# Chapter 5

## Short-term ethinylestradiol treatment suppresses inferior caval vein thrombosis in obese mice

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## Summary

*Background:* Obesity and oral estrogens are independent risk factors for venous thrombosis, and their combined effect is stronger than the sum of the isolated factors.

*Objective:* Investigate the interaction between obesity and estrogens at the level of coagulation, inflammation and the venous thrombotic tendency in a mouse model.

*Methods:* Female C57Bl/6J mice were fed a standard fat diet (SFD) or a high fat diet (HFD) to induce nutritional obesity. After 14 weeks, while maintaining their diet, mice were orally treated 8 days with 1 µg ethinylestradiol or vehicle (n=25 per group), and subsequently subjected to an inferior caval vein (ICV) thrombosis model.

*Results:* The ICV thrombosis model resulted in an increased thrombus weight in vehicle-treated HFD mice (3.0±0.7 mg) compared to vehicle-treated SFD mice (1.4±0.4 mg; p=0.064). Surprisingly, estrogens reduced the thrombus weight, which was significant for the HFD group (0.8±0.5 mg; p=0.013). As compared to SFD feeding, HFD feeding significantly increased plasma levels of coagulation factor VIII, combined factor II/VII/X (p<0.001), and plasminogen activator inhibitor-1 (p=0.009), causing a prothrombotic shift of the coagulation profile. Estrogens had no significant effects on this profile with either diet, whereas serum amyloid A and hepatic inflammatory cytokines were minimally affected.

*Conclusions:* The synergistic effect of obesity and estrogens on the venous thrombotic risk in women could not be translated into the mouse context. Short-term ethinylestradiol administration in a mouse ICV thrombosis model counteracts the prothrombotic phenotype associated with nutritionally-induced obesity, despite a comparable activated plasma coagulation profile in estrogen-treated and untreated obese mice.

## Introduction

Obesity and the use of oral estrogens, as in contraceptive use and hormone replacement therapy, are both associated with an enhanced activity of the coagulation system and an increased risk of venous thrombosis.[1-6] Interestingly, large case-control studies have shown that the combined effect of obesity and contraceptive use is higher than the sum of the separate factors, at least for the use of oral contraceptives, but not for transdermal patches.[4,7,8] This latter observation suggests that the first-pass effect of the liver, the main site for the production of coagulation factors, is important for the interaction of obesity and estrogens.

Besides the joint effects of obesity and estrogens on the coagulation profile, they both affect fibrinolysis, the lipid profile and inflammatory state, although these effects are predominantly opposite. For example, whereas estrogens have beneficial effects on the lipid profile, improve fibrinolysis and have anti-inflammatory actions, obesity is associated with lipid disorders, reduced fibrinolysis and it is considered to be a chronic, low-grade inflammatory state which may contribute to the venous thrombotic risk.[2,9-12]

Animal studies, including ferric chloride injury of the mouse femoral artery, have demonstrated that obesity enhances the arterial thrombotic tendency.[13-16] However, the effects of obesity or oral estrogens on the venous thrombogenicity are less well characterized. In the present study, we have evaluated the effects of nutritionally induced obesity and short-term oral estrogen administration, alone and in combination, on venous thrombosis using a murine inferior caval vein thrombosis model. To study their effects on hemostasis regulation, we analysed the plasma coagulation profile and the hepatic mRNA levels of coagulation factors and of inflammatory cytokines.

## Materials and methods

*Dose finding*

Female C57Bl/6J mice (Charles River, Maastricht, The Netherlands) were fed a standard chow diet (Tecnilab-BMI, Someren, The Netherlands) and at eight weeks of age, they were bilaterally ovariectomized under isoflurane anesthesia. After a two-week recovery period, mice were treated with increasing doses of ethinylestradiol (EE; Sigma Aldrich Chemie, Steinheim, Germany), administered daily at a fixed time-point as 100 µl arachid oil containing 0, 0.03, 0.1, 0.3, 1, or 3 µg EE by oral gavage (n=6 per group). In a separate experiment, ovariectomized mice were treated orally with 1 µg EE per day, in the presence or absence of 100

µg non-selective estrogen receptor antagonist (ICI 182780; Sigma Aldrich Chemie, Steinheim, Germany) which was subcutaneously injected. For both experiments, mice were treated for 10 days after which a blood sample on acid citrate was drawn and plasma was obtained by centrifugation and stored at -80°C.[17] Subsequently, mice were sacrificed by cervical dislocation and the liver was isolated and snap frozen in liquid nitrogen for RNA analyses. The uterus was collected and weighed to monitor the biological activity of the EE.

