVA) was used with Bonferroni correction as post-test for multiple comparisons of stimulated vs. unstimulated cells. All statistical testing were conducted using the software Graphpad Prism 5.0. A probability value of $\leq .05$ was considered as significant.

5 Results

Sex steroids, both endogenous and exogenous are associated with changes of lipoprotein pattern (16, 23, 42, 47-49). However, to the best of our knowledge, the effect of HRT on lipoprotein ratios remains to be studied so as to better reflect the overall atherogenic lipoprotein burden in serum (32, 35). Oral HRT which was used in EVTET, is associated with increased circulating levels of CRP, -TG and clotting factors (92, 93) and decreased LDL-C/HDL-C ratio (66, 87).

High levels of circulating NAMPT is associated with an atherogenic metabolic profile (19) and inflammatory atherothrombotic diseases (105, 106). Regulative mechanisms for circulating NAMPT are not well studied (101). Human hepatocytes have been proposed as a potential source for circulating NAMPT (130). To the best of our knowledge, the effect of HRT on circulating NAMPT and the effect of EE2 on NAMPT cell release have not been studied before.

5.1 Effects of hormone replacement therapy

To investigate the effect of HRT on NAMPT and lipid profile, plasma/serum from 138 postmenopausal women exposed to HRT, were analyzed.

As published in EVTET (84), there were no significant differences in baseline parameters. In addition to the published baseline parameters, there were no significant differences in NAMPT, APOA1, APOB (total), Lp (a), LDL-C, TC/HDL-C-, LDL-C/HDL-C- and APOB/APOA1 ratio between the treatment- and the placebo group at baseline (Table 3).

Table 3. Baseline characteristic of lipid profile and NAMPT, n=138 women						
Biomarker	Baseline					
	n=68	Placebo	Treatment	n=70	Reference range	Recommended level
NAMPT (pg/ml)	60	455 (218-1108)	443 (213- 1022)	61	-	-
Lp (a) (mg/L)	65	288 (144- 838)	267 (164- 743)	66	<250	<150
ApoA1 (g/L) $\bar{x}$	66	1.44 (1.38- 1.50)	1.48 (1.41- 1.56)	68	1.1-2	-
ApoB (g/L)	66	1.33 (1.16- 1.53)	1.27 (1.09- 1.45)	68	0.5-1.4	-
ApoB/ApoA1	66	0.89 (0.73-1.16)	0.84 (0.74-1.09)	68	-	<1
TC (mmol/L) $\bar{x}$	68	6.61 (6.32- 6.90)	6.51 (6.28- 6.74)	69	3.9-7.8 (> 50 yrs.)	<5
HDL-C (mmol/L) $\bar{x}$	68	1.63 (1.53- 1.73)	1.76 (1.65- 1.86)	69	0.95 – 2.79	>1.3
TC/HDL-C	68	3.76 (3.27-4.80)	3.79 (3.07-4.78)	69	-	<5
LDL-C (mmol/L) $\bar{x}$	68	4.20 (3.90-4.52)	4.15 (3.92-4.38)	69	1.95 – 5.34	<3
LDL-C/ HDL-C	68	2.55 (2.02-3.32)	2.40 (1.72-3.17)	69	<5	-
TG (mmol/L)	68	1.36 (1.00- 1.86)	1.24 (0.84- 1.68)	69	<2.6	<1.7

Values are presented as median (Q1-Q3)

$\bar{x}$ =values are presented as mean (95 % confidence interval)

Reference ranges and recommended level apply to women and are based on the Norwegian guidelines for individual primary prevention of CVD 2009 (11)

5.1.1 Effect on NAMPT and lipid profile

In order to study the effect of HRT on plasma NAMPT and lipid profile after 3 months of treatment, the change from baseline to the 3 month follow-up time-point was calculated. Lp (a), APOA1, APOB, TC, HDL-C, LDL-C, TC/HDL-C- and LDL-C/HDL-C ratio, was significantly more reduced after 3 months of HRT treatment than with placebo treatment (Table 4). Estradiol, was as expected, significantly increased in the HRT exposed group. We found no significant difference in NAMPT, APOB/APOA1 ratio and TG between the groups. Median values for each group at each time-point, are presented in (Table 5).

Table 4. Change from baseline to the 3 month follow-up time-point, n=138 women					
Biomarker	Placebo, n=68		Treatment, n=70		P value
	n	score	score	n	
Estradiol (nmol/L)	59	1.00 (-2.00- 6.00)	129 (63- 206)	63	<0.001
NAMPT (pg/ml)	47	-83.46 (-244-239)	87.47 (-289-245)	48	0.49
Lp (a) (mg/L)	55	-29.0 (-0.70- 14.0)	-72.0 (-197- (-) 24.0)	61	0.001
ApoA1 (g/L) $\bar{x}$	59	0.02 (-0.01- 0.06)	-0.09 (-0.13- (-) 0.05)	63	<0.001
ApoB (g/L)	59	-0.02 (-0.09- 0.08)	-0.11 (-0.20- 0.00)	63	<0.001
ApoB/ApoA1	59	-0.02 (-0.10-0.05)	-0.03 (-0.11-0.07)	63	0.93
TC (mmol/L) $\bar{x}$	60	0.04 (-0.15- 0.23)	-0.93 (-1.10- (-) 0.77)	63	<0.001
HDL-C (mmol/L) $\bar{x}$	60	0.03 (-0.03-0.10)	-0.16 (-0.23- (-) 0.09)	63	<0.001
TC/HDL-C	60	0.02 (-0.34-0.30)	-0.29 (-0.60-0.10)	63	0.013
LDL-C (mmol/L) $\bar{x}$	60	-0.02 (-0.19-0.15)	-0.72 (-0.86- (-) 0.57)	63	<0.001
LDL-C/HDL-C	60	-0.06 (-0.37-0.21)	-0.27 (-0.59-0.12)	63	0.03
TG (mmol/L)	60	0.03 (-0.17- 0.31)	-0.05 (-0.31- 0.13)	63	0.06

Values are presented as median (Q1-Q3)

$\bar{x}$ =values given in mean (95 % confidence interval)

Score= values after 3 months HRT exposure-values at baseline

Table 5. Median values for the placebo and treatment group at baseline and at the 3 month follow-up time-point, n=138 women

Biomarker	Placebo, n=68						Treatment, n=70			
	Baseline		3 months		Reference range	Recommended level	Baseline		3 months	
	n			n			n			n
Estradiol (nmol/L)	66	14.0 (10.0- 18.0)	16.0 (11.0-22.0)	59	-	-	68	14.5 (10.0- 23.0)	166 (90.0- 223)	63
NAMPT (pg/ml)	60	455 (218-1108)	428 (182- 1057)	50	-	-	61	443 (213- 1022)	628 (389- 923)	52
Lp (a) (mg/L)	65	288 (144- 838)	382 (149- 880)	56	<250	<150	66	267 (164- 743)	221 (124- 539)	61
ApoA1 (g/L) $\bar{x}$	66	1.44 (1.38- 1.50)	1.47 (1.41- 1.53)	59	1.1-2	-	68	1.48 (1.41- 1.56)	1.37 (1.31- 1.43)	63
ApoB (g/L)	66	1.33 (1.16- 1.53)	1.27 (1.14- 1.50)	59	0.5-1.4	-	68	1.27 (1.09- 1.45)	1.16 (1.03- 1.34)	63
ApoB/ ApoA1	66	0.89 (0.73-1.16)	0.88 (0.72-1.06)	59	-	<1	68	0.84 (0.74-1.09)	0.84 (0.75-1.00)	63
TC (mmol/L) $\bar{x}$	68	6.61 (6.32- 6.90)	6.55 (6.19- 6.91)	60	3.9-7.8 (> 50 yrs.)	<5	69	6.51 (6.28- 6.74)	5.46 (5.25- 5.66)	64
HDL-C (mmol/L) $\bar{x}$	68	1.63 (1.53- 1.73)	1.69 (1.56- 1.82)	60	0.95 – 2.79	>1.3	69	1.76 (1.65- 1.86)	1.57 (1.47- 1.66)	63
TC/HDL-C	68	3.76 (3.27-4.80)	3.84 (3.13-4.92)	60	-	<5	69	3.79 (3.07-4.78)	3.53 (3.03-4.19)	63
LDL-C (mmol/L) $\bar{x}$	68	4.20 (3.90-4.52)	4.06 (3.74-4.38)	60	1.95 – 5.34	<3	69	4.15 (3.92-4.38)	3.36(3.17-3.56)	63
LDL-C/ HDL-C	68	2.55 (2.02-3.32)	2.32 (1.72-3.06)	60	<5	-	69	2.40 (1.72-3.17)	2.22 (1.80-2.57)	63
TG (mmol/L)	68	1.36 (1.00- 1.86)	1.32 (0.99- 1.91)	60	<2.6	<1.7	69	1.24 (0.84- 1.68)	1.07 (0.88- 1.49)	64

Values are presented as median (Q1-Q3)

$\bar{x}$ =values given in mean (95 % confidence interval)

Reference ranges and recommended level apply to women and are based on the Norwegian guidelines for individual primary prevention of CVD 2009 (11)

5.2 Hepatic effect of estradiol

Cell experiments were set up, in order to study estradiol's effect on the liver. More specifically, we studied cell release/secretion and gene expression of NAMPT and various proteins/lipids involved in inflammation and lipid homeostasis in HepG2 cells after stimulation with EE2.

5.2.1 Effect on NAMPT

The level of NAMPT in conditioned medium was significantly reduced by EE2. A dosage of 0.1 nM EE2 reduced the release of NAMPT from HepG2 cells after 24 hours exposure, as compared to unstimulated cells. However, EE2 did not affect mRNA expression of NAMPT (Figure 9).

