Understanding the biological mechanisms underlying acquired risk factors for venous thrombosis: Studies in mice

Audrey C.A. Cleuren

Understanding the biological mechanisms underlying acquired risk factors for venous thrombosis: Studies in mice

Proefschrift

ter verkrijging van
de graad van Doctor aan de Universiteit Leiden,
op gezag van Rector Magnificus prof.mr. P.F. van der Heijden
volgens besluit van het College voor Promoties
te verdedigen op donderdag 7 juni 2012
klokke 13.45 uur

door

Audrey Corrie Andrea Cleuren

geboren te Maastricht
in 1984

Promotiecommissie

Promotor:
Prof. dr. P.H. Reitsma

Co-promotor:
dr. B.J.M. van Vlijmen

Overige leden commissie:
Prof. dr. H. ten Cate (Maastricht University)
Prof. dr. F.M. Helmerhorst
Prof. dr. J.W.A. Smit

The research described in this thesis was supported by a grant of the Dutch Heart Foundation (DHF-2006B045) and was performed at the Einthoven Laboratory for Experimental Vascular Medicine, Department of Thrombosis and Hemostasis of the Leiden University Medical Center in Leiden, the Netherlands.

Financial support by the Dutch Heart Foundation for the publication of this thesis is gratefully acknowledged.

Cover illustration: sagital section (hematoxylin/eosin staining) of a one-day old factor V Leiden hemizygous mouse.

ISBN: 978-90-8891-424-9
Printed by: Boxpress BV

“Don’t dream your life, but live your dream”
S.L. Clemens (1835-1910)

Table of contents

Chapter 1

General introduction

Blood coagulation

The basis for our modern understanding of the cardiovascular system was set in 1628 when William Harvey first described the cardiovasculature as a closed circulatory system.[1] Nowadays, it is known that this system has a total surface of about 1000 m^2 and consists of approximately 100.000 km of vessels which transports blood to provide cells with oxygen and nutrients, and removes metabolic waste products.[2]

Hemostasis is the process that maintains blood in a clot-free state under physiological circumstances and prevents excessive blood loss upon vascular injury. When vascular injury occurs, the vessel constricts, platelets aggregate and form a platelet plug, and proteins of the procoagulant system will stabilize the clot via a dense fibrin network, while proteins of the anticoagulant and fibrinolytic systems prevent uncontrolled clot formation.[3] This balance between procoagulant, anticoagulant and fibrinolytic factors has to be tightly regulated to ensure rapid and localized clot formation, and disturbance of this balance can lead to either a hypocoagulable or hypercoagulable state, which can subsequently result in bleeding or thrombosis, respectively. Although thrombus formation can occur in both the arterial and venous system, the work described in this thesis will focus on factors leading to an increased risk for venous thrombosis.

Risk factors for venous thrombosis

In 1856, Rudolf Virchow postulated 3 causes of thrombosis: changes in blood flow, changes in the vessel wall and changes in blood composition.[4] Although this classification is still valid, epidemiological studies have shown that risk factors for venous thrombosis relate to changes in either blood flow or blood composition, while changes in the vessel wall seem to be of lesser importance.[5] This led to a new classification, which separates genetic and acquired risk factors for venous thrombosis.

Genetic risk factors

Genetic risk factors often relate to changes in blood composition, resulting in a hypercoagulable state. It is therefore not surprising that the first genetic risk factors for venous thrombosis identified were partial deficiencies in the anticoagulant factors antithrombin, protein C and protein S. Complete deficiencies of these factors result in severe hemostatic dysfunctions that are not compatible with life. On the other hand, partial deficiencies due to heterozygosity are rare in the general

population with prevalences of less than 0.5% and confer an approximately 10-fold increased risk for venous thrombosis.[6]

Besides deficiencies in anticoagulant factors, mutations in procoagulant factors have also been reported. The factor V Leiden mutation is the most common genetic risk factor for venous thrombosis with a prevalence of 5% in the Caucasian population.[6] This gain-of-function mutation renders a factor V protein that has an increased procoagulant and a decreased anticoagulant activity, resulting in a 7-fold increased thrombosis risk in heterozygotes, which can increase up to an 80-fold higher risk in homozygotes.[7] Another common genetic risk factor is the G20210A mutation in the prothrombin gene, which is associated with increased prothrombin levels in the plasma, thereby leading to a 2- to 5-fold higher risk for venous thrombosis.[6]

In addition to these mutations within coagulation genes, plasma coagulation factor levels can also be indirectly influenced by other genes. For example, ABO blood group affects von Willebrand factor levels in the plasma, which subsequently influences FVIII levels. This in turn can increase the risk for venous thrombosis since high plasma FVIII levels have been identified as an independent risk factor.[8]

Acquired risk factors

Over the last years, large epidemiological studies have identified a number of acquired risk factors for venous thrombosis that result in stasis and/or a hypercoagulable state.[5,9] The first category includes factors such as immobilization, trauma, plaster casts and major surgery. On the other hand, hypercoagulability may be caused by obesity, malignancies, female hormones as in oral contraceptive use or pregnancy, and several endocrine disorders like hyperthyroidism, acromegaly and the metabolic syndrome are also associated with a hypercoagulable state.[10-13] However, the underlying mechanisms how these acquired factors lead to a hypercoagulable state are largely unknown.

Besides the association with a hypercoagulable state, another common feature of this latter group of acquired risk factors is that they are all, at least to a certain extent, able to induce changes in liver homeostasis. For example, estrogens, thyroid hormones and obesity can regulate lipid and glucose metabolism in the liver via affecting gene transcription of key players involved in these metabolic pathways.[14-16] Since the liver is also the main site of production of plasma coagulation factors we therefore hypothesized that acquired risk factors for venous

thrombosis associated with a hypercoagulable state also modulate transcription of hepatically expressed genes belonging to the plasma coagulation system

Regulation of hepatic coagulation gene transcription

There are several ways by which transcription of coagulation genes can be modulated, ranging from a direct modulation via nuclear receptors that bind to a response element in the coagulation gene, to an indirect modulation where transcription is affected as a bystander effect of changes in liver homeostasis.

A number of acquired risk factors that are associated with a hypercoagulable state are hormonal in origin, like the use of oral contraceptives and hyperthyroidism. It is known that hormones can affect transcription, as they exert their actions via binding to nuclear hormone receptors. When liganded, the hormone receptors can bind to the hormone response element in the promoter region of a target gene, thereby acting as a transcriptional modulator of transcription.[17,18] For example, it has been reported that the FVII and FXII genes have functional estrogen response elements, which may account for the changes in plasma levels of these factors observed during contraceptive use.[19,20]

A second mechanism to modulate transcription is by interfering with constitutive expression of coagulation genes. To ensure adequate coagulation upon vascular damage, coagulation factors have to be continuously produced in order to achieve the required steady-state levels in the circulation. This production of coagulation factors depends, amongst others, on transcription factors of the hepatic nuclear factor (HNF) and the CCAAT/enhancer-binding protein (CEBP) family. HNF4α is of particular interest since it is not only the most abundantly expressed transcription factor in the liver and is important for the transcription of a number of pro- and anticoagulant genes,[21,22] but also because hormones like estrogens and thyroid hormone can interact with HNF4α.

Acquired risk factors may also modulate transcription of coagulation genes via transcription factors that play a minor role in constitutive expression, but have a highly inducible activity. For instance, a hallmark of obesity is an increased insulin level and a chronic inflammatory state, which coincides with an increased transcriptional activity of nuclear factor (NF)-κB.[23] *In vitro* studies have demonstrated that NF-κB can induce the expression of fibrinogen, factor VIII and PAI-1,[23,24] and this mechanism may also extent to other coagulation genes that form part of the hypercoagulable state associated with obesity.

Finally, risk factors can induce changes in liver homeostasis, resulting in a long chain of events in which transcriptional modulation of coagulation genes can be considered as a bystander effect. For example, estrogens, thyroid hormone and obesity can affect the lipid metabolism and triglyceride accumulation in the liver, which in the case of obesity can lead to steatohepatitis. Due to this effect on liver metabolism and physiology, it is not difficult to imagine that transcription of numerous genes, including coagulation genes may be affected as well.
Thus, there are several levels by which transcription of hepatically expressed coagulation genes can be modulated. We hypothesize that transcriptional modulation of coagulation genes by hormones like estrogens and thyroid hormone will be direct, i.e. that liganded hormone receptors directly interact with their respective hormone response elements in the promoter region of coagulation genes. On the other hand, obesity is more likely to modulate coagulation gene transcription through an indirect multistep process, as the result from changes in liver physiology.