*Nutritionally-induced obesity and estrogen treatment*

Five-week-old female C57Bl/6J mice (generated at the K.U. Leuven animal facilities) were fed a standard fat diet (SFD; KM-04-k12, Muracon, Carfil, Oud-Turnhout, Belgium, containing 13% kcal as fat with a caloric value of 10.9 kJ per g) or a high fat diet (HFD; TD88137, Zeist, The Netherlands, containing 42% kcal as fat with a caloric value of 20.1 kJ per g) to induce nutritional obesity (n=50 mice per group). After 14 weeks, while maintaining their diet, they were treated by oral gavage for eight days with 1 µg EE per day or a vehicle (n=25 per group). Subsequently, mice were subjected to an inferior caval vein thrombosis model as described by Singh et al.[18] In brief, under isoflurane anesthesia, a midline laparotomy incision was made and the inferior caval vein (ICV) was exposed. After applying a neurosurgical vascular clip to the dissected ICV, the ICV was ligated upon deposition of a Prolene thread.[19] By removing the Prolene thread after ligation, slow flow was restituted, triggering platelet activation, stasis and thrombus formation. The abdomen was closed, and 24 hours later the ICV was harvested and the thrombus was isolated and weighed after overnight drying. In each group, the ICV from 2-3 animals subjected to the venous thrombosis model were formalin-fixed, paraffin-embedded and stained with hematoxylin/eosin for histological analyses. Furthermore, the uterus and liver were isolated and weighed, as well as the epididymal (gonadal) and inguinal (subcutaneous) fat pads. Before the start of oral EE/vehicle treatment and before the thrombosis experiment, a blood sample on acid citrate was taken from the retro-orbital sinus after overnight fasting and plasma was stored at -80°C. All animals were housed in micro-isolation cages under a 12 hour day/night cycle, and had free access to drinking water and (experimental) diet. All experimental procedures were approved by local ethical committees.

*Plasma assays*

Alkaline phosphatases (ALP), aspartate transaminase (AST) and alanine aminotransferase (ALT), as well as the total, HDL and LDL cholesterol and triglyceride levels were determined using routine clinical assays. Antithrombin antigen was measured by one-dimensional electrophoresis using an anti-human antithrombin polyclonal antibody.[15] Plasminogen activator inhibitor-1 (PAI-1) antigen levels were measured with a specific ELISA[20] and fibrinogen antigen levels were assessed using an ELISA with an anti-human fibrinogen polyclonal antibody.

Coagulation factor V, factor VIII and combined factor II/VII/X plasma activity levels were measured as described by Nagai et al.[15] Antithrombin activity levels and thrombin-antithrombin (TAT) complexes were determined with commercial kits (Coamatic Antithrombin kit, Chromogenix, Mölndal, Sweden and Enzygnost TAT micro, Dade Behring, Marburg GmbH, Marburg, Germany, respectively).

6-keto Prostaglandin $F1_{\alpha}$ (6-keto $PGF_{1\alpha}$) was assessed with a commercially available kit (Cayman Chemical Company, Ann Arbor, MI, USA); serum amyloid A and interleukin-6 measurements were performed using commercial immunoassay kits (BioSource Europe, Nivelles, Belgium) according to the manufacturer's protocol.

*RNA analyses*

In each group, six animals were sacrificed without being subjected to the venous thrombosis model. Livers were isolated and snap frozen in liquid nitrogen for quantitative RNA analyses. RNA isolation and real-time qPCR was performed on 20 to 30 mg of liver tissue, according to the method previously described by Ter Horst et al. for lung tissue.[21] Gene-specific primers were designed with the Primer Express software (Applied Biosystems) and are presented in table 1. Data were analyzed with the supporting ABI Prism software package using the comparative threshold cycle method with β-actin as an internal control. The standard fat diet group treated with the vehicle was set as the reference group and the $\Delta C_t$ values of the individual samples were related to the mean $\Delta C_t$ of the reference group.

*Statistical analyses*

Data are expressed as mean ± standard error of the mean (SEM). Statistical significance for differences between groups was analyzed by non-parametric t-testing (Mann-Whitney). Analyses were performed using GraphPad Instat (San Diego, CA, USA) and p-values $<0.05$ were considered to be statistically significant.

**Table 1: RT-PCR primer sequences**

| Gene | Forward primer | Reverse primer |
|---|---|---|
| β-actin | AGGTCATCACTATTGGCAACGA | CCAAGAAGGAAGGCTGGAAAA |
| Factor II | GGACGCTGAGAAGGGTATCG | CCCCACACAGCAGCTCTTG |
| Factor V | CATGGAAACCTTACCGACAGAAA | CATGTGCCCCTTGGTATTGC |
| Factor VII | CGTCTGCTTCTGCCTAGA | ATTTGCACAGATCAGCTGCTCAT |
| Factor VIII | CTTCACCTCCAGGGAAGGACTA | TCCACTTGCAACCATTGTTTTG |
| Factor X | GTGGCCGGGAATGCAA | AACCCTTCATTGTCTTCGTTAATGA |
| Antithrombin | TGGGCCTCATTGATCTCTTCA | CCTGCCTCCAGCAACGAT |
| Fibrinogen-γ | TGCTGCCTGCTTTTACTGTTCTC | TCTAGGATGCAACAGTTATCTCTGGTA |
| PAI-1 | CGCTGCACCCTTTGAGAAAG | TGATGAGTTCAGCATCCAAGATG |
| IL-6 | ACAAGTCGGAGGCTTAATTACACAT | AATCAGAATTGCCATTGCACAA |
| TNF-α | CCCCAAGGGATGAGAAGTTC | TGTGAGGGTCTGGGCCATAG |
| MMP-12 | TGTGGAGTGCCCGATGTACA | AGTGAGGTACCGCTTCATCCAT |
| MCP-1 | CAGCACCTTTGAATGTGAAGTTG | TGCTTGAGGTGGTTGTGGAA |

PAI-1 : plasminogen activator inhibitor-1 ; IL-6 : interleukin-6 ; TNF-α : tumor necrosis factor- α ; MMP-12 : matrix metalloproteinase-12 ; MCP-1 : monocyte chemoattractant protein-1.