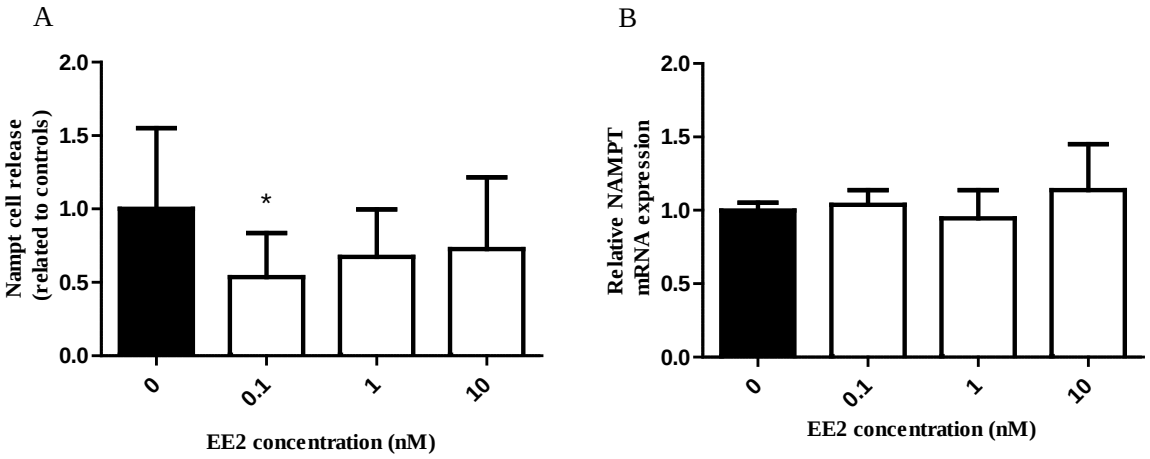

Figure 9. Effects of EE2 on NAMPT secretion and mRNA expression in HepG2 cells. The cells were treated with 0-10 nM EE2. Controls were given DMEM. The bars represent the relative mean ±SD. (A) the cell supernatant was measured after 24 hours incubation using the bioplex suspension array system. The average cell release at baseline was 1027 pg/ml (n=4). (B) The mRNA expression was measured after 6 hours incubation using qRT-PCR (n=3). *p< 0.05 relative to unstimulated cells using one-way ANOVA with Bonferroni correction

5.2.2 Effect on inflammation

To elucidate potential inflammatory effects of EE2 in hepatocytes, factors that are associated with liver inflammation were studied. The level of MCP-1, IL-6, TNF-α and CXCL16 were analyzed in conditioned medium, in addition to CXCL16 mRNA expression. HepG2 cells were treated with 0.1-10 nM EE2 for 24 hours. Controls were given DMEM.

There were no significant differences either in CXCL16 secretion or gene expression after EE2 stimulation (Figure 10). The levels of MCP-1, IL-6 and TNF-α were under the detection limits for instrumental assessment.

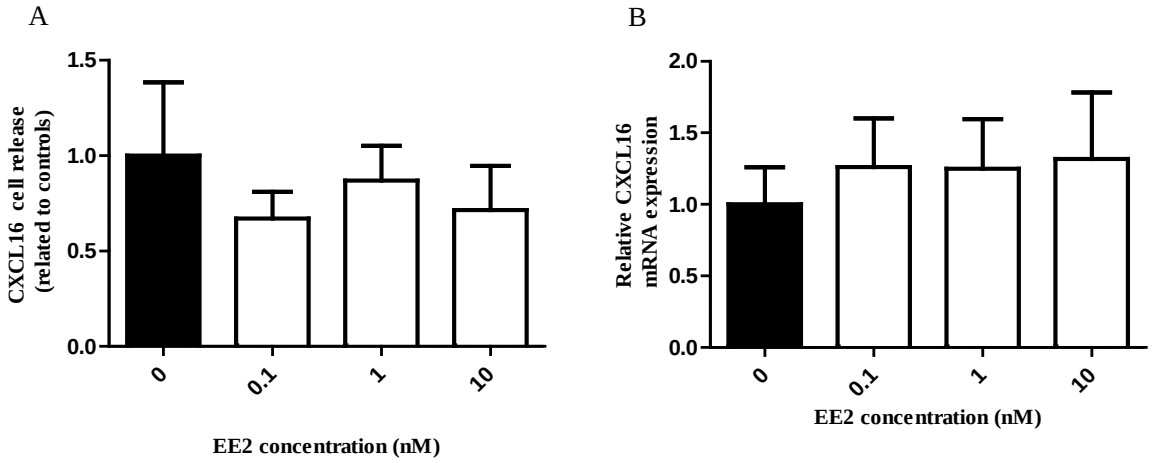

Figure 10. Effects of EE2 on CXCL16 secretion and mRNA expression in HepG2 cells. The cells were treated with 0-10 nM EE2. Controls were given DMEM. The bars represent the relative mean ±SD. (A) the cell supernatant was measured after 24 hours incubation using the bioplex suspension array system. The average cell release at baseline was 158 pg/ml (n=2). (B) The mRNA expression was measured after 6 hours incubation using qRT-PCR (n=3).

5.2.3 Effect on lipoprotein and bile acid metabolism

As depicted in Figure 11, there was no significant difference in secreted apo lipoproteins i.e. APOA1 and APOB100 in conditioned medium as compared to unstimulated cells. HepG2 cells were treated with 0.1-10 nM EE2 for 24 hours. Controls were given DMEM. TG secretion from the same experimental conditions, were also analyzed, but to be too low for instrumental detection.

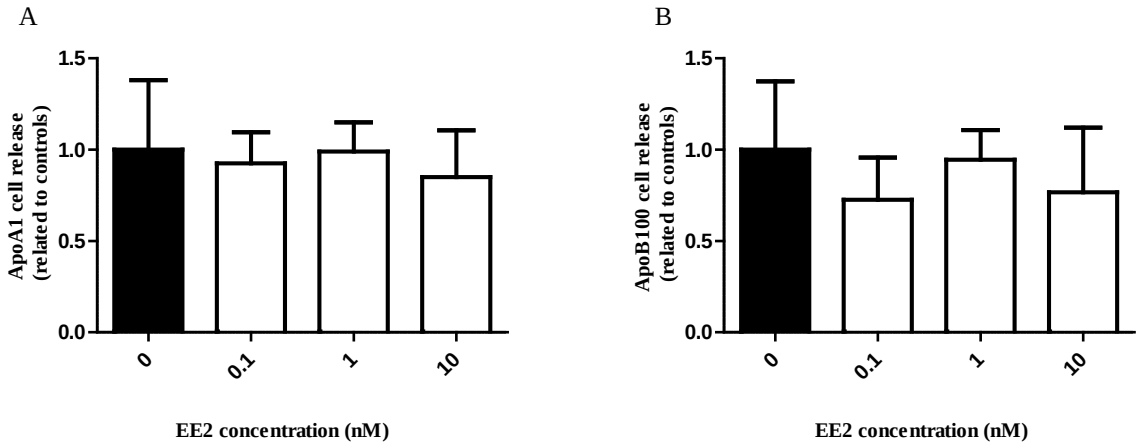

Figure 11. Effects of EE2 on APOA1 (A) and APOB100 (B) cell release in HepG2 cells. The cells were treated with 0-10 nM EE2 for 24 hours. Controls were given DMEM. The cell supernatant was measured after 24 hours incubation using the bioplex suspension array system. The bars represent the relative mean ±SD (n=2). The average cell release at baseline was 63 pg/m (A) and 14.7 pg/ml (B).

Interestingly, EE2 induced gene expression of genes involved in *de novo* lipogenesis, reverse cholesterol transport and bile acid metabolism. A dosage of 10 nM EE2 increased the relative mRNA levels of ABCG8, ABCA1, CYP7A and FAS in HepG2 cells after 6 hours incubation (Figure 12).

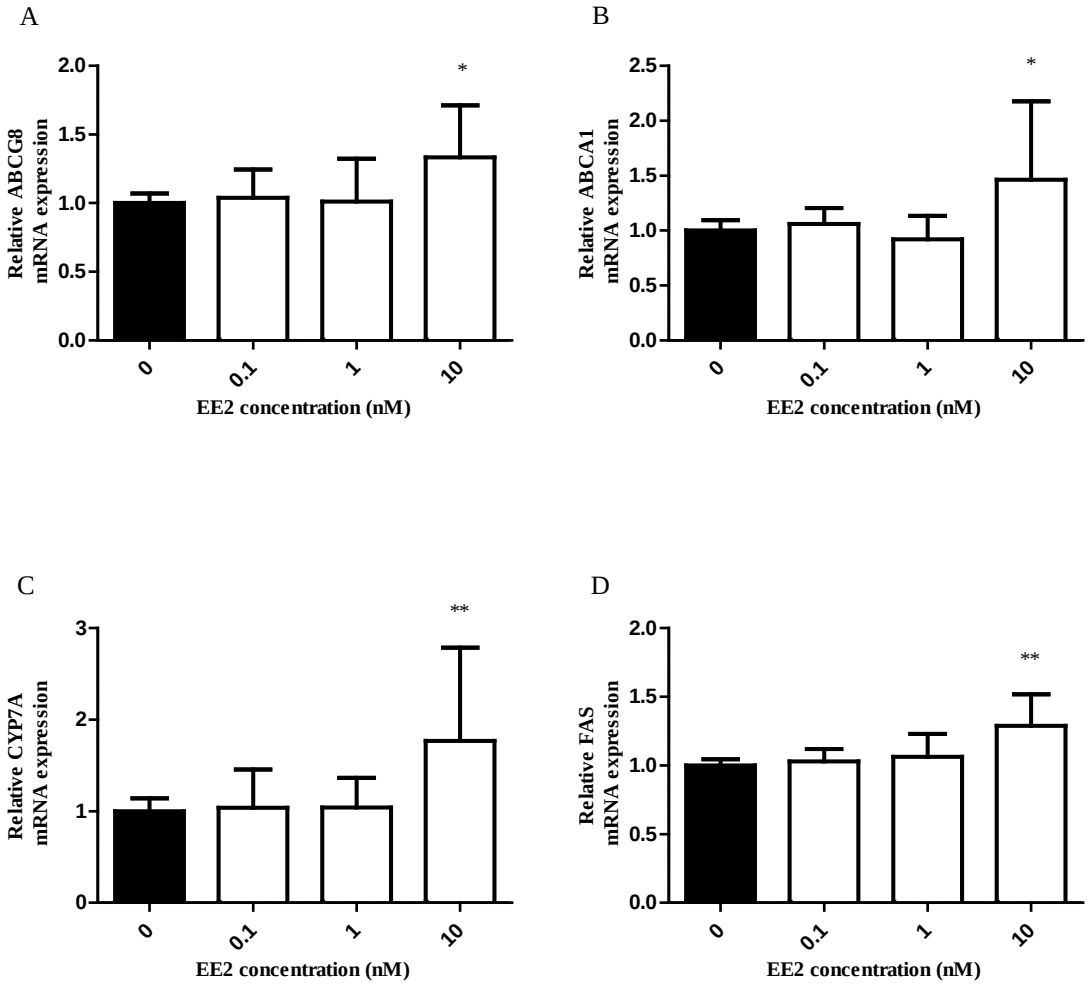

Figure 12. Effects of EE2 on mRNA expression of ABCG8 (A), ABCA1 (B), CYP7A (C) and FAS (D). HepG2 cells were treated with 0-10 nM EE2. Controls were given DMEM. The mRNA expression was measured after 6 hours incubation using qRT-PCR. The bars represent the mean ±SD. *p< 0.05, **p<0.01 relative to unstimulated cells using one-way ANOVA with Bonferroni correction (n=3).

Other genes involved in lipid homeostasis such as VLDLr, ABCG5, ADRP, LDLr, L-FABP and LXRα, did not exhibit significant mRNA expression after 6 hours incubation with EE2 dosages ranging from 0.1- to10 nM (Figure 13).