Strategies to study transcriptional modulation of coagulation genes

Several strategies to study transcriptional modulation can be used to test our hypotheses. With *in silico* approaches, transcription factor binding sites can be predicted by combining sequence databases and algorithms, like consensus sequences for hormone response elements. This method has been applied to determine estrogen response elements in both the human and mouse genome and indentified response elements in a number of coagulation genes.[25] However, an important limitation of predicting transcription factor binding sites *in silico* is that they are not necessarily functional *in vivo*. The opposite can also occur: functional response elements do not always contain the consensus sequence used by an algorithm and may therefore erroneously be overlooked as a response element. These limitations are nicely illustrated by comparing an *in silico* study performed by Bourdeau and colleagues which identified estrogen response elements in the murine FVII, FX, FXIII and α_2-antiplasmin genes, with an *in vivo* ChIP-on-chip study that showed that fibrinogen, FII, FXI, antithrombin, heparin cofactor II, protein S, protein Z and plasminogen were directly bound to the estrogen receptor α.[15,25]
In contrast to *in silico* studies, *in vitro* studies can be used for functional testing of e.g. hormone response elements, as has been done for coagulation factors VII, IX and XII.[19,20,26] However, the disadvantage of *in vitro* approaches is that they often focus on a specific cell type, which makes it difficult to reproduce physiological

processes since they often require multiple interactions between cells of different origins. In addition, some risk factors like obesity cannot be mimicked *in vitro*, as the key intermediates leading to obesity, and the resulting hypercoagulable state, are not known.
Therefore, it is important to have a complete physiological system that provides all the known and unknown but essential factors necessary to modulate transcription. Another advantage of *in vivo* models is that they provide the opportunity to study whether the modulating effects on transcription are translated in the plasma protein levels and subsequently in a thrombosis phenotype. Hence, the use of an *in vivo* model is warranted.

Animal models

Mice are by far the most used animals in thrombosis research. They are not only appealing because of their small size and short generation time, but also because of the relative ease of genetic manipulation. Furthermore, liver-specific gene regulation is fairly conserved between humans and mice, suggesting a highly comparable regulation of hepatic gene transcription.[27] As previously mentioned, it is also important to evaluate whether transcriptional effects translate into altered plasma protein levels and a thrombotic phenotype. Although there are some differences between human and mouse coagulation on the plasma level, e.g. the fact that protein S in mice does not bind to C4BP as it does in humans[28], in general murine coagulation factors and their functions are quite similar to their human counterparts.[29,30] This has the advantage that a large number of assays used to determine the functional levels of human plasma coagulation factors can be relatively easy adapted for their application in mouse research.
To evaluate changes in the coagulation profile and its impact on the thrombotic phenotype, the use of mice has its limitations, as they do not develop thrombosis spontaneously. To overcome this problem, several experimental models have been developed in which thrombus formation is triggered either by stasis or by vessel wall damage due to ferric chloride, photochemical injury, mechanical trauma, or by applying an electric current.[31-33] In addition to these experimental models, transgenic mouse models have been generated by disrupting or manipulating coagulation factors, like the factor V Leiden and thrombomodulin proline mutant mice. As a result of these mutations, mice display tissue fibrin depositions which are considered to be a marker for a thrombotic phenotype. Moreover, they also have the potential to develop macrovascular thrombosis.[34,35]

As the use of mice can provide information on the mechanisms of transcriptional modulation following risk factor exposure, and whether these transcriptional changes alter the plasma coagulation profile and subsequently the thrombotic tendency, murine *in vivo* models were used in the experiments described in this thesis.

Aim and outline of the thesis

The aim of this thesis was to obtain a better understanding of the biological mechanisms underlying acquired risk factors that lead to an increased risk for venous thrombosis. The risk factors studied, i.e. female hormones, thyroid hormone and obesity, are all associated with a hypercoagulable state and are all capable of interfering with liver metabolism. Therefore, we hypothesized that these risk factors modulate transcription of coagulation genes, either directly via nuclear hormone receptors and hormone response elements in the target gene (female hormones and thyroid hormone), or indirectly by affecting liver homeostasis (obesity). This in turn may result in a hypercoagulable state and subsequent increased venous thrombotic risk. To test these hypotheses, we used an *in vivo* approach which enabled us to not only study transcriptional modulation but also the resulting effects on both the plasma protein levels and the thrombotic phenotype, which we assessed by using both experimental and genetic thrombosis models.

As we considered mice to be a valuable tool to study risk factor-induced effects on hepatic transcription, plasma coagulation and thrombosis, **chapter 2** reviews the literature with respect to the available genetic mouse models of venous thrombosis, of which two models are chosen to use in studies described later in this thesis.

In **chapter 3**, the effects of oral ethinylestradiol on the mouse plasma coagulation profile are presented. In addition, the underlying mechanism of estrogen-induced changes in coagulation is elucidated. As the acquired risk factors of oral contraceptive use and hormone replacement therapy contain both an estrogen and progestin component, the effect of this combined therapy on transcript levels of hepatically expressed coagulation genes is described in **chapter 4**.

Experiments in **chapter 5** were initiated to study the effects of oral ethinylestradiol and obesity on experimentally induced thrombosis. In addition, as venous thrombosis is considered to be a multicausal disorder, the interaction between these two risk factors with respect to the thrombotic tendency was also evaluated. The long-term effects of estrogen administration on plasma coagulation parameters

and the subsequent effect on a spontaneous thrombotic phenotype, i.e. fibrin deposition, of two different transgenic mouse models are presented in **chapter 6**.
In addition to chapter 5, **chapter 7** focuses specifically on obesity and describes the relation between coagulation and the development and regression of nutritionally-induced obesity. The effects of hyperthyroidism on coagulation and part of the underlying mechanism by which thyroid hormone modulates the coagulation profile are the topic of **chapter 8**.
In **chapter 9**, data on the generation and evaluation of factor V Leiden hemizygous mice are presented, showing the impact of an additional genetic factor instead of an acquired risk factor on the thrombotic phenotype of factor V Leiden mice.
Finally, **chapter 10** discusses the overall findings presented in this thesis.

References

1 Harvey W. Excercitatio anatomica de motu cordis et sanguinis in animalibus. 1628.
2 van de Graaff K, Fox SI. Concepts of human anatomy and physiology. 1989.
3 Monroe DM, Hoffman M. What does it take to make the perfect clot? Arterioscler Thromb Vasc Biol 2006;26:41-8.
4 Virchow R. Phlogose und Thrombose im Gefäßsystem; Gesammelte Abhandlungen zur Wissenschaftlichen Medizin. 1856.
5 Rosendaal FR. Venous thrombosis: the role of genes, environment, and behavior. Hematology Am Soc Hematol Educ Program 2005;1-12.
6 Franco RF, Reitsma PH. Genetic risk factors of venous thrombosis. Hum Genet 2001;109:369-84.
7 Rosendaal FR, Koster T, Vandenbroucke JP, et al. High risk of thrombosis in patients homozygous for factor V Leiden (activated protein C resistance). Blood 1995;85:1504-8.
8 Rosendaal FR. Venous thrombosis: a multicausal disease. Lancet 1999;353:1167-73.
9 Anderson FA, Spencer FA. Risk factors for venous thromboembolism. Circulation 2003;107:I9-16.
10 Franchini M, Lippi G, Manzato F, et al. Hemostatic abnormalities in endocrine and metabolic disorders. Eur J Endocrinol 2010;162:439-51.
11 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.
12 Tchaikovski SN, Rosing J. Mechanisms of estrogen-induced venous thromboembolism. Thromb Res 2010;126:5-11.
13 Vandenbroucke JP, Rosing J, Bloemenkamp KW, et al. Oral contraceptives and the risk of venous thrombosis. N Engl J Med 2001;344:1527-35.
14 Flores-Morales A, Gullberg H, Fernandez L, et al. Patterns of liver gene expression governed by TRbeta. Mol Endocrinol 2002;16:1257-68.
15 Gao H, Falt S, Sandelin A, et al. Genome-wide identification of estrogen receptor alpha-binding sites in mouse liver. Mol Endocrinol 2008;22:10-22.