## Results

*Dose-finding*

A dose-finding study in ovariectomized mice was performed prior to the obesity / estrogen experiment in order to determine the minimal oral dose of ethinylestradiol (EE) required to induce changes in the coagulation profile. Oral EE dose-dependently induced an increase in the uterine wet weight, and changes in antithrombin levels (figure 1a), combined factor II/VII/X, factor VIII activity and PAI-1 levels, without affecting plasma ALT, AST or ALP levels (data not shown). The reduced plasma factor II/VII/X activity and antithrombin was paralleled by dose-dependent decreases of hepatic mRNA levels (figure 1 panel b). A dose of 1 µg EE per mouse per day caused significant reductions in antithrombin antigen and activity levels (120±2% of normal pooled plasma (NPP) vs. 105±5% NPP antithrombin antigen levels for vehicle-treated and estrogen-treated animals respectively, p=0.013; 123±2% NPP vs. 102±5% NPP, p=0.006 for activity levels), and combined factor II/VII/X activity (105±4% NPP vs. 87±3% NPP, p=0.006), whereas PAI-1 plasma levels were significantly increased as compared to vehicle-treated mice (1.48±0.10 ng/ml vs. 2.48±0.32 ng/ml, p=0.009). Significant changes in the hepatic transcript levels of estrogen-treated mice included a reduction of antithrombin (relative transcript level: 1±0.08 vs. 0.46±.15, p=0.025), factor V (1±0.04 vs. 0.69±0.04, p=0.037), factor VII (1±0.04 vs. 0.67±0.03, p=0.004) and

factor X (1±0.04 vs. 0.66±0.03, p=0.016). Additional treatment with the estrogen receptor antagonist ICI182780 resulted in significant counteraction of the estrogen induced effects, including the plasma antithrombin levels (figure 1c; 93.1±1.9% NPP for control treated animals, 84.2±2.4% NPP for estrogen treatment and 94.6±1.9% NPP for both estrogen and ICI182780 administration) and combined factor II/VII/X activity (91.6±2.1% NPP vs. 82.7±2.2% NPP vs. 95.1±2.2% NPP, respectively). Also on hepatic mRNA levels, estrogen receptor antagonist administration caused normalization of the effects of the oral estrogens in antithrombin (figure 1d; 1±0.15 for vehicle-treated animals vs. 0.66±0.06 for estrogen treated mice vs. 0.91±0.07 for combined estrogen and estrogen receptor antagonist treatment), factor VII (1±0.06 vs. 0.61±0.04 vs. 0.98±0.10, respectively) and factor X (1±0.11 vs. 0.52±0.05 vs. 0.91±0.06). Therefore, a dose of 1 µg EE per mouse per day was selected for further studies, being the lowest oral dose that resulted in significant and estrogen-specific changes in plasma coagulation parameters and in the hepatic mRNA levels of coagulation genes.

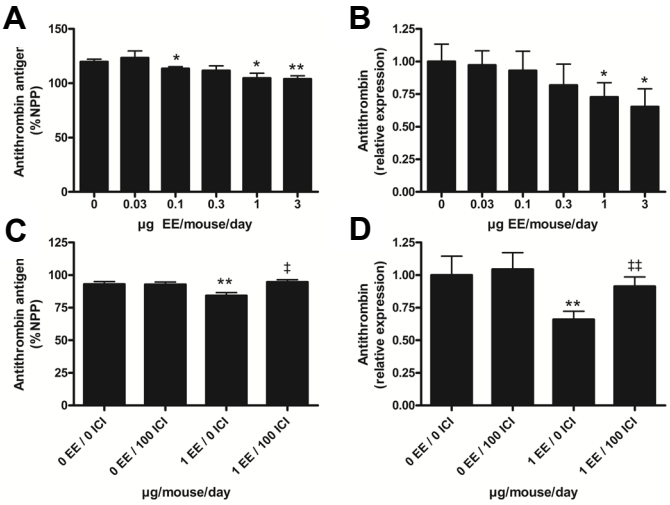


**Figure1:** Dose-dependent effects of oral ethinylestradiol (EE) on the plasma antithrombin antigen (panel a) and hepatic mRNA levels related to ß-actin (b). The effects of 1 µg EE per mouse per day on plasma and hepatic mRNA levels of antithrombin are normalized by additional treatment with 100 µg of the estrogen receptor antagonist ICI 182780 (ICI; panel c and d). NPP: normal pooled plasma. Data are represented as mean±SEM. *p<0.05 and **p<0.01 versus vehicle treatment, ‡p<0.01 and ‡‡p<0.001 versus estrogen treatment alone.

*Nutritionally induced obesity*

After 15 weeks, mice on a HFD receiving the vehicle treatment showed a significant increase in body weight, liver weight and gonadal and subcutaneous fat pads (all p<0.001) as compared to vehicle-treated mice on SFD (table 2). Plasma lipids including total plasma cholesterol, HDL and LDL cholesterol and triglyceride levels,

as well as plasma levels of the liver enzymes AST and ALT, were significantly higher in vehicle-treated HFD mice than in vehicle-treated SFD mice (table 3). Levels of 6-keto $PGF_{1\alpha}$ showed a non significant decrease in nutritionally-induced obese vehicle-treated mice as compared to mice on a standard fat diet without estrogen treatment (100±36.1% vs. 42.7±23.1%, p=n.s.).