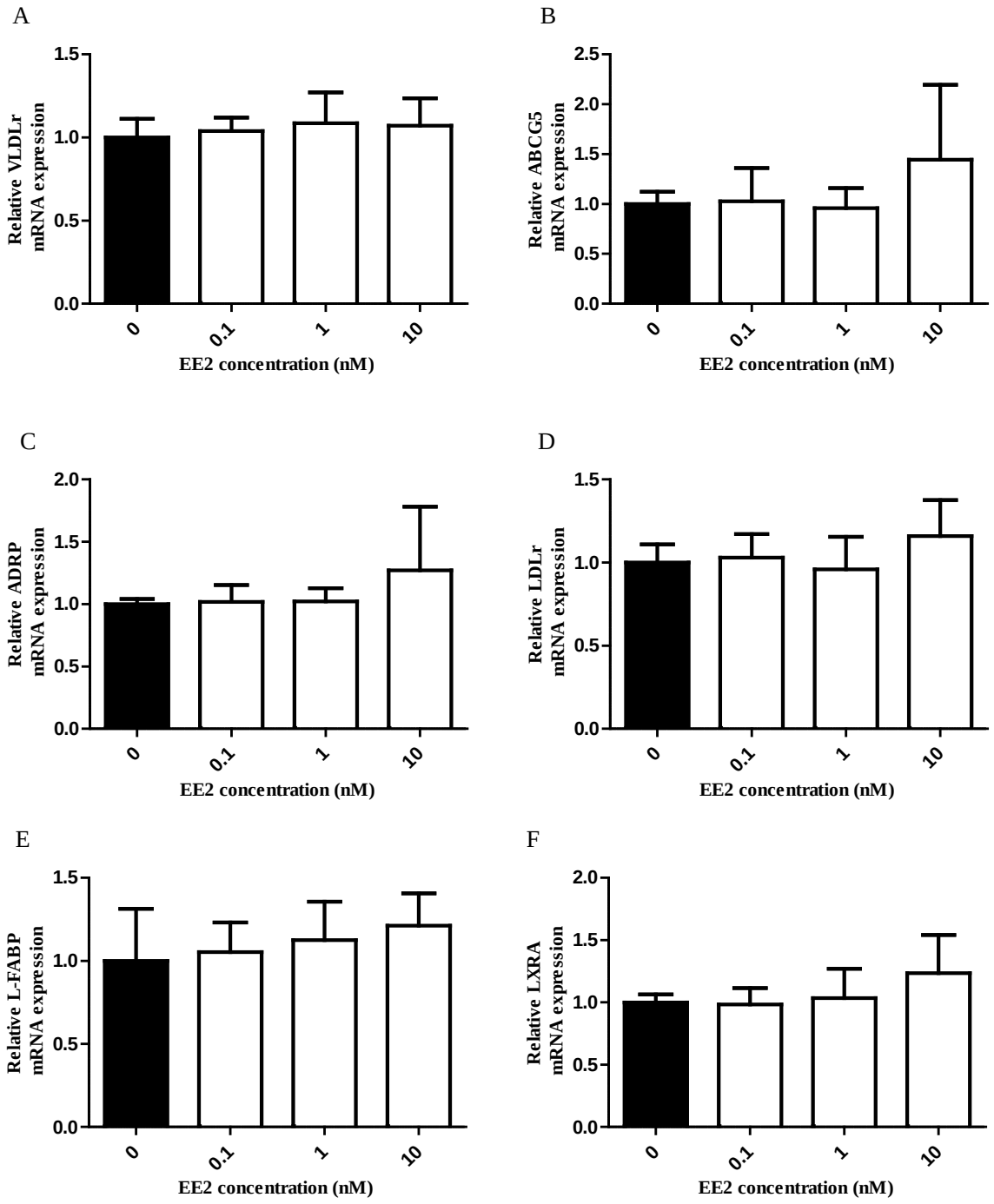

Figure 13. Effects of EE2 on VLDLr (A), ABCG5 (B), ADRP (C), LDLr (D), L-FABP (E) and LXRα (F) mRNA expression. HepG2 cells were treated with 0-10 nM EE2. Controls were given DMEM. The mRNA expression was measured after 6 hours incubation using qRT-PCR. The bars represent the mean ±SD (n=3).

6 Discussion

Women in menopause experience a state of estrogen deficiency, which is associated with cardiovascular complications (45, 46) and proatherogenic changes in lipoprotein pattern (23, 42, 47) in addition to enhanced pathobiology in other body systems (50, 52, 53). HRT (estrogen or estrogen combined with progesterone) has been vividly debated in the recent decade as it has shown both favorable (66) and adverse (45, 82-86) effects on CVD. However, in recent years our understanding about HRT has improved and we know today that the effects may depend on the chosen regime, the right timing and the route of administration. Although estrogen is known to have cardioprotective effects on the vascular endothelium (54, 55) and HRT has shown anti-atherogenic effects (66, 87), large RCTs have failed to demonstrate a benefit of HRT on CHD risk, except in younger postmenopausal women (63, 66, 67, 131). Thus an improved understanding regarding the physiological effects of female sex steroids are important to create HRT that only produce the desired effects.

In the first part of the project, we analyzed circulating NAMPT, lipoproteins and apolipoproteins in 138 postmenopausal women randomized into either HRT or placebo for three months. Next, we calculated their lipoprotein ratios in an attempt to gain more in depth information about their lipid profile.

The second part of the project was a study of gene expression and cell release of NAMPT, lipid homeostasis and inflammation in a hepatocyte cell line after exposure to different doses of EE2.

6.1 Methodological considerations

The work in this thesis is based on an experimental approach in which variables are manipulated to create the context relevant for the study of interest in contrast to observational studies. When this approach is used in medical research, a human being or a surrogate system undergoes some kind of intervention in order to evaluate its impact.

We have utilized both an *In vitro* model system, a hepatocyte cell line named HepG2, and an RCT for our experiments. In the following, methodological advantages and disadvantages concerning the conducted experiments will be discussed.

6.1.1 The human trial EVTET

The study design in EVTET was a randomized, double-blind, and placebo-controlled clinical trial with a double triangular sequential design. A more detailed discussion about the methodological aspects of EVTET (20) can be found in the appendix 3.

RCTs are considered as gold standard for clinical research (132). The design is made to resemble the objectiveness of laboratory research where the researcher controls the exposure. RCT's consists of at least a control group and an experimental group. In order to eliminate systematic errors, the design may have several safeguards. In its simplest form, randomization is executed, so as to ensure that confounding factors will be evenly distributed between the treatment and the no treatment group and not affect the results. However, confounding in RCTs, may still occur (133). Another safeguard is blinding, wherein the patient and sometimes the researcher and staff in contact with the patients and samples does not know which treatment the patient receives during the trial. Blinding is achieved by the use of placebo in order to prevent that the patient/staff/researchers behavior changes due to awareness of treatment and thus erroneously affect the outcome (134, 135). Even though RCT is the strongest research design and has the potential for confirming causal relationships, it has its limitations. In addition to ethical considerations, high costs, potential conflict of interests, most limitations are related to external validity i.e. to what extent is the effect applicable to a population outside of the study (135).

The lipid profile of the women in the EVTET study (Table 3) was at baseline within reference range for individual primary prevention of CVD according to the Norwegian guidelines 2009, except for Lp (a) which was above the range (11). Although the average level of TC and LDL-C was within reference range, the levels were above recommended level for prevention of CVD (11).

Furthermore, based on risk calculations from the same guidelines, the women in the EVTET study that smoked had on average a 2 % 10 year risk of dying of CVD whereas the non-smokers had a 1 % 10 year risk of dying from CVD. The calculations are based on baseline parameters like gender, age, smoking/non-smoker status, TC and blood pressure. Baseline parameters for age, body mass index, smoking and more can be found in the EVTET article in the appendix. The 10 year risk range, spans from <1 % and up to 6 % for the age range of the women in EVTET.

To put the risk of the women in EVTET in perspective, women within the same age range as the women in EVTET with a value ≥5 %, are eligible for primary prevention of CVD with pharmaceuticals (11). In summary, the women in EVTET had on average a relatively low 10 year risk of dying from CVD.

A weakness of the study design in EVTET in relation to the aims of the present thesis is that the results are limited to patients with high risk of VTE. If the opportunity would have been available for us to design the RCT the way we wanted for the purpose of this study, we would not have selected women with high risk of VTE. Instead healthy postmenopausal women would have been selected or perhaps women with a stronger atherogenic profile, as our aims in this study are more related to the atherosclerotic process underlying athero thrombosis than the purely coagulative underlying VTE.

6.1.2 Detection of NAMPT concentration in plasma

We used the Bio-Plex suspension array technology assay to quantify the protein concentration of NAMPT in plasma of postmenopausal women at high risk of recurrent VTE, after HRT exposure. NAMPT was detected in a singleplex format. This method is based on ELISA and a common methodology in clinical routine laboratories. Maximum precision while working is required throughout the process. It is also important to optimize the standard curve before using it as a reference to calculate the results. In addition, the precision when loading the plasma samples is pivotal. In order to attain reliable results, immunoassays are dependent on the specificity of the antibodies.

Most immunoassays for detecting NAMPT do not discriminate between eNAMPT and iNAMPT. It is not known, whether the intracellular and extracellular form have the same conformation and this could be crucial for the correct quantification of NAMPT (99, 102). Reported levels of serum NAMPT are highly variable and can vary according to age, race and assay methodology (136, 137). Korner et al. (138) compared different immunoassays and found the ELISA method to be the most specific assay to reliably quantify NAMPT in human serum samples. The plasma levels of NAMPT found in this master thesis were similar to the levels Korner et al. found in his serum samples with the ELISA method (139, 140).

In order to estimate the precision of the immunoassays, an intra- and inter-assay assessment was done, as explained in section 4.1.1. Assays are considered precise when intra- and inter-

assay CV is below 20% (141). The intra-assay CV during NAMPT detection in plasma was 6 % and the inter-assay CV was 24 %, suggesting plate-to plate variability. One possibility as to why the inter-assay CV was so high may have been due to too few plates as there was only enough plasma for 4 plates.

Pre-analytical conditions like different storage and thawing cycles might also be potential causes of plate-to-plate variability. All the samples used in our study were subject to the same storage conditions, thus eliminating storage conditions as a source of the plate-to-plate variability. However, the plasma samples were badly marked in regards to their number of freeze thaw cycles when we received them from the bio bank at Oslo University hospital HF, Ullevål. Hence, there exist uncertainty the standardization of the the number of freeze thaw cycles (maximum two). Therefore freeze thaw effects might be a source for the observed plate-to-plate variability.

NAMPT is a clock gene, involved in the circadian timing system (142) . Circulating NAMPT concentrations follow a diurnal rhythm, peaking in the afternoon (143). The time of blood sampling may have varied between the women and between measurements. Consequently, this may have caused a potential bias in our results.