16 Radonjic M, de Haan JR, van Erk MJ, et al. Genome-wide mRNA expression analysis of hepatic adaptation to high-fat diets reveals switch from an inflammatory to steatotic transcriptional program. PLoS One 2009;4:e6646.
17 Bagamasbad P, Denver RJ. Mechanisms and significance of nuclear receptor auto- and cross-regulation. Gen Comp Endocrinol 2011;170:3-17.
18 Glass CK. Going nuclear in metabolic and cardiovascular disease. J Clin Invest 2006;116:556-60.
19 Di Bitondo R, Hall AJ, Peake IR, et al. Oestrogenic repression of human coagulation factor VII expression mediated through an oestrogen response element sequence motif in the promoter region. Hum Mol Genet 2002;11:723-31.
20 Farsetti A, Misiti S, Citarella F, et al. Molecular basis of estrogen regulation of Hageman factor XII gene expression. Endocrinology 1995;136:5076-83.
21 Inoue Y, Peters LL, Yim SH, et al. Role of hepatocyte nuclear factor 4alpha in control of blood coagulation factor gene expression. J Mol Med (Berl) 2006;84:334-44.
22 Safdar H, Inoue Y, van Puijvelde GH, et al. The role of hepatocyte nuclear factor 4alpha in regulating mouse hepatic anticoagulation and fibrinolysis gene transcript levels. J Thromb Haemost;8:2839-41.
23 Iwasaki Y, Nishiyama M, Taguchi T, et al. Insulin exhibits short-term anti-inflammatory but long-term proinflammatory effects in vitro. Mol Cell Endocrinol 2009;298:25-32.
24 Liu SF, Malik AB. NF-kappa B activation as a pathological mechanism of septic shock and inflammation. Am J Physiol Lung Cell Mol Physiol 2006;290:L622-L645.
25 Bourdeau V, Deschenes J, Metivier R, et al. Genome-wide identification of high-affinity estrogen response elements in human and mouse. Mol Endocrinol 2004;18:1411-27.
26 Crossley M, Ludwig M, Stowell KM, et al. Recovery from hemophilia B Leyden: an androgen-responsive element in the factor IX promoter. Science 1992;257:377-9.
27 Schmidt D, Wilson MD, Ballester B, et al. Five-vertebrate ChIP-seq reveals the evolutionary dynamics of transcription factor binding. Science 2010;328:1036-40.
28 Tchaikovski SN, Van Vlijmen BJ, Cleuren AC, et al. Pregnancy-associated changes in the hemostatic system in wild-type and factor V Leiden mice. J Thromb Haemost 2009;7:312-8.
29 Emeis JJ, Jirouskova M, Muchitsch EM, et al. A guide to murine coagulation factor structure, function, assays, and genetic alterations. J Thromb Haemost 2007;5:670-9.
30 Tsakiris DA, Scudder L, Hodivala-Dilke K, et al. Hemostasis in the mouse (Mus musculus): a review. Thromb Haemost 1999;81:177-88.
31 Day SM, Reeve JL, Myers DD, et al. Murine thrombosis models. Thromb Haemost 2004;92:486-94.
32 Dorffler-Melly J, Schwarte LA, Ince C, et al. Mouse models of focal arterial and venous thrombosis. Basic Res Cardiol 2000;95:503-9.
33 Diaz JA, Hawley AE, Alvarado CM, et al. Thrombogenesis with continuous blood flow in the inferior vena cava. A novel mouse model. Thromb Haemost 2010;104:366-75.
34 Cui J, Eitzman DT, Westrick RJ, et al. Spontaneous thrombosis in mice carrying the factor V Leiden mutation. Blood 2000;96:4222-6.
35 Weiler-Guettler H, Christie PD, Beeler DL, et al. A targeted point mutation in thrombomodulin generates viable mice with a prethrombotic state. J Clin Invest 1998;101:1983-91.

Chapter 2

Transgenic mouse models of venous thrombosis: fulfilling the expectations?

Audrey CA Cleuren, Bart JM van Vlijmen, Pieter H Reitsma

Semin Thromb Hemost. 2007 Sep;33(6):610-616

Summary

Over the past 15 years, transgenic mice have been generated that carry defective and/or mutant alleles of the natural anticoagulant genes, thereby displaying a spontaneous thrombotic phenotype. With the generation of these mouse lines, better opportunities became available for investigating both existing and novel risk factors for venous thrombosis. In addition, these models could serve as a tool for evaluating novel antithrombotic strategies. In this review, we will summarize these mouse models and evaluate whether they have fulfilled the expectations.

Introduction

Mice are at present the most frequently used experimental animals. Their popularity is mainly due to their short breeding time, and easy low-cost maintenance. More importantly, as we have gained extensive knowledge of the mouse genome, mice have also proven to be extremely useful for carrying out genetic modification like transgenesis and gene targeting. However, because regular inbred mice do not develop venous thrombosis spontaneously, their use in animal research on venous thrombosis has been quite limited. A thrombotic phenotype could only be obtained by crude experimental approaches like ferric chloride induced injury, mechanical injury, photochemical injury or ligation induced stasis (reviewed in [1,2]). Although such models provide some insight into the thrombotic tendency under different genetic and environmental circumstances, there are important limitations. The major drawback is that these models lead to thrombus formation in normal blood vessels by inducing endothelial damage (in a largely platelet dependent process), whereas venous thrombi in humans are formed under conditions of stasis and/or hypercoagulability. In addition, subtle effects on the thrombotic tendency, which are a hall-mark of human studies, cannot be detected in these inducible mouse models. Finally, these models do not allow evaluation of the effects of prolonged exposure to certain inherited and acquired risk factors.

A potential breakthrough to circumvent these limitations came with the development of transgenesis and gene targeting in mice. These techniques allow the introduction of genes, the mutation of genes, or the knock-out of complete genes in the mouse genome. This technological advance opened an avenue to study risk factors for venous thrombosis in a more clinically relevant manner. In the past 15 years, most genes known to be involved in primary hemostasis, secondary hemostasis and fibrinolysis have indeed been targeted in mice, resulting in complete deficiencies, mutated gene products or altered protein levels, and these models have been extensively reviewed elsewhere.[3-11]

This review will focus on genetic mouse models for venous thrombosis and therefore we evaluate the results of deletions and modifications of natural anticoagulant factors, as they are key elements in altering the venous thrombotic risk. Gene modifications in murine tissue factor pathway inhibitor, antithrombin and the proteins involved in the protein C pathway, including protein C, thrombomodulin, the endothelial protein C receptor and factor V (factor V Leiden) yielded mouse lines with spontaneous thrombotic phenotypes. These phenotypes

range from mildly enhanced tissue fibrin deposition, to lethal thrombosis-induced consumptive coagulopathy.
With the generation of these mouse lines, better opportunities became available for investigating both existing and novel risk factors for venous thrombosis. In addition, these models could serve as a tool for evaluating novel antithrombotic strategies. Here we will summarize the mouse models that lack or carry mutant anticoagulant factors and display a spontaneous thrombotic phenotype. Furthermore, we evaluate whether these mouse models based on genetic modification have fulfilled the expectations, i.e. whether these models contributed to our understanding of risk factors that modulate the venous thrombotic risk, and if they have provided novel strategies for evaluating antithrombotic therapies.

Tissue factor pathway inhibitor

Tissue factor pathway inhibitor (TFPI) directly inhibits activated factor X (FXa), and subsequently inhibits the tissue factor/factor VIIa (TF/FVIIa) complex which is responsible for the initiation of coagulation. Mice deficient in TFPI were generated by disruption of the Kunitz domain 1 ($TFPI^{K1-/-}$), which is required for TF/FVIIa inhibition.[12-14] Homozygosity for this mutation results in embryonic lethality and examination of $TFPI^{K1-/-}$ embryos revealed a severe thrombotic phenotype including signs of yolk sac hemorrhage, immunoreactive fibrin(ogen) in the liver and intravascular thrombi, symptoms that are indicative for a consumptive coagulopathy. Mice heterozygous for the TFPI mutation have plasma TFPI activity levels about 50% of wild-type mice, are fertile and do not display a thrombotic phenotype.[12-14]
Chan et al. showed that the survival rate of $TFPI^{K1-/-}$ mice depends on the level of FVII.[15] When FVII levels are decreased genetically - by crossing with FVII knock-out mice - homozygous TFPI deficient mice survive the embryonic period. However, after birth the combined knock-out still succumbs due to a perinatal consumptive coagulopathy. In line with this study, it appeared that a low level of tissue factor (TF) (obtained by crossing the TFPI mutant mice with mice expressing a low levels of human TF) also rescues the intrauterine lethality of TFPI deficient embryos.[15,16] Surprisingly, in contrast to the perinatal death observed in TFPI deficient mice having a decreased FVII level, $TFPI^{-/-}$/low TF mice are viable and show no signs of thrombosis. Also, $TFPI^{-/-}$/low TF mice do not show signs of hemorrhage in the lung nor cardiac fibrosis, which is seen in pure low TF mice.