*Estrogen treatment*

Eight days of treatment with an oral dose of 1 μg EE did not cause significant changes in the body weight although the uterus weight in estrogen-treated mice was significantly increased as compared to vehicle-treated mice on the same diet (for both groups, p<0.001; table 2). The liver weight showed a significant increase due to estrogen administration in the SFD group only (p<0.001), whereas estrogens induced a reduction in gonadal as well as subcutaneous fat pads (p=0.010 for the subcutaneous fat pads in the HFD fed mice). Total plasma cholesterol and LDL cholesterol levels were significantly increased after estrogen treatment in mice on a SFD (both p<0.001), whereas no significant changes in plasma lipid levels were observed in the HFD group (table 3). AST levels decreased significantly in both diet groups upon orally administered estrogens (SFD p=0.010, HFD p=0.002), whereas ALT levels were significantly reduced in the HFD fed mice only (p=0.011). Estrogen administration in SFD as well as HFD fed mice did not had an effect on 6-keto $PGF_{1\alpha}$ levels as compared to the vehicle treatment (from 100±36.1% to 133.1±51.5% for estrogen-treated SFD mice and from 42.7±23.1% to 52.9±23.3% for estrogen-treated HFD mice; p=n.s.)

**Table 2: Organ and adipose tissue weights of mice kept on a standard (SFD) or high fat (HFD) diet, treated with ethinylestradiol (EE+) or vehicle (EE-).**

| | SFD | | HFD | |
|---|---|---|---|---|
| | EE- (n=23) | EE+ (n=19) | EE- (n=23) | EE+ (n=24) |
| Body weight (g) | 23.5±0.3 | 24.1±0.4 | 33.2±1.1‡ | 31.1±0.6 |
| Liver weight (mg) | 982±17 | 1209±24** | 1348±60‡ | 1385±38 |
| Uterus wet weight (mg) | 69±5 | 114±3** | 75±5 | 109±3** |
| Gonadal fat (mg) | 302±29 | 246±25 | 1822±174‡ | 1430±122 |
| Subcutaneous fat (mg) | 233±18 | 185±17 | 1109±76‡ | 850±51* |

Data represented as mean±SEM of n animals.

*p<0.05 and **p<0.001 versus vehicle treatment same diet, ‡p<0.001 versus SFD vehicle treatment.

**Table 3: Plasma metabolic parameters and liver enzymes of mice kept on a standard (SFD) or high fat (HFD) diet, treated with ethinylestradiol (EE+) or vehicle (EE-).**

| | SFD | | HFD | |
|---|---|---|---|---|
| | EE- (n=17) | EE+ (n=13) | EE- (n=16) | EE+ (n=17) |
| Total cholesterol (mg/dl) | 98±3 | 113±2*** | 185±9‡‡ | 177±7 |
| HDL cholesterol (mg/dl) | 76±3 | 81±2 | 132±6‡‡ | 124±5 |
| LDL cholesterol (mg/dl) | 22±2 | 32±1*** | 63±6‡‡ | 68±4 |
| Triglycerides (mg/dl) | 53±3 | 59±3 | 66±5‡ | 58±5 |
| Alkaline phosphatases (U/l) | 136±6 | 161±8 | 129±6 | 123±3 |
| Aspartate transaminase (U/l) | 274±20 | 196±28** | 378±36‡ | 234±23** |
| Alanine aminotransferase (U/l) | 92±10 | 92±20 | 155±22‡‡‡ | 93±11* |

Data represented as mean±SEM of n animals.
*p<0.05, **p<0.005 and ***p<0.001 versus vehicle treatment same diet, ‡p<0.05, ‡‡p<0.005, ‡‡‡p<0.001 versus SFD vehicle treatment.

*Inferior caval vein thrombosis*

In total, 69 mice were subjected to the inferior caval vein thrombosis model, of which 5 mice died during the surgical procedure. Ligation of the inferior vena cava for 24 hours resulted in a thrombus weight of 1.4±0.4 mg for mice on SFD receiving vehicle treatment, and a borderline (in)significant increase in thrombus weight to 3.0±0.7 mg for vehicle-treated mice on a high fat diet (p=0.064; figure 2). Surprisingly, estrogen treatment resulted in both diet groups in a reduction in thrombus weight, which was statistically significant for the HFD group (3.0±0.7 mg vs. 0.8±0.5 mg; p=0.013), resulting in a thrombus weight comparable to vehicle-treated SFD mice.