6.1.3 HepG2 cells as a model system

The use of *in vitro* model systems for studying physiological responses is widely used for understanding human physiology. One of the main advantages of *in vitro* model systems is that they make it possible to investigate signaling pathways and cellular responses to stimuli without affecting living subjects. Cell lines are widely used in screening research because they are affordable, easy to apply and standardize. However, the use of cell line experiments should be regarded as initial screening investigations and of being hypothesis generating. In order to verify that the measured effect *in vitro* also apply in primary cells *in vivo* the results must be repeated in other model systems.

In order to increase the functionality of cell lines and prolong their life span, genetic transformations are necessary. These genetic alteration, make them able to divide almost indefinitely in contrast to normal cells which are only able to divide a certain number of times before they lose their ability to proliferate (144). The genetically altered cells become what are termed a continuous cell line. Cells accumulate mutations with time and as normal cells

stop dividing after a certain amount of time the mutations are not passed on to the next generation. However, as immortal cells do not stop dividing, over time this may result in the development of cells phenotypically different from original cells. In order to avoid accumulation of mutations, it is advisable to use cells with as few passages as possible and to not subculture for very long periods of time. The HepG2 cells used in our experiments were only passaged for 3 months and with a maximum number of 28 sub divisions. Unaltered growth rate and phenotypic changes examined by macroscopic evaluations suggest that the cell were homogenous during the experimental period. Although cryopreservation and resuscitation protocols are supported (145), hepatocytes are extremely sensitive to damage after cryopreservation and need to be handled with the uttermost care (146).

The response of the cell screening system must have some resemblance to the intact organism in order for the experimental results to have any clinical relevance. Hepatocytes directly isolated from tissue and never sub cultured are termed primary hepatocytes. These are good for research as primary cell physiology is more similar to conditions *in vivo* and they have not undergone genetic modifications to the same extent as cell lines. Primary hepatocytes are difficult to attain and thus the continuous cell line HepG2 was used in our experiments. These cells are isolated from a well-differentiated hepatocellular carcinoma. Cell lines derived from cancer cells have a different metabolism and cell cycle regulation compared to cells normally occurring *in vivo*. In contrast to many other human hepatoma cell lines, no hepatitis B virus has been found integrated in the genome of the HepG2 cell line and neither have any viral antigens been found in the conditioned medium (147). In addition to its morphological appearance (148), the fact that the HepG2 cell line produces many liver specific proteins, including albumin, implies that the cell line is mature (149). The HepG2 cell line is generally considered a suitable *in vitro* model system for the study of polarized human hepatocytes and the role of the liver in metabolism (149).

6.1.4 Cell culture experiments

In the cellular experiments we aimed to study the effect of estradiol on gene expression and cell release in hepatocytes in relation to NAMPT, inflammation, lipoprotein and bile metabolism so as to better understand estrogens effect on the liver. The liver is central in relation to these targets of interest as it, among other metabolic functions, produces inflammatory proteins, converts cholesterol into bile acids, is the central regulator of lipoprotein metabolism (102, 150) and produces NAMPT (103, 151-153).

HepG2 cells, the selected human hepatocyte cell line, have previously been described as "a micro cosmos" for studying the role of the liver (149). This cell line seems to "exhibit most cellular features of normal hepatocytes" (148). Of interest in relation to the targets studied in this thesis, HepG2 cells produce apolipoproteins, lipoproteins and their receptors, cholesterol, TG and bile acids (149). In addition, the cells express LXRα (154) and the cytokines Il-6, TNF, MCP-1 and CXCL16 (155-159). NAMPT is produced and released by different hepatocyte cell lines, like Huh-7 cells (160) and HepG2 cells (130, 161).

According to previous research with HepG2 cells, this cell line seems appropriate regarding the targets studied in this experiment. However, in relation to the exposure agent estradiol, there exists an ongoing discussion regarding if HepG2 cells express the ER or not. Estradiol mediates its effect through either ERα, ERβ, membrane-bound sub- populations of ER-α and ER-β, G protein-coupled or a combination of these pathways (162-166). In HepG2 cells, estradiol have been found to mediate its effect through nuclear or extra nuclear pathways or both depending on the target of interest (167-173). In relation to the ongoing discussion about the ability of HepG2 cells to express ER, some report ERα and ERβ expression in HepG2 cells (167), others only ERβ expression (174) and others again, no ER expression (175). From the literature, there are reports about using what they call "ERα stabile HepG2 cells" (175) or HepG2 cells co-transfected with ERα (168, 176).

As mentioned in the method section 4.2.1, our HepG2 cells were isolated from a liver biopsy of a 15 year old Caucasian male (obtained from ATCC). There are reports that HepG2 cells are responsive to estradiol (13, 75), but even if there would be an adequate presence of ER in this cell line, we cannot rule out that the male origin of the HepG2 cells could influence the response, and probably by dampening it.

We found low expression of ERα and ERβ in our HepG2 cells relative to ERα and ERβ positive cells. Unfortunately, valuable information about the effect of the ERα inhibitor Fulvestrant was lost due to high cell toxicity with 100 nM EE2. Therefore, it is hard to tell if EE2 exhibited its effect through the ERα pathway or not. Nevertheless, regardless of the pathway EE2 mediated its effect through in our HepG2 cells; we received a weak effect on targets. The HepG2 cell line, unaltered in any case, may not have been the best model system to study the effect of estradiol and to answer the aim of the cellular experiment.

FBS may contain endogenous sex steroids (171) thus the HepG2 cells were stimulated with EE2 and research medium without FBS. Phenol red has been found to have estrogenic effects on breast cancer cells (100). Yet others have not found estrogenic effects of phenol red (171). However, to be on the safe side, phenol red was not used in our research medium.

Pretreatment of cells with estradiol can cause an estrogen memory effect which changes the subsequent response of the cells to estrogen (177). In addition, it resembles *in vivo* conditions. Hence, pretreatment of cells with estradiol *in vitro* may be important for the clinical relevance of the results. We did not pretreat the HepG2 cells with EE2 and this may be a potential weakness with our cell experiment.

We selected a 6 and a 24 hour time-point for cell harvest and a dose-response design with dosages ranging from 0.1 to 100 nM EE2 dosages based on previous experience with estradiol, HepG2 cells, mRNA expression and cell release, in the research group (125, 151). The right time-points and exposure dosages to ensure an effect of EE2 seem to be totally dependent on the target of interest (125, 167, 168, 171, 178, 179). However, the appropriate exposure dosage is not dependent on the dosage which mediates expression of the target of interest, but of which questions we want answered by the experiment e.g. investigating hepatic supraphysiological estradiol exposure to simulate the effect of oral HRT or investigating the effect of endogenous levels of estradiol or the effect of estradiol in general. We have results reflecting the effect of 0.1 to 10 nM EE2 on HepG2 cells which is close to the hepatic endogenous E2 range of 1 to10 nmol/L (112, 125, 180). However, EE2 is reported from previous studies to exert higher potency than its naturally occurring analogue E2 (125).

6.1.5 Analyses of gene expression

For reliable results from gene expression analysis, it is important to pay attention to the various pitfalls along the process. The RNA isolation process is prone to errors which require precision and accuracy throughout the process. In addition, it is important to avoid RNA degradation by RNases by taking precautions regarding keeping an RNase free environment. A limitation with the column based technology used in the experiments, is that it is easy to clog the column by overloading it. Thus it is important to load with a volume recommended by the manufacturer. To avoid DNA contamination of RNA an on –column DNase treatment was performed.

Quantification of RNA is a critical step for the end result as it is essential that the amount of RNA is equal in each sample during cDNA synthesis. In addition, nucleic acids can be contaminated with other molecules like proteins and other organic compounds which can create RNA with a low quality. Low quality RNA can cause unreliable results. An absorption ratio between 260 and 280 nm was calculated by the Nano-Drop system to assess the purity of the nucleic acids. A sample is generally considered pure when the ratio is 2.0. The expected ratio when using the RNA purification kits from 5 prime is 1.8 to 2.2. All the samples used in the experiments were within the expected range.

The qRT-PCR technique is a well-established method for quantification of gene expression. It is sensitive enough to detect the presence of a single copy of mRNA transcript in an experimental sample (123). The process is therefore prone to errors due to contamination. Only solutions and reagents that were tested to be free of contaminants that could interfere with the assay were used.

The well loading of RNA during cDNA synthesis is a critical step for the correct quantification of mRNA and very high accuracy is essential. Precautions were taken with a meticulous and standardized pipetting technique.

Two different technologies were used in this experiment to detect the amplified target sequence, the SYBR green dyes and the TaqMan probe system. The SYBR green dyes are used with specific primers for the target sequence just like the Taqman probe system, but amplification of primer-dimers, non-target sequences and DNA contamination might also be reported by the SYBR green dyes as they report all present double stranded DNA. The Taqman probe system is more specific in its reporting as it only reports target cDNA.

However, same as with the SYBR green dyes, it is also prone to unspecific primers with the consequence of amplification of non-target sequences. The specificity of the primers for the target sequence is thus of outmost importance. All primers used with the SYBR green technology were designed and tested by the research group before used in these experiment (126).

In order to detect amplification of potential contaminants, a dissociation curve can be run at the end of each PCR when using the SYBR green dyes. This is not possible with the Taqman probe system. The SYBR green primers are more affordable than the primers used for Taqman and the two different reporting technologies have been classified as equally reliable (181).

The comparative Ct or comparative threshold method was used to calculate the relative amount of target mRNA in each sample. The mRNA was calculated and the Ct values for each sample was normalized against the endogenous housekeeping gene, with the formula $2^{-\Delta\Delta C}$. A reference- or housekeeping gene is used to standardize the Ct value of the target gene to minimize possible internal errors, such as differences in RNA loading during cDNA synthesis (182).The internal reference gene is ideally not regulated under the experimental conditions and the use of an appropriate endogenous control is important when using the 2-ΔΔCt method. The Exponential phase of amplification is above any background noise within the assay and is the most accurate place to set the threshold and measure the amplicon.

The comparative Ct or comparative threshold method is based on several assumptions. One assumption is that the efficiency of the PCR reaction is close to 100 % and another that the PCR efficiency of the target gene is similar to that of the internal control gene (182). However, these assumptions are only valid if the threshold is set at the exponential phase of amplification and if the target and the reference gene have similar efficiency (183).

6.1.6 Detection of protein- and lipid secretion in cell supernatant

The commercially available Bio-Plex suspension array technology assay was used to quantify the protein level of NAMPT, TNF, MCP-1, IL-6, CXCL16, APOA1 and APOB100 in cell supernatants of EE2 exposed HepG2 cells. Quantification of secreted TG was determined enzymatically with the triglyceride Enzymatic PAP 150 kit from BioMerieux.