These studies demonstrated that the severe thrombotic phenotype seen in tissue factor pathway inhibitor deficient mice can partly be rescued by restoring the hemostatic balance, i.e. by lowering tissue factor or FVII levels.

Antithrombin

Antithrombin (AT) is a serine protease inhibitor that inactivates several coagulation proteases that are generated during blood coagulation, particularly thrombin and FXa. AT deficient mice ($AT^{-/-}$) die in utero and the embryos show extensive subcutaneous and intracranial hemorrhages. Fibrin deposition was present in the liver and myocardium but no fibrin was present at the site of hemorrhage, which might be due to consumptive coagulopathy and/or liver dysfunction, indicating a severe thrombotic phenotype.[17]

Although $AT^{+/-}$ mice have significantly reduced plasma antithrombin antigen and activity levels, their external appearance is normal and they do not show signs of spontaneous thrombosis.[17] However, an additional thrombogenic stimulus results in a mild thrombotic phenotype with increased tissue fibrin deposition as compared to challenged wild-type mice.[18]

Because heparin is an important enhancer of antithrombin activity, mice have been generated that carry a mutation in the heparin binding site of AT, which corresponds to the R47 mutation frequently seen in AT deficient patients.[19] Mice homozygous for this mutation ($AT^{m/m}$) are born, but exhibit extensive fibrin deposition and develop spontaneous life-threatening thrombosis, predominantly in the heart and liver.

To evaluate whether diminished tissue factor activity could rescue the hypercoagulability of $AT^{-/-}$ mice, AT deficiency was introduced on a low TF background.[20] However, the resulting $AT^{-/-}$/low TF mice die in utero and exhibit disseminated thrombosis in the liver and heart.

The rather mild thrombotic phenotype of $AT^{+/-}$ mice and the more severe phenotype of $AT^{m/m}$ mice, make these mice a potentially suitable model for studying risk factors of thrombosis and antithrombotic strategies. Until now, only $AT^{m/m}$ mice have been used for this purpose. Dewerchin et al. have shown that the human monoclonal anti-FVIII antibody LCL-mAb-LE2E9 efficiently prevents thrombosis (presented as priapism) in male $AT^{m/m}$ mice, indicating that FVIII is a potential target for anticoagulation therapy.[21]

Protein C pathway

The protein C pathway consists of two circulating proteins, protein C (PC) and protein S, and two endothelial transmembrane proteins, thrombomodulin (TM) and the endothelial protein C receptor (EPCR). Furthermore, the factor V Leiden (FVL) mutation also affects the protein C pathway as FVL leads to an increased APC resistance. All of these genes, except protein S, have been inactivated and modified in mice, and will be discussed below.

Protein C

Activated protein C, in association with its cofactor protein S, can cleave FVIIIa and FVa, thereby inhibiting coagulation. Homozygous PC deficient embryos show early signs of thrombosis, beginning at the embryonic age of 12.5 days (E12.5).[22] Although some $PC^{-/-}$ pups are born, they do not survive beyond 24 hours after delivery. Microscopically, scattered microvascular thrombosis in the brain and focal liver necrosis was seen, as well as hemorrhages in the brain. This combination of thrombosis and bleeding in the brain is indicative of a consumptive coagulopathy.

Whereas complete PC deficiency leads to embryonic lethality, $PC^{+/-}$ mice appear healthy and reproduce normally.[22] As is seen in heterozygous AT deficient mice, heterozygous PC deficient mice also exhibit more pronounced intravascular fibrin deposition, but only after a thrombogenic stimulus.[23]

Mice with severe PC deficiency (PC levels less than 4%; these mice were generated by introducing a PC gene construct back into the PC deficient mice) survive beyond birth and develop spontaneous thrombosis characterized by large thrombi and fibrin depositions in the lungs, liver and heart.[24] The survival of these mice, and the onset and severity of the thrombotic phenotype, strongly depends on the actual plasma PC levels.

To determine whether the coagulopathy in $PC^{-/-}$ embryos can be compensated by additional deletion of the FVII gene, double deficient mice were generated.[25] $PC^{-/-}$ embryos with heterozygous FVII deficiency are born but die of consumptive coagulopathy. Double homozygous deficient embryos show from day E12.5 intra- and extravascular coagulopathy, resulting in hemorrhages and immediate death after birth.

However, PC deficient mice can be rescued from embryonic lethality by a combination with FXI deficiency.[26] The resulting double homozygous mice can live up to 3 months but eventually die of thrombotic diseases as is demonstrated by massive systemic fibrin depositions.

Taken together, these data show that interruption of the protein C pathway by deleting protein C itself generates several prothrombotic phenotypes. These phenotypes range from a slightly increased thrombotic risk as is seen in $PC^{+/-}$ mice, to severe spontaneous thrombotic events present in mice with very low PC levels. Several gene-gene interactions have been studied, resulting in the rescue of completely PC deficient mice when it is combined with FXI deficiency, although these mice develop spontaneous thrombosis later in life. This has led to the opportunity to study protein C deficiency in adult mice, and suggests a role for the intrinsic pathway to manipulate the thrombotic outcome.

Endothelial protein C receptor

EPCR can bind protein C, thereby facilitating the rate of PC activation by the thrombin-thrombomodulin complex. Total EPCR deficiency leads to intrauterine death.[27] It has been suggested that thrombosis at the maternal-embryonic interface might contribute to embryonic death, as the developing placenta is positively stained for fibrin.

$EPCR^{+/-}$ mice develop normally, appear healthy and do not show apparent thrombotic complications upon a thrombogenic stimulus.[27,28] In contrast to the $EPCR^{-/-}$ embryos, mice with very low EPCR levels are viable, and do not show increased fibrin deposition after a challenge as compared to wild-type mice.[29]

In an attempt to determine the cause of the embryonic lethality, Li et al. used conditional knock-out strategies to selectively express EPCR in the embryo or in the placenta.[30] Embryonic EPCR expression in the absence of placental expression could not circumvent the embryonic lethality. In contrast, ablation of EPCR in the embryo but not in the placenta resulted in viable EPCR deficient mice with an age-dependent spontaneous thrombotic phenotype. In addition, the combination of EPCR deficiency with low TF activity (less than 1%) is also able to rescue EPCR deficient mice by preventing local thrombin generation at the maternal-embryonic interface.[30]

Summarizing, EPCR expression in the placenta (in particular trophoblast cells) is essential for the maintenance of pregnancy, as only embryonic EPCR can be selectively knocked-out without resulting in embryonic lethality. A low level of EPCR expression is able to maintain a normal phenotype and only a total deficiency of EPCR results in a severe thrombotic phenotype.

Table 1: transgenic mouse models of venous thrombosis.

Gene	Genotype¶	Thrombotic phenotype#	Reference
Tissue factor pathway inhibitor	$TFPI^{K1-/-}$	Embryonic lethality, consumptive coagulopathy	12,13
	$TFPI^{K1+/-}$	Normal phenotype	12,13
	$TFPI^{K1-/-}/FVII^{+/-}$ or $TFPI^{K1-/-}/FVII^{-/-}$	Embryonic lethality, consumptive coagulopathy	15
	$TFPI^{K1-/-}$/low TF	Normal phenotype	16
Antithrombin	$AT^{-/-}$	Embryonic lethality, consumptive coagulopathy	17
	$AT^{+/-}$	Normal phenotype, unless challenge	18
	$AT^{m/m}$	Fatal thrombosis at later age	19
	$AT^{-/-}$/low TF	Embryonic lethality, consumptive coagulopathy	20
Protein C	$PC^{-/-}$	Embryonic lethality, consumptive coagulopathy	22
	$PC^{+/-}$	Normal phenotype, unless challenge	23
	Low PC (<4%)	Fatal thrombosis at later age	24
	$PC^{-/-}/FVII^{-/-}$ or $PC^{-/-}/FVII^{+/-}$	Perinatal death, consumptive coagulopathy	25
	$PC^{-/-}/FXI^{-/-}$	Fatal thrombosis at later age	26
Endothelial protein C receptor	$EPCR^{-/-}$	Embryonic lethality, placental thrombosis	27
	$EPCR^{+/-}$	Normal phenotype	27
	Low EPCR	Normal phenotype	29
	$EPCR^{-/-}$/low TF	Normal phenotype, unless challenge	30
Thrombomodulin	$TM^{-/-}$	Embryonic lethality, developmental retardation	31,32
	$TM^{+/-}$	Normal phenotype	31,32
	$*TM^{pro/pro}$	Fibrin deposition	32-34
	TMLox	Partial embryonic lethality, Fatal thrombosis at later age	38
Factor V Leiden	$* FV^{Q/Q}$	Fibrin deposition	42
	$FV^{Q/Q}/FVIII^{-/-}$ or $FV^{Q/Q}/FIX^{-/-}$	Restore ability clot formation	43
	$FV^{Q/Q}/GLA^{-/-}$	Fibrin deposition, non-fatal occlusive thrombi	44
	$FV^{Q/Q}/TFPI^{+/-}$	Perinatal lethality	45
	$FV^{Q/Q}/PZ^{-/-}$	Perinatal lethality	46-48

¶ Additional abbreviations: $TFPI^{K1}$: tissue factor pathway inhibitor with a disrupted Kunitz 1 domain; low TF: reduced tissue factor levels; $AT^{m/m}$: antithrombin with R48C mutation; $TM^{pro/pro}$: thrombomodulin with E387P mutation; TMLox: endothelium-specific thrombomodulin loss; $FV^{Q/Q}$: factor V with R504Q mutation (factor V Leiden); GLA: α-galactidose A; PZ: protein Z.
# Normal phenotype implicates no thrombosis present, evaluated or challenged. * Phenotypes depend on genetic strain.