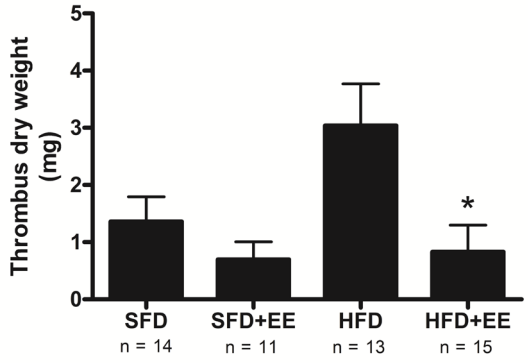


**Figure 2:** Thrombus weight for mice kept on a standard (SFD) or high fat (HFD) diet, treated with ethinylestradiol (EE+) or vehicle (EE-). 69 animals were subjected to the inferior caval vein thrombosis model (see Methods) of which 11 veins were embedded for histological analyses and 5 animals died during surgery. Data represented as mean±SEM of n animals.
*p<0.05 versus vehicle treatment same diet.

Histological analyses of the ligated veins revealed that the thrombi in all groups, with the exception of the EE-treated SFD group in which the harvested veins did not contain a thrombus, showed lamination with the presence of erythrocytes, platelets and nucleated cells, which is typical for venous thrombi. The platelet content of the thrombi of vehicle-treated HFD fed mice was decreased as compared to those of the vehicle-treated SFD mice (29±5% for the HFD and 41±10% for the SFD; p=n.s.). Thrombi of EE-treated HFD mice consisted of 23±2% platelets, which was comparable for the vehicle-treated HFD group.

*Coagulation profile*

Plasma factor VIII and combined factor II/VII/X activity levels were significantly higher in the vehicle-treated HFD fed mice than in vehicle-treated SFD fed mice (both $p<0.001$; table 4). Oral estrogen treatment resulted in both diet groups in a trend towards decreased plasma factor II/VII/X activity levels. This decreasing trend under estrogen treatment was also observed for factor V activity and for plasma antithrombin activity levels. Antithrombin antigen levels were significantly increased in the HFD group that received the vehicle treatment as compared to the vehicle-treated SFD fed mice ($p<0.001$), whereas oral estrogens in the HFD group resulted in a significant reduction in antithrombin antigen levels ($p=0.002$). Thrombin-antithrombin complexes were somewhat higher in HFD fed mice, without an effect of estrogen treatment. PAI-1 plasma levels showed a significant increase in nutritional obese mice compared to the vehicle-treated SFD group ($p=0.009$), whereas oral estrogens did not cause changes in PAI-1 levels in mice either on a SFD or HFD. Finally, the HFD resulted in an increasing trend in plasma fibrinogen levels, but administration of estrogens had no effect on the fibrinogen levels in both diet groups.

In contrast to the plasma data, nutritionally-induced obesity resulted in significantly reduced relative mRNA levels of coagulation factors II, V, VII, VIII, X and antithrombin compared to vehicle-treated SFD fed mice ($p<0.01$ for factor II, V, VII, X and antithrombin, $p=0.015$ for factor VIII; table 5). Compared to the SFD group receiving the vehicle treatment, vehicle-treated HFD mice had significantly increased levels of hepatic PAI-1 transcripts ($p=0.021$), which paralleled the plasma data. Orally administered estrogens caused significant reductions in hepatic mRNA levels of factor II, V, VII, X, antithrombin and fibrinogen-$\gamma$ in both diet groups (table 5), which coincided with non-significant decreases in plasma levels (table 4).

**Table 4: Plasma coagulation profile for mice kept on a standard (SFD) or high fat (HFD) diet, treated with ethinylestradiol (EE+) or vehicle (EE-).**

| | SFD | | HFD | |
|---|---|---|---|---|
| | EE- (n=17) | EE+ (n=17) | EE- (n=16) | EE+ (n=17) |
| Factor V Act (nM) | 30±3 | 22±2 | 38±4 | 31±3 |
| Factor VIII Act (%NPP) | 91±4 | 80±5 | 155±13‡‡ | 160±11 |
| Factor II/VII/X Act (%NPP) | 101±5 | 96±5 | 126±5‡‡ | 115±7 |
| Antithrombin Act (%NPP) | 126±6 | 114±4 | 137±7 | 132±4 |
| Antithrombin Ant (%NPP) | 110±4 | 103±2 | 129±3‡‡ | 114±3* |
| TAT (ng/ml) | 86±35 | 86±27 | 122±40 | 111±31 |
| Fibrinogen (mg/ml) | 2.6±0.2 | 2.5±0.2 | 3.2±0.1 | 3.8±0.3 |
| PAI-1 (ng/ml) | 1.8±0.4 | 2.0±0.3 | 4.0±0.7‡ | 3.7±0.5 |

Act: activity level; Ant: antigen level; TAT: thrombin-antithrombin complexes; PAI-1: plasminogen activator inhibitor-1; %NPP: percentage of value normal mouse pool plasma.

Data represented as mean±SEM of n animals.

*p<0.005 versus vehicle treatment same diet, ‡p<0.005 and ‡‡p<0.001 versus SFD vehicle treatment.