NAMPT was detected in a singleplex format as explained in section 6.1.2. All the other proteins were multiplexed. Multiplexing allows for the detection of several proteins at once. A great advantage of this method is that it makes it possible to use only small amounts of sample. In addition to the limitations of the singleplex method explained previously, there are some specific theoretical limitations concerning multiplexing e.g. anti-body cross-reactivity and intra-well interference (184). Inter- and intra-assay analysis was not done for these analyses. However, in contrast to the plasma samples from EVTET, there was more control with the samples from the start of the experiment to the end as the samples were our property for the whole testing period. A standardized confluence across the experiments and wells within each experiment is important in order to detect the correct cell release by exposure. Cellular death might cause a leakage of cell content into the cell supernatant and thus distort the results about cellular release. In order to avoid this potential pitfall we quantified the amount of cellular death with a LDH based cytotoxicity kit and rejected data with abnormal death relative to controls.

6.2 General discussion

In the work presented in this thesis, the effect of exogenous female sex steroids was studied. First we analyzed NAMPT in plasma from postmenopausal women. We found no significant effect on plasma NAMPT after three months of HRT treatment. Next, we studied the effect of HRT on lipid profile. We found both favorable and adverse effects on apolipoproteins and lipoproteins, but only favorable effects on lipoprotein ratios. Lastly, we studied the effect of EE2 in the hepatocyte cell line HepG2. We found a suppressive effect of EE2 on the cell release of NAMPT and no effect on NAMPT mRNA expression. We also studied cell release of apolipoproteins, TG and proinflammatory cytokines, but found no significant effect of EE2 on their secretion nor on the gene expression of the proinflammatory protein CXCL16. In terms of effect on hepatic lipid homeostasis, we found an inducing effect of EE2 on genes involved in reverse cholesterol transport, bile acid metabolism and *de novo* lipogenesis such as: ABCG8, ABCA1, CYP7A and FAS. No effect was found on the gene expression of LXRα, VLDLr, LDLr, ABCG5, ADRP and L-FABP. In the following section these results will be discussed in relation to the aims of thesis and other relevant literature.

6.2.1 Effect of hormone replacement therapy on plasma NAMPT

We found no significant effect of HRT on plasma NAMPT in postmenopausal women at high risk of recurrent VTE. This is new knowledge as the effect of female sex steroids on circulating NAMPT has, as far as we are aware, not been studied before. Actually, very little information about NAMPT and sex steroids exists in general. To our knowledge, the only previous research done on NAMPT and female sex steroids has been *in vitro*, investigating NAMPT gene expression in 3T3-L1 pre-adipocytes and adipocytes (185, 186). However, the previous research does not entail cell stimulation with the hormone combination (estrogen and progesterone) that was used in the HRT in our trial. Hence, there exist no data about the effect of the combination estrogen/progesterone on NAMPT that can shed light on the effect of HRT on NAMPT.

It is not yet determined which level of NAMPT that is abnormal (187). Hence, in the scenario that we had found a significant effect of HRT on plasma NAMPT, we would not have known if the plasma level had been clinically relevant or not. In our analysis, there was an approximately 50 % (not significant) higher plasma NAMPT post-treatment compared to pre-

treatment and although the difference was not significant, there was no increase in plasma NAMPT in the placebo group pre- and post-treatment. We cannot exclude the possibility that a larger study population would have been able to detect a significant difference between the intervention groups, as a larger sample would have yielded more statistical power to detect any differences. In addition, whether HRT has an effect on circulating NAMPT after longer exposure than three months, remains to be elucidated.

6.2.2 Effect of estradiol on NAMPT mRNA expression and cell release

As discussed previously and to the best of our knowledge, investigations on NAMPT and sex steroids are limited to the study of NAMPT gene expression in 3T3-L1 pre-adipocytes and adipocytes (185, 186). These studies are interesting as adipocytes are known to secrete NAMPT and be a source of circulating NAMPT (99, 101). Nevertheless, no one has, as far as we are aware, investigated cell release of NAMPT after stimulation with sex steroids. We are the first to investigate this and to study the effect of estrogen on NAMPT in hepatocytes.

We found no effect of EE2 on NAMPT gene expression in HepG2 cells which is in line with the findings of Zhou et al. and Maclaren et al. in 3T3-L1 adipocytes exposed to E2 (185, 186). However, we found significant suppression of NAMPT release in HepG2 cells exposed to EE2.

Garten et al. and Schuster et al., has previously found, although with other exposure agents than sex steroids, a constitutive and positively regulated secretion of NAMPT in HepG2 cells (130, 161). Nonetheless, we are the first to find a negative regulation of NAMPT secretion in HepG2 cells.

Uncertainty exists around if NAMPT is actually released by the cell or if it’s released into the supernatant as a result of cell lysis. Tanaka et al. suggest NAMPT to be released via an ER–Golgi or microvesicles independent pathway in 3T3-L1 adipocytes (188). In addition, actual cell release of NAMPT is reported in other cell types too (160, 188, 189), but the exact mechanism remains to be determined. In order to ensure that the cell release was not due to cell lysis, but to an actual release of NAMPT, a cytotoxicity test was performed and there was found no significant cytotoxicity related to the presented results on cellular release.

It was previously hypothesized that circulating NAMPT was reflected only by the amount of visceral adipose tissue (152). However, several studies report no relationship between circulating NAMPT and body mass index (138, 151, 190). Different tissues in addition to adipose tissue are now reported to contribute to circulating NAMPT (103, 153, 191). Recently, hepatocytes were also suggested as a potential source of circulating levels of NAMPT (130, 151). However, uncertainty exists around which tissues are the main contributor to the circulating levels. Interestingly, circulatory levels of NAMPT have been found to be down regulated concurrently with impaired liver function (151, 192, 193). This may indicate that the liver is one of the main contributors to circulatory levels of NAMPT and in which case a down regulation of NAMPT secretion by estradiol which was found in this thesis, would create reduced circulatory levels of NAMPT. However, the effect of estradiol on NAMPT secretion in other cells contributing to circulatory NAMPT has not been studied before. Therefore, how estradiol will affect the secretion in those cells and how that will affect circulatory levels of NAMPT is at present unknown.

6.2.3 Effect of oral hormone replacement therapy on lipid profile

Associations between oral HRT and lipid profile have been studied extensively before and HRT is known to have both favorable and adverse effects on lipid profile (23, 39, 66, 95-98). However, to the best of our knowledge, most studies have focused on lipoproteins and apolipoproteins in isolation and not considered the ratios between them. In this thesis, we looked at the effect of HRT on lipoproteins, apolipoproteins and their corresponding ratios in order to better evaluate the atherogenic lipid burden in serum.

We found HRT to decrease Lp (a), APOA1, APOB, TC, HDL-C, LDL-C after three months of treatment in postmenopausal women at high risk of VTE .These findings are supported by other studies where the same type of HRT that was used in EVTET have been used (39, 95-98). In addition, LDL-C is reduced by HRT treatment as is HDL-C when HRT is supplemented with an androgenic progestin (23, 39, 66, 95-98).

Some progestogens are associated with an estrogen opposing effect and suppress the estrogen induced increase in HDL-C and TG levels. The degree of androgenicity seems to determine the estrogen opposing effect of the progestogen (97, 98, 194). The progesterone used in EVTET was Norethisterone Acetate (NET-Ac) which is considered one of the most

androgenic progestogens. We found no effect of HRT on TG and decreased HDL-C levels which may be explained by the opposing effect of the androgenic NET-Ac (23, 97, 98, 194).

In addition to finding an opposing effect of HRT (with NET-Ac) on HDL-C and TG, Kwok et al. found NET-Ac to reduce lipoprotein (a) more effectively than less androgenic progestogens like Desogestrel, but less effective in opposing the estrogen related increase in CRP (23). In support of the findings by Kwok et al. (23) and Farish et al. (195), we also found a decrease in serum Lp (a) after HRT, and Eilertsen et al. found an increase in serum CRP after HRT in the women from our study (82).

Lp (a) is not modified by external factors like medication e.g. statin therapy and lifestyle changes e.g. diet and exercise, except for niacin administration. However, it is difficult to tolerate Niacin in the doses needed to decrease Lp (a) (27-30). It is thus particularly interesting that HRT decreased Lp (a) levels in our study. Though, this is not a new finding and supported by other studies (39, 66, 95-98).

We found a favorable effect of HRT on the TC/HDL-C and LDL-C/HDL-C ratio despite a decrease in HDL-C. The reason may be that the concomitant reduction in TC (probably due to decreased LDL-C levels as the TG level was not changed) and LDL-C was larger than the reduction in HDL-C.

On average and limited to the assessed surrogate markers of CHD risk, the plasma atherogenicity was reduced after 3 months of HRT. In addition, Lp (a) levels changed from being above reference range to be within reference range. Furthermore, when keeping blood pressure and age constant, the 10 year risk of dying from CVD changed from 2 % to 1 % for smokers and from 1 % to < 1% for non-smokers after 3 months of HRT (11). These changes were not seen in the placebo group.

6.2.4 Effect of estradiol on hepatic lipoprotein- and bile acid metabolism

We found EE2 to induce gene expression in our HepG2 cells of genes involved in the reverse cholesterol transport. The influence of sex steroids on the reverse cholesterol transport process is at present not completely elucidated (196). However, a less effective reverse cholesterol transport is associated with increased cardiovascular risk (16).

The affected genes by EE2 were ABCG8, ABCA1 and CYP7A. The transporter proteins ABCG5 and ABCG8 mediate cholesterol efflux from the liver into the bile canaliculi and CYP7A is the rate limiting enzyme involved in converting cholesterol and 27-hydroxycholesterol into bile acids (197). ABCA1 mediates lipid efflux from peripheral tissue to lipid-poor pre-β HDL which promotes the formation of nascent HDL, containing more lipids (198). Increased expression of ABCA1 which we found in our study, is associated with increased HDL-C levels (199). Unopposed estradiol is associated with increased HDL-C levels (23, 39, 66, 95-98). Taken together, it may be plausible that estradiol increase HDL-C by inducing ABCA1 expression. However, this needs further clarification in future studies to be determined.

The gene expression of one of the enzymes catalyzing *de novo* fatty acid synthesis, FAS, was also upregulated after EE2 treatment. LXR transcriptionally regulate *de novo* lipogenesis through FAS, bile acid synthesis via CYP7A, sterol efflux via ABCA1, ABCG5 and ABCG8, and glucose metabolism (200). However, LXRα was not significantly increased in our study. Neither was L-FABP, which is a fatty acids binding protein and may play a key role in fatty acid homeostasis (201).

EE2 did not affect the cell release of ApoB100 and APOA1. The dosage (0.1-10 nM EE2) and time-point (24 hours) of cell harvest may have been conclusive. Cell harvesting time-points between 24-48 hours have previously been found to induce the highest release of ApoA1 and ApoB100 in HepG2 cells (178). In terms of dosage, Tam et al. found a maximal increase in APOA1 secretion with 20 nM E2 and a maximal increase in APOB100 secretion with 500 nM E2 in HepG2 cells, both peaking at 48 hours. However, Tam et a. also found an increase in APOA1 secretion with 1-2 nM E2 in HepG2 cells, but only at 50 % of the level obtained with 20 nM E2 (177). As the physiological range may span from 1–10 nmol/L E2 (112), APOB100 may not be affected by E2.