Thrombomodulin

Endothelial membrane bound thrombomodulin (TM) forms a complex with thrombin and inhibits the amplification loop of the coagulation cascade by activating protein C. Mice with a complete TM deficiency die before embryonic day 9.5 and it is suggested that this is due to an inhibition of growth and development, secondary to a defect in the parietal yolk sac.[31,32] $TM^{+/-}$ mice show a 50% reduction in TM levels compared to wild-type mice, but they appear normal and are free of thrombotic events.[31,32]

The knock-in of a TM mutation at position 387 (glutamic acid to proline), which is thought to cause a loss of protein C co-factor activity, bypasses embryonic lethality.[32] The $TM^{pro/pro}$ mutation leads to a severely reduced capacity to generate activated PC and to inhibit thrombin. This in turn results in a mild thrombotic phenotype with fibrin depositions in the spleen, heart and lung.[32-35] Overt thrombosis occurs only in the presence of an additional pathological challenge.[34,36] Interestingly, these results depend on the genetic background of the mouse. $TM^{pro/pro}$ mice with a mixed 129Sv/C57BL/6J background show intravascular fibrin depositions whereas these depositions are not present in $TM^{pro/pro}$ mice with a C57BL/6J background.[34]

Using tetraploid aggregation, Isermann et al. showed that restoring TM expression in extraembryonic tissue can rescue $TM^{-/-}$ mice from early embryonic lethality. However, absence of TM from the endothelium still causes late embryonic lethality due to consumptive coagulopathy.[37] Furthermore, using a conditional (Cre/LoxP) approach the same research group showed that the endothelium-specific loss of TM results in partial embryonic lethality.[38,39] Mice that survive to birth, and beyond, are born healthy and lack a visible thrombotic phenotype until the age of two to three weeks. After this period, all mutant mice develop severe thrombosis, terminating in a lethal consumptive coagulopathy.

In conclusion, these studies have been extremely valuable in unraveling the functions of thrombomodulin. As the thrombotic phenotype of $TM^{pro/pro}$ mice is variable and dependent on interaction with secondary genetic modifiers, this model is attractive as a tool for future research on risk factors that modulate the venous thrombotic risk and to study novel strategies for antithrombotic therapies.

Factor V Leiden

Normally, factor V (FV) is degraded by activated protein C (APC), but due to an arginine to glutamine substation at position 506 in the human FV gene (also known

as the factor V Leiden (FVL) mutation[40]), FV is more resistant to the actions of APC. This mutation of the FV gene is the most common genetic risk factor for venous thrombosis in humans.

In 1998 Yang et al. showed that the murine R504Q mutation in the FV gene, which corresponds to the human FVL mutation, results in partial APC resistance.[41] Cui et al. generated mice carrying this homologous mutation in the FV gene by a gene-targeting knock-in approach.[42] Adult hetero- and homozygous mice ($FV^{+/Q}$ and $FV^{Q/Q}$) are fertile and exhibit normal survival, but $FV^{Q/Q}$ mice reveal evidence for chronic low-grade thrombin generation, resulting in fibrin deposition in multiple tissues. As with the $TM^{pro/pro}$ mice, the genetic background of the mouse is important for the thrombotic phenotype, with the mixed 129Sv/C57BL/6J background being more prone to developing fibrin depositions than C57BL/6J mice.

$FV^{Q/Q}$ mice have been crossed with hemophilia A ($FVIII^{-/-}$) and hemophilia B ($FIX^{-/-}$) mice.[43] The hemophilia mice show spontaneous hemorrhages and are not able to form thrombi after vascular injuries. However, an additional FVL mutation ($FV^{+/Q}$ or $FV^{Q/Q}$) can restore the ability to form a thrombus after injury.

Fabry disease, a deficiency in α-galactidose A (GLA), is associated with premature vascular events that, in human, may be thrombotic in nature.[44] When $GLA^{-/-}$ mice are mated with $FV^{Q/Q}$ mice, the offspring shows greatly increased fibrin deposition and occlusive thrombus formation as compared with mice with only one defect. This indicates a synergistic interaction between GLA deficiency and factor V Leiden.

Eitzman et al. demonstrated that a modest reduction in TFPI levels, which is by itself not sufficient to cause a thrombotic phenotype, combined with $FV^{Q/Q}$ is nearly completely fatal in the early perinatal period.[45] $FV^{Q/Q}$/ $TFPI^{+/-}$ mice show increased fibrin deposition, suggesting disseminated thrombosis. The same holds for protein Z (PZ) deficiency: $PZ^{-/-}$ mice appear healthy and have no signs of vascular thrombosis or hepatic fibrin depositions, whereas in combination with $FV^{Q/Q}$ mutation intrauterine and perinatal thrombosis results, eventually leading to mortality.[46-48]

Recently, Sood et al. described a mouse model in which TM and EPCR variants are experimentally validated as modulators of pregnancy success in $FV^{Q/Q}$ mothers.[49] It appears that the interaction between these thrombotic risk factors has different consequences on disease manifestation, depending on the vascular bed in which the interaction occurs.

In the FVL mouse model, the severity and phenotypic expression of spontaneous thrombosis is proven to be variable and dependent on interaction with secondary genetic or environmental modifiers, including the genetic background of the mouse, FVIII, FIX, α-galactidose, TFPI and protein Z. Thus, the FVL model represents important aspects of thrombophilia and thrombosis in humans, and has proven to be valuable to validate existing, and develop novel, concepts of disease mechanisms in human.

Discussion

Mouse models that lack or carry mutant anticoagulant factors yielded several insights regarding the role of these factors and provided several mouse models that display a spontaneous thrombotic phenotype. In mice, complete deficiencies of natural anticoagulants results in many cases in embryonic lethality, mostly related to hemodynamic dysfunction. This demonstrates that anticoagulant factors are crucial for embryonic survival, and explains why complete deficiencies are rare in humans.[3] In contrast to complete deficiencies, heterozygous deficiencies coincide with normal survival. For antithrombin and protein C, deletion of one allele results in an increased thrombotic tendency, however, only when an additional thrombotic stimulus is applied. These latter two models, as well as the $TM^{pro/pro}$ and $FV^{Q/Q}$ mice, display a mild thrombotic phenotype characterized by tissue fibrin deposition as a sign of chronic low-grade thrombosis.

To circumvent embryonic lethality, different models have been developed, resulting in viable mice with a strong thrombotic tendency or a spontaneous thrombotic phenotype. These models include the homozygous mutation in the heparin binding site of AT, severe protein C deficiency, combined complete PC/FXI deficiency, $TM^{pro/pro}$ and deletion of TM expression on endothelial cells. Interestingly, some of the thrombotic phenotypes in these models are dependent on the genetic background of the mice.

The generation of genetic thrombosis models was expected to provide better opportunities to study risk factors for venous thrombosis, and novel antithrombotic drugs. The question is whether the models discussed in this review indeed have fulfilled these expectations. Severe spontaneous thrombosis models can be useful for testing new antithrombotic strategies. However, to our knowledge, Dewerchin et al. were the only to employ such a model for evaluating the use of a monoclonal anti-FVIII antibody in preventing thrombosis.[21]

Of the mouse thrombosis models that followed from deletion and/or mutation of anticoagulant factors, the $FV^{Q/Q}$ model appears to be particularly successful in studying risk factors for venous thrombosis. The $FV^{Q/Q}$ model demonstrates that the intensity of the thrombotic phenotype can be modified by other apparently neutral prothrombotic factors. Examples are TFPI and protein Z; since they emerged as candidate modifying genes for thrombotic risk in human FV Leiden carriers.