**Table 5: Hepatic mRNA levels for mice kept on a standard (SFD) or high fat (HFD) diet, treated with ethinylestradiol (EE+) or vehicle (EE-).**

| | SFD | | HFD | |
|---|---|---|---|---|
| | EE- (n=6) | EE+ (n=6) | EE- (n=6) | EE+ (n=6) |
| Antithrombin | 1±0.10 | 0.47±0.02** | 0.60 ±0.03‡‡ | 0.40±0.02** |
| Factor II | 1±0.07 | 0.56±0.04** | 0.70±0.04‡‡ | 0.46±0.03** |
| Factor V | 1±0.10 | 0.47±0.03** | 0.57±0.04‡‡ | 0.37±0.03** |
| Factor VII | 1±0.10 | 0.47±0.03** | 0.52±0.05‡‡ | 0.39±0.02* |
| Factor VIII | 1±0.16 | 0.73±0.07 | 0.57±0.06‡ | 0.56±0.04 |
| Factor X | 1±0.10 | 0.50±0.03** | 0.51±0.04‡‡ | 0.37±0.02* |
| Fibrinogen-γ | 1±0.11 | 0.53±0.04** | 0.81±0.39 | 0.40±0.03* |
| PAI-1 | 1±0.24 | 1.18±0.30 | 2.37±0.51‡ | 1.64±0.84 |
| MMP-12 | 1±0.30 | 1.24±0.44 | 3.73±2.20‡ | 5.09±2.01 |
| MCP-1 | 1±0.21 | 2.16±0.26 | 1.95±0.57 | 3.54±0.34 |

PAI-1: plasminogen activator inhibitor-1; MMP-12: matrix metalloproteinase-12; MCP-1: monocyte chemoattractant protein-1.

Data represented as mean±SEM; data normalized for β-actin with the vehicle-treated SFD group set as reference.

*p<0.05 and **p<0.005 versus vehicle treatment same diet, ‡p<0.05 and ‡‡p<0.005 versus SFD vehicle treatment.

*Inflammation*

To determine whether the decrease in thrombus weight in HFD fed mice following estrogen treatment was related to estrogen-mediated regulation of inflammatory pathways, systemic inflammation was monitored by measuring serum amyloid A (SAA) and interleukin-6 (IL-6) levels in the plasma. SAA levels of vehicle-treated mice on HFD were comparable to vehicle-treated SFD fed mice (13.7±3.9 μg/ml vs. 12.7±3.7 μg/ml, respectively). Estrogen administration resulted in both diet groups in increased SAA levels, which reached statistical significance in the SFD group (12.7±3.7 μg/ml vs. 25.3±3.9 μg/ml; p=0.003), but not in the HFD group (13.7±3.9 μg/ml vs. 21.8±5.7 μg/ml; p=0.430). Plasma IL-6 levels, with the exception of a few mice, were undetectable and thereby precluded statistical analysis.

Hepatic mRNA levels of IL-6, tumor necrosis factor-α (TNF-α), matrix metalloproteinase-12 (MMP-12) and monocyte chemoattractant protein-1 (MCP-1) were measured to further evaluate liver inflammation. Expression of IL-6 and TNF-α were not detectable before $C_t$ 35 and therefore considered not to be present in significant amounts. The MMP-12 transcript level was significantly increased in vehicle-treated HFD fed mice as compared to vehicle-treated SFD fed mice (p=0.0411; table 5) and in both diet groups, estrogen treatment caused a further non-significant increase. MCP-1 mRNA levels were slightly enhanced by estrogen treatment of mice both on a SFD and on a HFD.

## Discussion

In the present study, we found that nutritionally-induced obesity results in increased plasma levels of factor II/VII/X, factor VIII and PAI-1 and causes a trend towards an increased thrombus weight in a caval vein thrombosis model. Surprisingly, under obese conditions, short-term oral estrogen administration results in a significant lower thrombogenicity, which is not paralleled by a reversal of the obesity-induced procoagulant state, nor does it coincide with changes in the inflammatory state.

The present mouse study was designed to further investigate the epidemiological observation on the synergistic effect of obesity and oral estrogen use on the venous thrombotic risk. However, the finding that oral estrogens in women are prothrombotic cannot simply be translated into the mouse context, which may have several causes. First, matching an established thrombotic risk in epidemiological studies versus the detection of a thrombotic tendency in acute models of experimental thrombosis in mice, may not simulate the cumulative risk for thrombosis correctly, since this is a chronic risk. Second, human data show that

both obesity and oral estrogens result in increased plasma levels of factor II and factor VII.[1,4,7,9,11] Together with an increased resistance against activated protein C under these conditions, this may explain the thrombophilic interaction between obesity and estrogens in women. However, although the effects of estrogens observed are dose-dependent and receptor specific, our present data show that mice respond differently to oral estrogen administration, i.e. rather tend to induce a reduction in plasma factor II/VII/X activity, suggesting a species difference,. Finally, the duration and dose of exposure to estrogens is important for its actions.[7,8,22,23] The EE dose used in the present study was based on a preliminary dose-finding study in ovariectomized mice; in the obesity / estrogen study, the selected dose showed a similar effect on the plasma coagulation profile in non-ovariectomized mice, but less pronounced. As the uterus weight under ovariectomized and non-ovariectomized conditions increased to a similar level, the duration of treatment (10 versus 8 days) might contribute to the more pronounced changes in the plasma coagulation profile in the dose-finding study. Although this suggests the need for long-term treatment to achieve results more in line with human exposure and risk development, preliminary data of 7 week treatment in SFD fed ovariectomized mice showed comparable results on the plasma coagulation profile as observed for short-term EE-treated controls (i.e. non-significant antithrombotic shift). These data suggest that a more prolonged exposure does not affect the thrombotic tendency differently than short-term exposure does. In addition, using a carotid artery photochemical injury model, Abu-Fanne and colleagues also showed that chronic treatment with estradiol in non-ovariectomized as well as ovariectomized mice suppressed thrombosis.[24] Bleeding time was also evaluated and revealed that OVX mice treated with estradiol had a longer bleeding time compared to vehicle-treated mice. Thus also in this mouse study, estrogen administration has antithrombotic effects. Nevertheless, by using mouse models displaying a more spontaneous venous thrombotic phenotype, like the Factor V Leiden mouse model, thrombomodulin or antithrombin mutant mice, in combination with a prolonged exposure to estrogens, the cumulative chronic risk in humans may be better represented. Despite this shortcoming, comparison of the plasma coagulation profile in estrogen-treated lean and obese mice, suggests other factors than coagulation factor regulation to explain the impact of estrogens on the thrombotic tendency.