In summary of the non-significant effects by EE2, we saw no effect on the secretion of APOA1 and APOB100, the gene expression of VLDLr, LDLr, ABCG5, ADRP, LXRα and L-FABP. As the HepG2 cells were exposed with EE2 doses within the physiological range we would at least expect an effect of EE2 on ABCG5 as it functions as a heterodimer with ABCG8 on the membrane of hepatocytes (197). Furthermore we would expect an effect on the LDLr as E2 has shown an effect on this receptor previously in HepG2 cells (202). Taken together, we are suspecting an underestimated effect of EE2 in our HepG2 cells and as discussed in section 6.1.4 and in this section, the presence of the ER receptors, the right exposure doses and the right time-points for cell harvest, are pivotal for the expression level of the targets of interest.

6.2.5 Effect of estradiol on hepatic inflammation

The inflammatory cytokine CXCL16 has been found up-regulated in chronic inflammatory liver diseases such as non-alcoholic fatty liver disease (NAFLD) (159, 203, 204). Eilertsen et al. found serum CRP to be positively affected by oral HRT in the EVTET study, but not IL-6 and TNF (82). The estradiol component of HRT is known to cause an increase in circulating CRP independent of the progesterone component (23, 205). The increase in serum CRP associated with estradiol is attributed to supraphysiological exposure by oral HRT on the liver (51, 82). Normally, CRP is induced by cytokines, predominantly cytokine IL-6 (206).

We did not measure CRP after EE2 treatment of the HepG2 cells, but other markers of hepatic inflammation such as IL-6, TNF, MCP-1 and CXCL16. In line with the findings of Eilertsen et al. on IL-6 and TNF, none of the studied proinflammatory cytokines was secreted at a high enough level to be detected in the cell supernatant after EE2 exposure for 24 hours and neither gene expression of CXCL16 after 6 hours EE2 exposure.

7 Conclusions

The findings from the experiments in the present thesis are presented in the following.

1. **3 months of oral HRT (2 mg E2 and 1 mg NET-Ac) in postmenopausal women at high risk of recurrent VTE exerted:**
    a. No significant effect on plasma NAMPT.
    b. A decrease in Lp (a), APOA1, APOB, TC, HDL-C, LDL-C, TC/HDL-C and LDL-C/HDL-C ratio and no significant effect on TG and APOB/APOA1 ratio.

In conclusion, our results indicate no effect of oral HRT on plasma NAMPT, but there is a possibility that we did not have a large enough sample size to detect a difference and this remains to be elucidated in future studies. HRT reduced the amount of both atherogenic and anti-atherogenic lipoproteins and apolipoproteins in serum, but had an overall favorable effect on lipid profile reflected in decreased lipoprotein ratios.

2. **Stimulation of hepatocytes (HepG2 cells) with 0.1-10 nM EE2 for 6 h. for gene expression and 24 h. for cell release exerted:**
    a. A suppressive effect on cell release of NAMPT
    b. An increased mRNA expression of genes involved in the reverse cholesterol transport, bile acid metabolism and *de novo* lipogenesis such as: ABCG8, ABCA1, CYP7A and FAS.
    c. No significant effect on cell release of A) TG, the apolipoproteins: APOB100 and APOA1. B) proinflammatory cytokines: MCP-1, IL-6, TNF and CXCL16.
    d. No significant effect on the gene expression of: NAMPT, CXCL16, LXRα, VLDLr, LDLr, ABCG5, ADRP and L-FABP.

We expected more response from the cellular experience and suspect a dampened effect of estradiol in our HepG2 cells. In summary, we found an effect on NAMPT cell release and an effect on some genes related to reverse cholesterol transport, bile acid metabolism and *de novo* lipogenesis. Further studies with a cell line that is certain to be estradiol responsive are necessary to further elucidate the effect of estradiol in hepatocytes.

8 Future perspectives

Analysis of plasma NAMPT in EVTET samples from follow-up time points beyond three months remains to be studied and may reveal an effect of HRT.

In order to further exploit the data collected, a new cell experiment may be set-up, optimized for comparison of effects with the human trial i.e. use of a guaranteed estradiol responsive cell line, assessment of CRP and exposure agents with a dose and ratio of E2 and NET-Ac equivalent to the HRT used in the human trial. In this present thesis, a comparison would not have been possible as EE2 was used in the cellular experiments, whereas E2 was used in the human trial. EE2 has different pharmacokinetics than E2 and is not degraded in the liver to the same degree as E2 (207). In addition, considering that we treated with estradiol in the cellular experiment, and estradiol and progesterone in the human trial, it would have been impossible to compare the effects in the two experiments. Regardless, the comparison of experiments will be limited and only hypothesis generating as cells *in vitro*, isolated from their normal context and influence may not deduce a similar clinical outcome as *in vivo*.

In addition to the possible transfer value of comparing experiments when using estradiol and progesterone in both experiments, the effect of this combination on NAMPT expression has never been investigated before. Zhou et al. found a positive synergic effect on NAMPT gene expression by a hormone concentration and ratio between estriol, estradiol and progesterone, which is normally only present during pregnancy (185). Whether, a combination of progesterone and estradiol will produce a similar synergic effect on NAMPT expression as Zhou et al. found, remains to be elucidated.

Correlation analyses on data from the EVTET study are other ways the data can be further exploited. NAMPT is recently suggested to have a role in thrombosis (208), a regulative role in TG metabolism (102, 209) and is associated with induction of proinflammatory cytokines such as TNF and IL-6 (137). Hence correlation analyses between NAMPT and coagulation factors, lipoproteins, apolipoproteins, lipoprotein ratios and inflammation markers may contribute with new information to the field. Independent of NAMPT, correlation analysis between inflammation markers, lipids and coagulation factors are also interesting and should be studied further.

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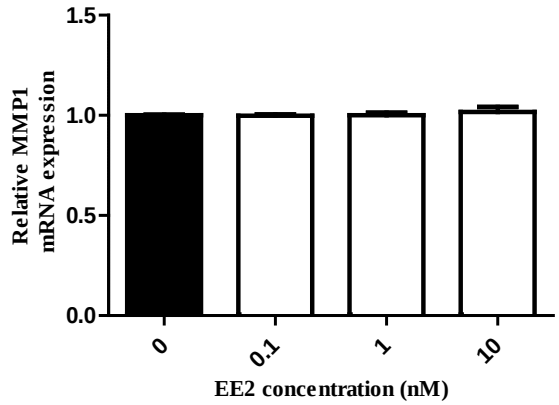
10 Appendix

Appendix 1. Intra and inter coefficients of variation

Assay	% CV	N
Intra	6.06	8
Inter	24.4	4

Appendix 2. Endogenous control gene after 6 hours exposure.



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Increased Risk of Recurrent Venous Thromboembolism during Hormone Replacement Therapy

Results of the Randomized, Double-blind, Placebo-controlled Estrogen in Venous Thromboembolism Trial (EVTET)

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Key words

Clinical trial, venous thromboembolism, hormone replacement therapy, estrogen

Summary

Recent observational studies suggest a 2-4 fold increased risk of venous thromboembolism (VTE) in women taking hormone replacement therapy (HRT). The present study was started before publication of these studies, and the aim was to determine if HRT alters the risk of VTE in high risk women. The study was a randomized, double-blind, and placebo-controlled clinical trial with a double-triangular sequential design. Females with previously verified VTE were randomized to 2 mg estradiol plus 1 mg norethisterone acetate, 1 tablet daily (n = 71) or placebo (n = 69). The primary outcome was recurrent deep venous thrombosis (DVT) or pulmonary embolism (PE). Between 1996 and 1998 a total of 140 women were included. The study was terminated prematurely based on the results of circumstantial evidence emerging during the trial. Eight women in the HRT group and one woman in the placebo group developed VTE. The incidence of VTE was 10.7% in the HRT group and 2.3% in the placebo group. In the HRT group, all events happened within 261 days after inclusion. The sequential design did not stop the study, but strongly indicated a difference between the two groups. Our data strongly suggests that women who have previously suffered a VTE have an increased risk of recurrence on HRT. This treatment should therefore be avoided in this patient group if possible. The results also support those of recent epidemiological studies, which also indicate increased risk of VTE in non-selected female populations during HRT.

Introduction

An increasing number of women are eligible for hormone replacement therapy (HRT), but the evaluation of the benefits and hazards of HRT still needs further investigation. The relief of climacteric symptoms, which improves quality of life, is recognized (1), and also the prevention of osteoporosis (2-4). HRT may on the other hand adversely increase the risk of breast and endometrial cancers (5, 6).

Numerous epidemiological studies strongly suggest that HRT may reduce the risk of arterial vascular thrombosis (7). However, the first randomized trial, the Heart and Estrogen/progestin Replacement Study (HERS), did not confirm a reduction in the overall rate of coronary heart disease events in women with established coronary artery disease (8).

The evidence on the effect of HRT on the risk of venous thromboembolism (VTE) is contradictory. Early epidemiological studies failed to show an increased risk of VTE among users (9-13), but recent studies suggest a 2-4 fold increased risk for current users (14-19). Five recent review articles have re-evaluated the risk associated with HRT use and the authors unanimously conclude that an association may exist, but that further investigations are required and clinical trials warranted (20-24).

The present randomized clinical trial was initiated to test whether estradiol treatment influences the risk of VTE. We chose to study individuals at high risk, since the much higher incidence of VTE in these individuals might help to detect a clinically relevant effect with a much smaller sample size than would have been required for low-risk females. The study was terminated prematurely as several novel epidemiological studies (14-19) and one randomized study published during execution of the study indicated increased risk of VTE (8).

Participants, Materials and Methods

Study Population

Participants were postmenopausal women younger than 70 years who had suffered previous DVT or PE. Previous VTE was verified by objective means, i.e., venography or ultrasound in cases of DVT, and lung-scan, helical computed tomography, or angiography in cases of PE. Women (n = 28) were also accepted for the study without objective testing if they had a typical history and had subsequently been treated for VTE. Postmenopausal was defined as no natural menstruation for at least one year.

Women were excluded for the following reasons: current use or use of anti-coagulants within the last three months; familial antithrombin deficiency; any type of malignant diseases including known, suspected or past history of carcinoma of the breast; acute or chronic liver disease or history of liver disease in which liver function tests had failed to return to normal; porphyria, known drug abuse or alcoholism; life expectancy less than two years; or women who had taken part in other clinical trials within 12 weeks before study entry.