It is possible that the use of mice displaying a spontaneous thrombotic phenotype will become more popular in studying gene-gene and gene-environment interactions. For example, pro- and anti-inflammatory genes are potential candidate genes in modulating thrombotic risk, and prothrombotic mice like $AT^{+/-}$, $PC^{+/-}$, $TM^{pro/pro}$ and $FV^{Q/Q}$ mice could be of great value in a first validation of these candidate genes. In addition, epidemiological findings in large case-control studies have yielded several environmental risk factors, like estrogens, progestins, obesity, pregnancy and malignancies. Perhaps it is possible to recapitulate the effect of these risk factors in prothrombotic mice, which would give a unique opportunity to dissect the underlying mechanisms.

A major breakthrough in the application of mice displaying spontaneous thrombotic phenotypes could also come from whole genome mutagenesis screens that are set up to identify genes that counteract the thrombotic phenotype. In these studies mice with a lethal prothrombotic phenotype are randomly mutagenized, in the expectation that some mutations will rescue the mice. To our knowledge such screens are currently ongoing but have not been published in full.[50] In the future, these screening programs can provide additional information on protective gene-gene and gene-environment interactions. This is of particular interest, as the mouse models also have taught us that thrombosis is a multi-causal disease that not only depends on prothrombotic risk factors but also on protective factors like the deficiency in a procoagulant factor.

Although the use of genetically altered mice in thrombosis research was thought to be a major breakthrough, providing excellent opportunities to study the interaction between different risk factors and to study novel antithrombotic strategies, we consider their application at present limited. However, we are convinced of the potential of these models and think that the limited use of these models is because they are time-consuming and labour-intensive, rather than that these are inappropriate for studying venous thrombosis.

References

1 Day SM, Reeve JL, Myers DD, et al. Murine thrombosis models. Thromb Haemost 2004;92:486-94.

2 Dorffler-Melly J, Schwarte LA, Ince C, et al. Mouse models of focal arterial and venous thrombosis. Basic Res Cardiol 2000;95:503-9.

3 Hogan KA, Weiler H, Lord ST. Mouse models in coagulation. Thromb Haemost 2002;87:563-74.

4 Mackman N. Mouse models in haemostasis and thrombosis. Thromb Haemost 2004;92:440-3.

5 Emeis JJ, Jirouskova M, Muchitsch EM, et al. A guide to murine coagulation factor structure, function, assays, and genetic alterations. J Thromb Haemost 2007;5:670-9.

6 Carmeliet P, Moons L, Collen D. Mouse models of angiogenesis, arterial stenosis, atherosclerosis and hemostasis. Cardiovasc Res 1998;39:8-33.

7 Sood R, Weiler H. Embryogenesis and gene targeting of coagulation factors in mice. Best Pract Res Clin Haematol 2003;16:169-81.

8 Mackman N. Tissue-specific hemostasis in mice. Arterioscler Thromb Vasc Biol 2005;25:2273-81.

9 Tsakiris DA, Scudder L, Hodivala-Dilke K, et al. Hemostasis in the mouse (Mus musculus): a review. Thromb Haemost 1999;81:177-88.

10 Preissner KT. Hemostatic protease receptors and endothelial cell function: insights from gene targeting in mice. Semin Thromb Hemost 2000;26:451-62.

11 Car BD, Eng VM. Special considerations in the evaluation of the hematology and hemostasis of mutant mice. Vet Pathol 2001;38:20-30.

12 Huang ZF, Broze G, Jr. Consequences of tissue factor pathway inhibitor gene-disruption in mice. Thromb Haemost 1997;78:699-704.

13 Huang ZF, Higuchi D, Lasky N, et al. Tissue factor pathway inhibitor gene disruption produces intrauterine lethality in mice. Blood 1997;90:944-51.

14 Dusse LM, Carvalho MG, Cooper AJ, et al. Tissue factor and tissue factor pathway inhibitor: a potential role in pregnancy and obstetric vascular complications? Clin Chim Acta 2006;372:43-6.

15 Chan JC, Carmeliet P, Moons L, et al. Factor VII deficiency rescues the intrauterine lethality in mice associated with a tissue factor pathway inhibitor deficit. J Clin Invest 1999;103:475-82.

16 Pedersen B, Holscher T, Sato Y, et al. A balance between tissue factor and tissue factor pathway inhibitor is required for embryonic development and hemostasis in adult mice. Blood 2005;105:2777-82.

17 Ishiguro K, Kojima T, Kadomatsu K, et al. Complete antithrombin deficiency in mice results in embryonic lethality. J Clin Invest 2000;106:873-8.

18 Yanada M, Kojima T, Ishiguro K, et al. Impact of antithrombin deficiency in thrombogenesis: lipopolysaccharide and stress-induced thrombus formation in heterozygous antithrombin-deficient mice. Blood 2002;99:2455-8.

19 Dewerchin M, Herault JP, Wallays G, et al. Life-threatening thrombosis in mice with targeted Arg48-to-Cys mutation of the heparin-binding domain of antithrombin. Circ Res 2003;93:1120-6.

20 Hayashi M, Matsushita T, Mackman N, et al. Fatal thrombosis of antithrombin-deficient mice is rescued differently in the heart and liver by intercrossing with low tissue factor mice. J Thromb Haemost 2006;4:177-85.
21 Dewerchin M, Van der Elst L, Singh I, et al. Inhibition of factor VIII with a partially inhibitory human recombinant monoclonal antibody prevents thrombotic events in a transgenic model of type II HBS antithrombin deficiency in mice. J Thromb Haemost 2004;2:77-84.
22 Jalbert LR, Rosen ED, Moons L, et al. Inactivation of the gene for anticoagulant protein C causes lethal perinatal consumptive coagulopathy in mice. J Clin Invest 1998 ;102:1481-8.
23 Levi M, Dorffler-Melly J, Reitsma P, et al. Aggravation of endotoxin-induced disseminated intravascular coagulation and cytokine activation in heterozygous protein-C-deficient mice. Blood 2003;101:4823-7.
24 Lay AJ, Liang Z, Rosen ED, et al. Mice with a severe deficiency in protein C display prothrombotic and proinflammatory phenotypes and compromised maternal reproductive capabilities. J Clin Invest 2005;115:1552-61.
25 Chan JC, Cornelissen I, Collen D, et al. Combined factor VII/protein C deficiency results in intrauterine coagulopathy in mice. J Clin Invest 2000;105:897-903.
26 Chan JC, Ganopolsky JG, Cornelissen I, et al. The characterization of mice with a targeted combined deficiency of protein c and factor XI. Am J Pathol 2001;158:469-79.
27 Gu JM, Crawley JT, Ferrell G, et al. Disruption of the endothelial cell protein C receptor gene in mice causes placental thrombosis and early embryonic lethality. J Biol Chem 2002;277:43335-43.
28 Zheng X, Li W, Gu JM, et al. Effects of membrane and soluble EPCR on the hemostatic balance and endotoxemia in mice. Blood 2007;109:1003-9.
29 Castellino FJ, Liang Z, Volkir SP, et al. Mice with a severe deficiency of the endothelial protein C receptor gene develop, survive, and reproduce normally, and do not present with enhanced arterial thrombosis after challenge. Thromb Haemost 2002;88:462-72.
30 Li W, Zheng X, Gu JM, et al. Extraembryonic expression of EPCR is essential for embryonic viability. Blood 2005;106:2716-22.
31 Healy AM, Rayburn HB, Rosenberg RD, et al. Absence of the blood-clotting regulator thrombomodulin causes embryonic lethality in mice before development of a functional cardiovascular system. Proc Natl Acad Sci U S A 1995;92:850-4.
32 Rosenberg RD. Thrombomodulin gene disruption and mutation in mice. Thromb Haemost 1997;78:705-9.
33 Weiler-Guettler H, Christie PD, Beeler DL, et al. A targeted point mutation in thrombomodulin generates viable mice with a prethrombotic state. J Clin Invest 1998;101:1983-91.
34 Weiler H, Lindner V, Kerlin B, et al. Characterization of a mouse model for thrombomodulin deficiency. Arterioscler Thromb Vasc Biol 2001;21:1531-7.
35 Weiler H, Isermann BH. Thrombomodulin. J Thromb Haemost 2003;1:1515-24.
36 Dorffler-Melly J, de Kruif M, Schwarte LA, et al. Functional thrombomodulin deficiency causes enhanced thrombus growth in a murine model of carotid artery thrombosis. Basic Res Cardiol 2003;98:347-52.
37 Isermann B, Hendrickson SB, Hutley K, et al. Tissue-restricted expression of thrombomodulin in the placenta rescues thrombomodulin-deficient mice from early lethality and reveals a secondary developmental block. Development 2001;128:827-38.