The trend towards an increased thrombus weight under obese conditions can be explained by a prothrombotic shift of the coagulation profile. On the other hand, the

observed protective effect of short-term estrogen administration on the thrombotic tendency could not be explained by a reversal of the prothrombotic shift in the plasma coagulation profile associated with obesity (table 4). Abdollahi et al. already showed that the interaction between obesity and oral estrogens on the thrombotic risk remained after adjustment for several coagulation factor levels, suggesting an alternative pathway affecting the risk of venous thrombosis.[1] Inflammation could be such an alternative pathway, as obesity and estrogen use affect the inflammatory state in an opposite direction, and inflammation and coagulation are closely related. However, inflammation and inflammation-related parameters did not provide evidence that could explain the unexpected antithrombotic effect of short-term estrogen administration under obese conditions. Several (*in vitro*) studies provide evidence for a role of estrogens in modulating endothelial cell function and/or gene expression and platelet function, both in a genomic and non-genomic manner.[24-30] We considered prostacyclin as a mediator of the suppressive effects of estrogens in the thrombosis model. However, plasma levels of 6-keto prostaglandin F1α were not significantly affected by estrogen administration.

Besides the thrombus weight, estrogen administration also (partially) counterbalanced the increased gonadal and subcutaneous fat pads and increased levels of circulating liver enzymes. This latter observation is in line with a study performed by Moorthy et al., who showed that estrogen treatment in naturally menopausal rats also results in a decrease in AST and ALT levels.[31] Strikingly, in our study the AST levels, and to a lesser extend ALT levels, parallel the thrombus weight under the different dietary and hormonal conditions. It therefore seems that the HFD induced liver damage is partially reversed by the estrogen treatment, an effect already apparent for AST in the SFD group. However, it is unclear whether the reduced thrombogenicity and liver damage are directly or indirectly coupled via metabolic estrogen-regulated pathways.

In the present study, changes in relative transcript levels of coagulation genes in the liver not always paralleled the observed plasma protein levels. Estrogen administration resulted in both diet groups in significant decreases of hepatic mRNA levels of coagulation factors, but this did not coincide with the plasma levels. On the other hand, vehicle-treated HFD fed mice had lower hepatic transcript levels as compared to vehicle-treated mice on a SFD, whereas the plasma coagulation factor levels were significantly increased under nutritionally induced obesity. These latter differences could be explained by the fact that the HFD caused a significant increase in liver weight compared to the SFD group. For each treatment group the

RNA recovery from liver tissue was equal, suggesting that the absolute expression is higher when the liver is enlarged. However, the liver weight of the vehicle- and estrogen-treated HFD fed mice were comparable, indicating that the reduced mRNA levels under estrogen administration are absolute, and no direct relation between expression and plasma levels exists. These data suggest that both obesity and estrogens induce changes in the plasma coagulation levels via mechanisms including posttranslational modifications and/or a decreased clearance capacity for these coagulation factors.

In summary, the synergistic effect of obesity and estrogen on the venous thrombotic risk in women could not be translated into the mouse context. However, nutritionally-induced obesity in mice resulted in an activated plasma coagulation profile and in a trend towards an enhanced venous thrombotic tendency. Short-term oral estrogen administration on the other hand, only had a very mild impact on the coagulation profile and did not result in aggravation of the thrombotic tendency but suppressed experimentally induced venous thrombosis in obese mice.

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## References

1 Abdollahi M, Cushman M, Rosendaal FR. Obesity: risk of venous thrombosis and the interaction with coagulation factor levels and oral contraceptive use. Thromb Haemost 2003;89:493-8.

2 Darvall KA, Sam RC, Silverman SH, et al. Obesity and thrombosis. Eur J Vasc Endovasc Surg 2007;33:223-33.

3 Oren E, Smith NL, Doggen CJ, et al. Body mass index and the risk of venous thrombosis among postmenopausal women. J Thromb Haemost 2006;4:2273-5.

4 Pomp ER, le Cessie S, Rosendaal FR, et al. Risk of venous thrombosis: obesity and its joint effect with oral contraceptive use and prothrombotic mutations. Br J Haematol 2007;139:289-96.