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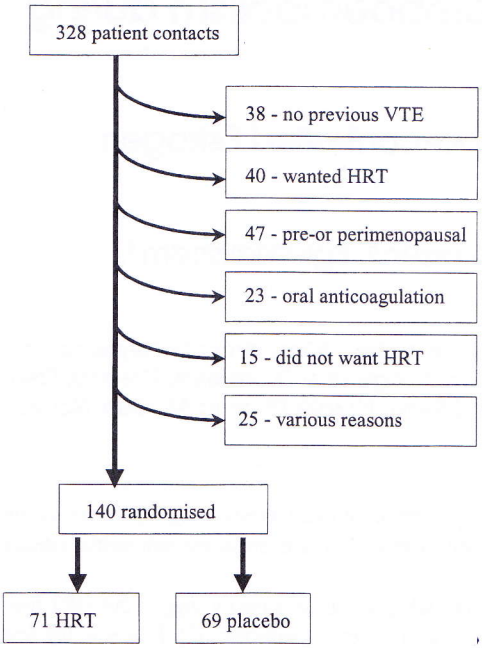

Fig. 1 Flow-chart showing the number of patient contacts and reasons for not being randomized in the study

Recruitment to the study was promoted by letters to family doctors, gynecologists, and hospitals. An invitation to participate was also made through health bulletins and media. The trial profile and reasons for exclusions are shown in Fig. 1.

The study protocol was approved by the Regional Ethical Committee and by the Norwegian Medicines Control Authority. Written, informed consent was obtained from all women. The study was carried out in accordance with the Helsinki Declaration and Good Clinical Practice.

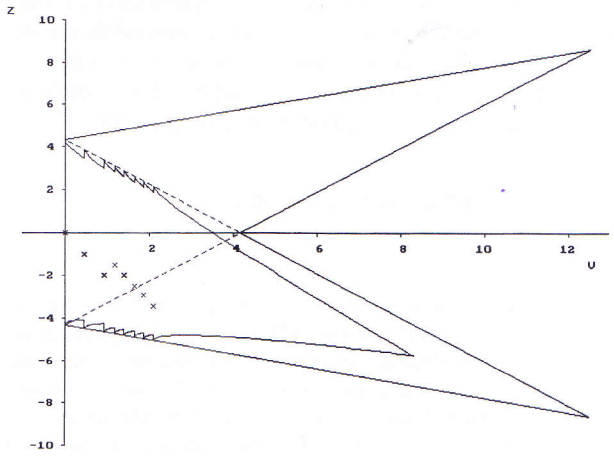

Fig. 2 The sequential design and development of recurrent venous thromboembolism (VTE). The effect of treatment *(z)* was calculated for every 10th patient reaching 3 months of follow-up. For each inspection Christmas tree correction was made, as indicated in the lower triangle. The *v* value is proportional to the sample path. The double triangles indicate the stop boundaries. A *z* value exceeding the upper or lower continuous lines of the triangles indicates a significant result in favor of one of the two treatment groups. The diagram is generated from the Pest 2.1 software (25)

Procedures

At the inclusion visit, data were collected on demographic characteristics, reproductive and health history, risk factors for VTE, and medication use. Participants had a clinical examination, including breast and pelvic examinations with cytological smear test and evaluation of the endometrium with transvaginal ultrasound. A screening mammogram was also performed.

Routine hematological and clinical chemistry screening including blood lipids were performed at baseline and at each follow-up visit. A baseline thrombophilia assessment, i.e., screening for antithrombin, protein C, and protein S deficiencies, activated protein C resistance, lupus anticoagulant and anti-cardiolipin antibodies, and the factor V Leiden mutation and the prothrombin gene 20210 GA allele variation were made.

All women were given detailed instructions on symptoms and signs of DVT and PE, and advised to contact their own physician, local hospital, the investigator, or a 24-h operated telephone number immediately if symptoms occurred.

Scheduled follow-up visits occurred after 3 and 12 months, and an end-of-study visit after 24 months. Women who withdrew their consent to participate were followed until the time of consent withdrawal. Every visit included general physical examination and venipuncture. The end-of study visit also included pelvic examination with cytological smear test and evaluation of the endometrium with transvaginal ultrasound and a screening mammogram. At 6 and 18 months, the women were followed up by postal questionnaires. Adverse events reported by the patient spontaneously, given in response to direct questioning, or observed on clinical examination were evaluated by the investigator.

Assignment

The study was carried out as a randomized, double-blind, and placebo-controlled study combined with a stratified double-triangular sequential design (Fig. 2) (25). The study was stratified for age (first stratum: <60 years of age, second stratum: >60 years of age), as age was considered the most important risk factor for VTE.

Within each of these strata, half the women were allocated to treatment with HRT containing 2 mg estradiol plus 1 mg norethisterone acetate 1 mg (Kliogest®, Novo Nordisk, Gentofte, Denmark) and the other half to equal-looking placebo tablets. Women were allocated to treatment by computer generated 1:1 block randomization with fixed block sizes of 10 women. To avoid early drop-outs due to the known adverse effect of breast tenderness, the dose regimen was one tablet every other day for the first two weeks, then one tablet daily. Every visit provided study medication refill and assessment of drug accountability.

Outcomes

The major outcome parameter was VTE verified by objective tests, i.e., venography or ultrasound in the case of DVT, and lung-scan, angiography, or helical computed tomography, in the case of PE. All primary end-points were independently and blindly examined by a radiologist and/or an internist/hematologist at the patient's local hospital. At the end of the trial all information on end-points including original venograms, CT-scans, and lung scans were independently and blindly evaluated by a radiologist, a specialist of nuclear medicine, and a hematologist not involved in the study. Secondary outcome parameters were acute myocardial infarction, transient ischaemic attacks, or stroke.

Statistical Analysis

For the safety of the women a double triangular sequential design was chosen to allow surveillance of the major end-points throughout the study (25). The expected two-year incidence of VTE was 7.5% in the placebo group (26-28). In case of an excess risk of HRT, we expected most VTEs to occur early. In analogy to the 3-4 fold increased risk of VTE associated with oral contraceptives (29), a three-fold change in the HRT group was assumed

Table 1 Baseline characteristics by treatment groups

	HRT (n=71)		Placebo (n=69)	
	no. or mean (SD)			
Previous/concomitant disease				
Myocardial infarction	0		1	
Angina pectoris	2		2	
Thromboembolic stroke	0		2	
Transient ischemic attack	2		2	
Hypertension	14		10	
Diabetes	3		0	
Smoking habits				
Never	25		29	
Previous	31		19	
1-10 cigarettes daily	9		11	
>10 cigarettes daily	6		9	
Baseline parameters				
Age , years	55.8	(7.0)	55.7	(5.9)
Body mass index (kg/m^2)	26.8	(4.3)	27.4	(4.0)
Weight (kg)	74.6	(12.8)	76.6	(11.4)
Total cholesterol (mmol/l)	6.5	(1.0)	6.6	(1.2)
HDL cholesterol (mmol/l)	1.7	(0.5)	1.6	(0.4)
LDL cholesterol (mmol/l)	4.2	(1.0)	4.3	(1.1)
Triglycerides (mmol/l)	1.4	(0.6)	1.7	(1.7)
Systolic blood pressure	137	(17)	139	(19)
Diastolic blood pressure	83	(10)	83	(8)

clinically relevant. At a significance level of 5% and a power of 90% the sample size was estimated to a maximum of 240 women (25).

In accordance with the trial plan, a sequential analysis was carried out for every 10th patient completing three months of treatment. For each sequential investigation, Christmas tree corrections of the two boundaries (Fig. 2) were carried out (25). All tests used were carried out two-tailed with a significance level of 5%. Continuously distributed factors and variables were presented by mean values with standard deviations (SD) in brackets. In case of extreme skewness, median with total range was used. Categorized factors and variables were presented in contingency tables. In order to visualize the thrombotic events as a function of time, a Kaplan-Meier plot was used. Comparison of the groups with regard to continuously distributed variables or factors were carried out using analysis of variance. The primary variable was analyzed by simple Binomial sequences and the other categorized factors and variables by contingency table analysis. Version 2.1 of PEST (Planning and Evaluation of Sequential Trials) was used in designing, and monitoring the study. The statistical package SAS(r) version 6.12 and PEST were used to perform the statistical analysis (25, 30-32).

Trial Termination

The first patient was randomized in February 1996. Novel epidemiological studies published during execution of our study (14-19) clearly indicated that HRT might increase the risk of VTE. After publication of the results of the randomized HERS study, which showed as a secondary end-point an increased risk of VTE (8), recruitment of women was discontinued in September 1998, until reviewed by the safety-monitoring committee. The committee was also concerned about a non-significant clustering of end-points in one study group, but without knowing treatment allocation (Fig. 2). The committee advised on premature termination of the study even though formal boundaries showing an excess risk of VTE were not reached. The final decision on termination of the study was made in February 1999, and by the end of March 1999, all the participants had completed a final follow-up visit.

Results

Previous Diseases and Baseline Parameters

Altogether 140 women were enrolled in the study: 71 were allocated to receive HRT and 69 to receive placebo (Fig. 1). Participants ranged in age from 42 to 69 years, with a mean of 55.8 years at baseline. Ninety-eight women were below 60 years old (first stratum), while 42 women were above 60 years old (second stratum). Distribution of demographics including age, body mass index, smoking habits, previous and concomitant illnesses, serum lipid levels, and blood pressure showed no significant differences between the two treatment groups (Table 1).

Before inclusion in the study all women had experienced at least one previous event of VTE. Type of previous VTE(s) and time elapsed since last VTE (Table 2) and risk factors for VTE (Table 3) were similar for HRT and placebo allocated women. A positive screening test for thrombophilia was detected in 28% (20/71) of the HRT women and 22% (15/69) in the placebo group (Table 4). Heterozygous factor V Leiden mutation was the most frequent finding, but no woman had antithrombin deficiency (exclusion criterium), protein C- or protein S deficiency, or lupus anticoagulant.

Adverse Events and Drop-Outs

The HRT group reported 137 adverse events while only 71 adverse events were reported in the placebo group. There was a significantly higher percentage of HRT women experiencing an adverse event from

Table 2 History of venous thromboembolism (VTE) by treatment groups

History of VTE	HRT (n=71)		Placebo (n=69)	
	no. (%) or median (range)			
Family history of VTE	25	(35)	18	(26)
Total number of previous DVT	52		49	
Total number of previous PE	28		29	
Coexisting VTE/PE	3		3	
Previous >1 VTE	6		6	
Years since last DVT	3	(0-32)	5	(0-37)
Years since last PE	4	(1-34)	6	(0-28)

Table 3 Risk factors for first venous thromboembolism by treatment group

Risk factor	HRT		Placebo	
	no. (%)			
Spontaneous	41	(58)	32	(46)
Pregnancy/delivery	6	(8)	11	(16)
Immobilisation/infection	1	(1)	8	(12)
Oral contraception	3	(4)	4	(6)
Surgery	20	(28)	14	(20)
Total	**71**	**(100)**	**69**	**(100)**

Table 4 Baseline thrombophilic states by treatment groups*

Thrombophilia	HRT	Placebo	p
	no. (%)		
Factor V Leiden mutation - homozygous	2 (3)	1 (1)	ns
Factor V Leiden mutation - heterozygous	13 (18)	10 (14)	ns
Prothrombin gene 20211 GA mutation	1 (1)	1 (1)	ns
Anti-cardiolipin antibodies	4 (6)	3 (4)	ns
Total	**20 (28)**	**15 (22)**	ns

*None of the patients had antithrombin, protein C, or protein S deficiencies, or lupus anticoagulant.

first (baseline) visit to second visit ($p < 0.001$) due to vaginal bleeding or breast tenderness, but this difference was not found at later visits. There was no statistically significant difference in the percentage of women with serious adverse events between groups. Excluding the women reaching end-points, a total of 16 women (7 in the HRT and 9 in the placebo group) were examined by venography or a lung-scan because of possible symptoms of VTE. The results of these examinations were normal.