38 Isermann B, Hendrickson SB, Zogg M, et al. Endothelium-specific loss of murine thrombomodulin disrupts the protein C anticoagulant pathway and causes juvenile-onset thrombosis. J Clin Invest 2001;108:537-46.
39 Weiler H. Mouse models of thrombosis: thrombomodulin. Thromb Haemost 2004;92:467-77.
40 Bertina RM, Koeleman BP, Koster T, et al. Mutation in blood coagulation factor V associated with resistance to activated protein C. Nature 1994;369:64-7.
41 Yang TL, Cui J, Rehumtulla A, et al. The structure and function of murine factor V and its inactivation by protein C. Blood 1998;91:4593-9.
42 Cui J, Eitzman DT, Westrick RJ, et al. Spontaneous thrombosis in mice carrying the factor V Leiden mutation. Blood 2000;96:4222-6.
43 Schlachterman A, Schuettrumpf J, Liu JH, et al. Factor V Leiden improves in vivo hemostasis in murine hemophilia models. J Thromb Haemost 2005;3:2730-7.
44 Shen Y, Bodary PF, Vargas FB, et al. Alpha-galactosidase A deficiency leads to increased tissue fibrin deposition and thrombosis in mice homozygous for the factor V Leiden mutation. Stroke 2006;37:1106-8.
45 Eitzman DT, Westrick RJ, Bi X, et al. Lethal perinatal thrombosis in mice resulting from the interaction of tissue factor pathway inhibitor deficiency and factor V Leiden. Circulation 2002;105:2139-42.
46 Yin ZF, Huang ZF, Cui J, et al. Prothrombotic phenotype of protein Z deficiency. Proc Natl Acad Sci U S A 2000;97:6734-8.
47 Corral J, Gonzalez-Conejero R, Hernandez-Espinosa D, et al. Protein Z/Z-dependent protease inhibitor (PZ/ZPI) anticoagulant system and thrombosis. Br J Haematol 2007;137:99-108.
48 Broze GJ, Jr. Protein Z-dependent regulation of coagulation. Thromb Haemost 2001;86:8-13.
49 Sood R, Zogg M, Westrick RJ, et al. Fetal gene defects precipitate platelet-mediated pregnancy failure in factor V Leiden mothers. J Exp Med 2007 Apr 16.
50 Westrick RJ, Manning SL, Dobies SL, et al. A Sensitized Genome-Wide ENU Mutagenesis Screen in the Mouse to Identify Genetic Modifiers of Thrombosis. ASH Annual Meeting Abstracts 2004;104:2.

Chapter 3

17α-Ethinylestradiol rapidly alters transcript levels of murine coagulation genes via estrogen receptor α

Audrey CA Cleuren, Irma K van der Linden,
Yvonne P de Visser, Gerry TM Wagenaar, Pieter H Reitsma,
Bart JM van Vlijmen

J Thromb Haemost. 2010 Aug;8(8):1838-1846

Summary

Background: Oral estrogen use is associated with changes in plasma levels of many coagulation proteins.

Objective: To gain more insight in the underlying mechanism of estrogen-induced changes in coagulation.

Methods: Ovariectomized female mice were used to study the impact of oral 17α-ethinylestradiol (EE) on plasma coagulation, hepatic coagulation gene transcript levels, and dependence on estrogen receptor (ER) α and β.

Results: 10 day oral EE treatment resulted in significant reduced plasma activity levels of factor (F) VIII, XII, combined FII/VII/X and antithrombin, while FIX activity significantly increased. Regarding hepatic transcript levels, oral EE caused significant decreases in fibrinogen-γ, FII, FV, FVII, FX, FXII, antithrombin, protein C, protein Z, protein Z inhibitor and heparin cofactor II mRNA levels, whereas FXI levels significantly increased and transcript levels of FVIII, FIX, protein S and α2-antiplasmin remained unaffected. All EE-induced coagulation-related changes were neutralized by co-administration of the non-specific estrogen receptor antagonist ICI182780. In addition, ERα deficient mice lacked the EE-induced changes in plasma coagulation and hepatic transcript profile whereas ERß deficient mice responded similarly as non-deficient littermate controls. A crucial role for the estrogen receptor was further demonstrated by its rapid effects on transcription, within 2.5 to 5 hours after EE administration, suggesting a short chain of events leading to its final effects.

Conclusions: Oral ethinylestradiol administration has a broad impact on the mouse coagulation profile at the level of both plasma and hepatic mRNA levels. The effects on transcription are rapidly induced, mostly down-regulating and principally mediated by estrogen receptor α.

Introduction

Estrogens in contraceptives and hormone replacement therapy cause changes in the plasma coagulation profile. These changes include increases in the levels of the procoagulant factors II, VII, IX, X, XII, XIII and fibrinogen, and reductions in the natural anticoagulants protein S and antithrombin, resulting in an increased risk for venous thrombosis (reviewed in [1,2]). Changes in coagulation were firstly identified in women taking oral contraceptives and not in women using transdermal patches,[3,4] suggesting that the first-pass effect by the liver is important for inducing these changes, presumably via affecting the transcription of hepatically expressed coagulation genes. However, several studies have demonstrated that ethinylestradiol-containing patches may also confer an increased venous thrombotic risk,[5,6] indicating that also the type of estrogen is of importance with respect to modulating coagulation and thereby the risk for venous thrombosis.

Estrogens largely mediate their effects via binding to the estrogen receptor (ER) α or β, which subsequently functions as a transcription factor by binding to estrogen response elements (EREs) in the promoter or enhancer regions of estrogen-responsive genes. Functional EREs have been identified with certainty for a limited number of coagulation factors, including factor VII and factor XII.[7,8] Recent human and mouse genome-wide searches for high-affinity estrogen response elements demonstrated that near-consensus EREs occur in many of the genes belonging to the pro- and anticoagulant pathways.[9] For the human genome, these include the liver-specific coagulation factors (F) II, FV, FVII, FIX, FX, FXI, FXII, protein S, protein Z and heparin cofactor II, whereas this list is less extensive for the mouse genome with FVII, FX, FXIII and α_2-antiplasmin. For the mouse, however, functional genome-wide screening of hepatic estrogen receptor α binding regions identified ERα-binding regions in the fibrinogen, factor II, factor XI, antithrombin, protein C, protein S, protein Z, plasminogen and heparin cofactor II genes.[10] These genome-wide results predict that estrogens potentially modulate transcription of a large number of coagulation genes expressed in the liver through the direct action of estrogen receptors. In this respect, it is surprising that coagulation-dedicated cDNA microarray analyses of livers of mice treated with estrogen revealed that none of the liver-specific coagulation genes were regulated by 17β-estradiol.[11]

Given the inconsistency between the results of these studies, we set out a series of *in vivo* experiments to directly study effects of oral 17α-ethinylestradiol (EE), the estrogenic and most thrombogenic component of oral contraceptives, on the plasma coagulation profile on the one hand, and the impact of a single and multiple

EE dose on hepatic coagulation gene transcription and its possible dependency on estrogen receptors on the other.

Methods

Animals

Heterozygous mice carrying an estrogen receptor α knockout allele (*Esr1*$^{+/-}$) or estrogen receptor ß knockout allele (*Esr2*$^{+/-}$) on a C57Black/6J background were obtained from the Jackson Laboratories (Bar Harbor, USA) and intercrossed to generate female mice with a complete receptor deficiency (*Esr1*$^{-/-}$ or *Esr2*$^{-/-}$ mice). To avoid possible baseline differences in plasma and mRNA expression levels due to the different origins of the mice, experiments with *Esr1* and *Esr2* mice included littermate wild-type controls as a reference (*Esr1*$^{+/+}$ or *Esr2*$^{+/+}$, respectively). Genotypes were confirmed by PCR analysis according to the protocol provided by the Jackson Laboratories. For experiments performed with wild-type female mice only, C57Bl/6J mice were purchased from Charles River (Maastricht, the Netherlands). All mice were housed under a 12-hour light/dark cycle and standard chow diet and drinking water were provided at libitum. At eight weeks of age, mice were bilaterally ovariectomized under isoflurane anaesthesia and after a two week recovery-period, they were randomly assigned to either the experimental or vehicle treatment group. For the *Esr1*$^{+/+}$ and *Esr1*$^{-/-}$ mice, ovariectomy was performed between an average of ten and twelve weeks of age.