5 Rosendaal FR, Helmerhorst FM, Vandenbroucke JP. Female hormones and thrombosis. Arterioscler Thromb Vasc Biol 2002;22:201-10.
6 Stein PD, Beemath A, Olson RE. Obesity as a risk factor in venous thromboembolism. Am J Med 2005;118:978-80.
7 Canonico M, Oger E, Conard J, et al. Obesity and risk of venous thromboembolism among postmenopausal women: differential impact of hormone therapy by route of estrogen administration. The ESTHER Study. J Thromb Haemost 2006;4:1259-65.
8 Canonico M, Oger E, Plu-Bureau G, et al. Hormone therapy and venous thromboembolism among postmenopausal women: impact of the route of estrogen administration and progestogens: the ESTHER study. Circulation 2007;115:840-5.
9 Koh KK, Yoon BK. Controversies regarding hormone therapy: Insights from inflammation and hemostasis. Cardiovasc Res 2006;70:22-30.
10 Silvestri A, Gebara O, Vitale C, et al. Increased levels of C-reactive protein after oral hormone replacement therapy may not be related to an increased inflammatory response. Circulation 2003;107:3165-9.
11 Vehkavaara S, Silveira A, Hakala-Ala-Pietila T, et al. Effects of oral and transdermal estrogen replacement therapy on markers of coagulation, fibrinolysis, inflammation and serum lipids and lipoproteins in postmenopausal women. Thromb Haemost 2001;85:619-25.
12 Zegura B, Guzic-Salobir B, Sebestjen M, et al. The effect of various menopausal hormone therapies on markers of inflammation, coagulation, fibrinolysis, lipids, and lipoproteins in healthy postmenopausal women. Menopause 2006;13:643-50.
13 Bodary PF, Vargas FB, King SA, et al. Pioglitazone protects against thrombosis in a mouse model of obesity and insulin resistance. J Thromb Haemost 2005;3:2149-53.
14 Konstantinides S, Schafer K, Neels JG, et al. Inhibition of endogenous leptin protects mice from arterial and venous thrombosis. Arterioscler Thromb Vasc Biol 2004;24:2196-201.
15 Nagai N, Lijnen HR, Cleuren AC, et al. Factor V Leiden mutation is associated with enhanced arterial thrombotic tendency in lean but not in obese mice. Thromb Haemost 2007;98:858-63.
16 Nagai N, Hoylaerts MF, Cleuren AC, et al. Obesity promotes injury induced femoral artery thrombosis in mice. Thromb Res 2008;122:549-55.
17 Tchaikovski SN, van Vlijmen BJ, Rosing J, et al. Development of a calibrated automated thrombography based thrombin generation test in mouse plasma. J Thromb Haemost 2007;5:2079-86.
18 Singh I, Smith A, Vanzieleghem B, et al. Antithrombotic effects of controlled inhibition of factor VIII with a partially inhibitory human monoclonal antibody in a murine vena cava thrombosis model. Blood 2002;99:3235-40.
19 Day SM, Reeve JL, Myers DD, et al. Murine thrombosis models. Thromb Haemost 2004;92:486-94.
20 Declerck PJ, Verstreken M, Collen D. Immunoassay of murine t-PA, u-PA and PAI-1 using monoclonal antibodies raised in gene-inactivated mice. Thromb Haemost 1995;74:1305-9.
21 ter Horst SA, Fijlstra M, Sengupta S, et al. Spatial and temporal expression of surfactant proteins in hyperoxia-induced neonatal rat lung injury. BMC Pulm Med 2006;6:8.
22 Moverare S, Skrtic S, Lindberg MK, et al. Estrogen increases coagulation factor V mRNA levels via both estrogen receptor-alpha and -beta in murine bone marrow/bone. Eur J Endocrinol 2004;151:259-63.

23 Yamamoto Y, Moore R, Hess HA, et al. Estrogen receptor alpha mediates 17alpha-ethynylestradiol causing hepatotoxicity. J Biol Chem 2006;281:16625-31.
24 Abu-Fanne R, Brzezinski A, Golomb M, et al. Effects of estradiol and raloxifene on arterial thrombosis in ovariectomized mice. Menopause 2007 Jun 1.
25 Koh KK. Effects of estrogen on the vascular wall: vasomotor function and inflammation. Cardiovasc Res 2002;55:714-26.
26 Leng XH, Zhang W, Nieswandt B, et al. Effects of estrogen replacement therapies on mouse platelet function and glycoprotein VI levels. Circ Res 2005;97:415-7.
27 Quehenberger P, Kapiotis S, Partan C, et al. Studies on oral contraceptive-induced changes in blood coagulation and fibrinolysis and the estrogen effect on endothelial cells. Ann Hematol 1993;67:33-6.
28 Simoncini T, Mannella P, Genazzani AR. Rapid estrogen actions in the cardiovascular system. Ann N Y Acad Sci 2006;1089:424-30.
29 Fox SW, Chambers TJ. The effect of oestrogen on megakaryocyte differentiation and platelet counts in vivo. Int J Cardiol 2006;109:359-66.
30 Moro L, Reineri S, Piranda D, et al. Nongenomic effects of 17beta-estradiol in human platelets: potentiation of thrombin-induced aggregation through estrogen receptor beta and Src kinase. Blood 2005;105:115-21.
31 Moorthy K, Sharma D, Basir SF, et al. Administration of estradiol and progesterone modulate the activities of antioxidant enzyme and aminotransferases in naturally menopausal rats. Exp Gerontol 2005;40:295-302.