Sixty-one women attended all visits per protocol. Thirty-seven (23 HRT and 14 placebo allocated women) did not attend all visits due to premature termination of the study. Nine women discontinued due to recurrent VTE. Thirty-three women withdrew consent (drop-outs), i.e., 10 women in the HRT group and 23 women in the placebo group. Thirteen of these (2 HRT and 11 placebo allocated women) left the study because they wanted to be certain of being treated with estrogen for their postmenopausal symptoms. In the placebo group, other reasons for withdrawal of consent were hot flushes (n = 5), anxiety (n = 3), chest pain (n = 1), and lack of compliance (n = 1). In the HRT group, the reasons were vaginal bleeds (n = 4), anxiety (n = 1), hypertension (n = 1), hematuria (n = 1), and acne (n = 1).

Primary and Secondary Outcomes

Mean duration of follow-up in the study was 485 days and 483 days in HRT and placebo allocated women, respectively. A total of eight women in the HRT group suffered recurrent VTE (Table 5). Three of these women had their DVT verified by venography, and one by ultrasound scanning. Three women suffered PE verified by lung scan (n = 2) or spiral computed tomography (n = 1). One patient suffered cerebral sinus vein thrombosis verified by magnetic resonance imaging. Only one primary end-point, a PE verified by a lung-scan, occurred in the placebo group. The incidence rates per 100 patient years were 8.5 (95% CI 2.6-14.4) in HRT allocated women and 1.1 (0-3.2) in the placebo group.

After 13 sequential analyses, the study was prematurely terminated without the stopping criteria being reached (Fig. 2). The incidence of VTE was found to be 10.7% in the HRT group and 2.3% in the placebo group. In the sequential analysis, this difference did not reach the level of significance, but is statistically significant ($p = 0.04$) if the sequential design is ignored. In spite of not reaching the stopping boundaries, the results are strongly indicative of significant inferiority of HRT with regard to the incidence of DVT and PE (25).

An early excess risk of VTE associated with HRT is evident from the Kaplan-Meier plot (Fig. 3). In the HRT group, all 8 primary end-points occurred within 261 days of treatment. In contrast, the only primary end-point of the placebo group occurred after 413 days on placebo.

Five of the women reaching a primary end-point, all in the HRT group, tested positive for thrombophilia (Table 5). Thrombophilia was a significant risk factor ($p = 0.04$) for recurrence on HRT with a relative risk (RR) of 2.6 (95% CI 1.3-5.4) as compared with no thrombophilia. Heterozygous factor V Leiden mutation was associated with a non-significant excess risk for recurrence on HRT (RR 1.4, 95% CI 0.4-5.3, as compared with no factor V Leiden). Seven of the 9 women with recurrent VTE had previously suffered spontaneous thrombosis, but all the recurrences occurred without precipitating risk factors. Previous spontaneous thrombosis was associated with a non-significant increased risk of recurrence on HRT (RR 1.4, 95% CI

Table 5 Characteristics of patients with recurrent venous thromboembolism

Type of VTE	Age (years)	Allocation	Time since last VTE (years)	Time to recurrent VTE (days)	Thrombophilia	Transient risk factor	
						First VTE	Recurrent VTE
Deep venous thrombosis	56	HRT	5	56	Factor V Leiden mutation – heterozygous	None	None
Pulmonary embolism	69	HRT	1	64	Factor V Leiden mutation – homozygous	None	None
Deep venous thrombosis	60	HRT	1	82	Factor V Leiden mutation – heterozygous	None	None
Pulmonary embolism	51	HRT	20	102	None detected	Surgery	None
Deep venous thrombosis	64	HRT	1	116	None detected	Surgery	None
Cerebral sinus vein thrombosis	54	HRT	2	170	Anti-cardiolipin antibodies	None	None
Pulmonary embolism	47	HRT	3	220	Anti-cardiolipin antibodies	None	None
Deep venous thrombosis	64	HRT	2	261	None detected	None	None
Pulmonary embolism	40	Placebo	3	413	None detected	None	None

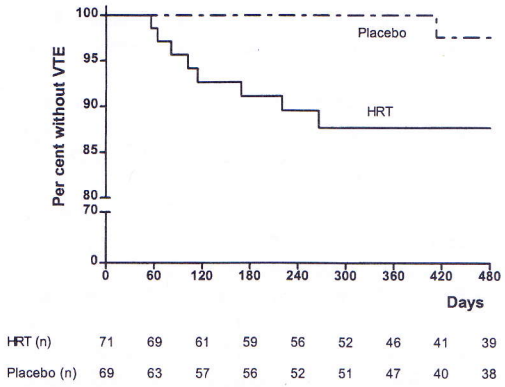

Fig. 3 Kaplan Meier plot indicating the proportion of patients without recurrent venous thromboembolism (VTE) as a function of time from randomization by treatment group, i.e., HRT (continuous line) and placebo

0.9-2.1 as compared with non-spontaneous previous thrombosis). All end points occurred within 5 years of prior VTE, except for one patient who had suffered DVT 20 years earlier (Table 5). The mean age of the women with end-points was 57.1 years compared with the mean age for the rest of the study population being 55.7 years (ns).

Only one patient in the study, allocated to placebo, experienced a secondary end-point. This patient had not experienced acute symptoms, but a cerebral computed tomography scan showed a small cerebral infarction.

Discussion

In this clinical trial, postmenopausal women younger than 70 years of age with prior VTE receiving continuous HRT had an increased risk of recurrent VTE. This is the first-ever randomized trial on the effect of HRT with VTE as a primary end-point. Our results are only valid for women with previous VTE, but they support the evidence of an early excess risk of VTE associated with use of HRT detected in recent epidemiological studies on healthy women (14-19) and with the excess incidence of VTE events observed in the randomized HERS-study (8).

The estimated 2-4 fold relative increased risk for VTE among HRT users is of the same magnitude as the risk associated with oral contraceptive use. Since the baseline incidence of VTE is much higher in postmenopausal women than in women of reproductive age, HRT may lead to a considerably higher number of women developing VTE (absolute risk) than does oral contraceptive use. In populations with high use of HRT, its impact on the overall frequency of VTE could therefore be substantial.

Our study is too small to carry out subgroup analysis, but it is known that two clinical factors appear to be important for the risk of recurrent thrombosis: presence or absence of transient risk factors for VTE (26, 28, 33-35) and the time elapsed since VTE. Our treatment groups were similar with regard to risk factors for VTE. Neither did time from previous VTE to inclusion in the study show statistically significant difference between groups.

The rate of identified thrombophilia was low. Only 35 (25%) had hereditary or acquired thrombophilia, which is lower than that reported in recent studies (27, 36). The reason for this may be due to patient selection. Women with recognized deficiencies or defects may have hesitated to take part in the study. Our study population may therefore represent a group at lower risk than an unselected population with previous VTE. Protein C and protein S deficiencies were not detected, but heterozygous factor V Leiden mutation was identified in 23 (16.5%) of the women. Only 4 of these women had already been identified prior to study entry.

Thrombophilia was associated with an excess risk of recurrent thrombosis, but thrombophilia can not completely explain the increased risk for recurrence in our study. Heterozygous factor V Leiden mutation was only a weak risk factor for recurrence. Three women with homozygous factor V Leiden mutation were included, and two of these were allocated HRT. One of the latter women, a 69-year old woman with severe osteoporosis, developed a PE 64 days after inclusion. Her only previous VTE was a spontaneous DVT 9 months prior to inclusion. The other patient was a 49-year old woman who had had a spontaneous DVT 10 years prior to the study. She completed the two-year period on HRT without adverse events. Homozygosity for the Leiden mutation has been reported to be associated with a 50-100 fold excess risk for VTE (37).

It is probable that estrogen acts in some women as an additional risk factor to generate a hypercoagulable state. In some individuals the pre-existing risk may be high and HRT may act as a trigger of thrombosis at an early stage of treatment. This hypothesis is supported by the early recurrences on estrogen as contrasted to the late recurrence on placebo in our study.

Although the compiled literature prior to 1996 did not give evidence for an increase in the risk of VTE on HRT (9, 10), we carefully considered the ethics of performing a randomized study on high-risk women. Firstly, our experience was that many physicians regularly prescribed estrogens in women with previous thrombosis. This is underlined by the fact that one of the major problems recruiting women to the study was that many women with previous VTE were already established on HRT, or they did not want to enter the study in fear of being allocated placebo (Figure 1). Secondly, it was emphasized that participants were well informed regarding symptoms and signs of VTE and encouraged to contact the investigator at any time during the study. Finally, the statistical model gave an opportunity to assess differences between groups throughout the study.

In conclusion, our study provides evidence, which strongly supports that initiating HRT in women with previous VTE most probably increases the risk of recurrent VTE. The incidence of recurrence was approximately 11% on HRT as compared to 2% on placebo. The increased risk is obviously clinically relevant, and prescribing HRT in such women should be avoided in most cases or only be given with great care. However, the net balance of risk and benefit of HRT use must also consider the potential beneficial health effects of relieving climacteric symptoms (1), reducing the risk of osteoporosis (2, 3), and possibly the risk of coronary heart disease (7). In women with no risk factors for VTE, the excess risk for VTE associated with HRT use would appear to be small as compared with the potentially stronger beneficial effects. Even in women with previous VTE or strong risk factors for VTE, the balance of risk and benefits might still favor the use of HRT in some cases. In such individuals, use of HRT in combination with oral anticoagulant treatment could be another safe approach, but this hypothesis needs confirmation in clinical trials.

Committees

Steering Committee: Dr. Per Morten Sandset (chairman), professor Harald Arnesen, professor Stig Larsen, professor Erik Qvigstad, and Dr. Egil Wickstrøm.

Safety-Monitoring Committee: Professor Ulrich Abildgaard (chairman, Aker Hospital, Department of Medicine, Oslo), professor Britt-Ingjerd Nesheim (Ullevål Hospital, Department of Gynecology), and professor Steinar Tretlie (Cancer Registry, Oslo).

End-Point Adjudication: Professor Nils-Einar Kløw (Ullevål Hospital, Department of Interventional Radiology), Dr. Carl Müller (Ullevål Hospital, Department of Nuclear Medicine), and Dr. Bernt Ly (Aker Hospital, Department of Medicine).

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