Hormone treatment

Ethinylestradiol (EE) and ICI182780 (both from Sigma Aldrich) stocks were prepared in ethanol and subsequently diluted in arachid oil with a final concentration of 1% ethanol. For estrogen treatment designated as 'multiple doses', mice received a daily gavage of 1 µg ethinylestradiol in 100 µl arachid oil for 10 consecutive days. In experiments designated as 'single dose', EE was given only once. The non-specific estrogen receptor antagonist ICI182780 was injected subcutaneously in a daily dose of 100 µg per mouse, starting one day prior to the ethinylestradiol administration in the case of the combined treatment. For the vehicle treatment, mice received 100 µl arachid oil with an ethanol concentration of 1% either orally or subcutaneously, as appropriate. In addition, separate experiments were performed to determine whether alternative estrogen treatment protocols yielded essentially different results and included subcutaneous treatment of ovariectomized mice with 1 µg 17ß-estradiol/day for 10 days, or oral treatment

with 1 µg EE/mouse/day for 50 days. Furthermore, non-ovariectomized female mice were treated with 1 µg EE/mouse/day for 10 days to determine the additional effect of exogenous estrogen administration. After the last administration, mice were anesthetized by an intraperitoneal injection with a mixture of ketamine (100 mg/kg), xylazine (12.5 mg/kg) and atropine (125 µg/kg) after which the abdomen was opened by a midline incision and a blood sample on sodium citrate (final concentration 0.32%) was drawn from the inferior caval vein. Plasma was obtained as previously described,[12] and stored at -80°C until use. Part of the left liver lobule was isolated and snap-frozen for mRNA analyses and the uterus was collected and weighed to determine the biological activity of both EE and ICI182780.
All experimental procedures were approved by the animal welfare committee of the Leiden University.

Plasma analyses
Alkaline phosphatases (ALP), aspartate transaminase (AST) and alanine aminotransferase (ALT) levels were determined using routine clinical chemistry assays.
Plasma activity levels of factor VIII, IX, XI and XII were determined in an APTT-based assay[13] by mixing individual mouse plasmas with human plasma deficient for the respective factor (STA factor VIII from Diagnostica Stago, factor IX and factor XI deficient plasma from Biopool and HemosIL factor XII from Instrumentation Laboratories) and automated APTT-reagent (Biomerieux). Combined factor II/VII/X activity was assessed by using Thrombotest (Axis-Shield). For all these activity assays, clotting times were measured with a semi-automated coagulometer (ACL-300, Instrumentation Laboratories).
In addition to the combined activity, FII, FVII and FX were also measured separately. Factor II activity was determined after complete activation with ecarin (Sigma Aldrich) followed by determination of the FII amidolytic activity using the chromogenic substrate S2238 (Chromogenix). For FX activity, plasma was activated with Russell's Viper Venom (Haematologic Technologies Inc.) and activity was measured by S2765 substrate (Chromogenix) conversion. Factor VII and antithrombin activity were determined using commercially available kits (Biophen FVII, Hyphen Biomed and Coamatic Antithrombin kit, Chromogenix, respectively) according to the manufacturer's protocol. Plasma antithrombin antigen and fibrinogen antigen levels were assessed by using commercial murine ELISA kits from Affinity Biologicals.

For all plasma assays performed, mouse calibration curves paralleled human calibration curves, indicating specific reactions between mouse and human proteins. Pooled normal mouse plasma was used to generate standard curves which were used to calculate the activity or antigen levels and subsequently the wild-type vehicle-treated group was set as a reference (100%).

RNA isolation and real-time RT-PCR

Individual liver samples (20-30 mg) of 10 animals per group were homogenized in RNAzol (Tel-Test) and RNA isolation and cDNA synthesis was performed as previously described,[14] with minor adjustments. Gene specific QPCR primers were designed with the Primer Express software (Applied Biosystems) and are presented in supplemental table 1. Quantitative real-time PCR was performed on the ABI Prism 7900 HT Fast Real-Time PCR System from Applied Biosystems and data were analysed using the accompanying Sequence Detection System software. The comparative threshold cycle method with β-actin as internal control was used for quantification and normalization. The vehicle-treated wild-type group was set as a reference and the ΔC_t values of the individual samples were related to the mean ΔC_t of the reference group.

Statistical analyses

Data are expressed as mean ± standard error of the mean (SEM) or as the difference between the experimental and the vehicle-treated group with the standard error of the difference, as appropriate. Data were analysed with the GraphPad Instat software and statistical differences between groups were evaluated using a one-way analysis of variance (ANOVA) with a Dunnett post-hoc test to evaluate the dose-finding study and a Bonferroni post-hoc test in the case of experiments performed with ethinylestradiol and ICI182780. For the estrogen receptor deficient mice, a Student's t-test was used to compare the estrogen-treated with the vehicle-treated mice of the same genotype. P-values <0.05 were considered to be significant.

Results

Dose finding

A dose-finding study was performed as previously described[15] and an in depth analyses is presented here, showing a hormone-dependent increase in uterus wet weight (fig. 1A), with a dose of 0.1 µg EE/mouse/day restoring the uterus wet

weight in ovariectomized mice to that observed in non-ovariectomized female mice, thus mimicking the endogenous estrogen levels. Analyses of circulating liver enzyme levels revealed a slight, but significant increase in ALP and ALT levels in mice treated with 10 µg EE/day, whereas these levels, and the levels of AST were normal for all other EE doses tested (data not shown).

Oral EE dose-dependently increased factor IX activity (fig. 1B) whereas the levels of factor VIII, XII, combined factor II/VII/X (fig. 1C) and antithrombin antigen levels were decreased. For hepatic mRNA analyses, glutathione peroxidase 3 (Gpx3) and the mannose receptor (Mrc1) were used as positive controls, as it has been shown that these genes are estrogen-responsive,[16,17] and as expected a dose-dependent increase in hepatic transcript levels was observed (fig. 1D). The changes in plasma coagulation factor levels of combined FII/VII/X and antithrombin coincided with dose-dependent changes in hepatic mRNA levels of these factors (fig. 1E), while factor IX remained constant in transcript levels. In addition, although FXI activity in plasma was not significantly altered by EE treatment, mRNA levels were dose-dependently increased after EE administration (fig. 1F).

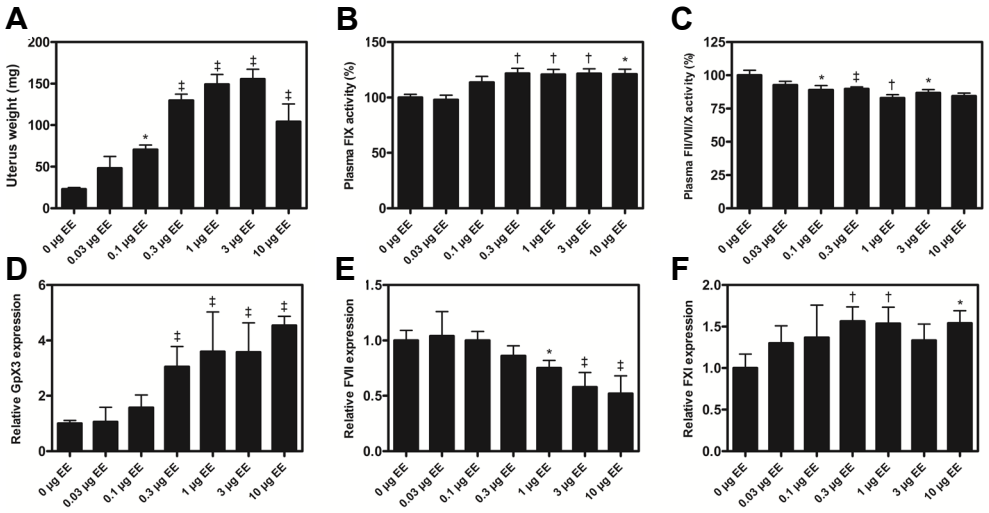

Figure 1: Dose-dependent effects of oral ethinylestradiol administration in ovariectomized female mice.

Effects of 10-day oral ethinylestradiol (EE) treatment on the uterus wet weight (A) and plasma coagulation factor activity levels (B-C) and hepatic transcript levels (D-F) in ovariectomized female mice (N=6 per group). Hepatic transcript levels are relative to ß-actin as an internal control and data are presented as mean±SEM. Original data presented in supplemental tables 2 and 3. *p-value<0.05, †p-value<0.01 and ‡p-value<0.001 versus vehicle-treated animals.

A dose of 1 µg EE/mouse/day was selected for further evaluation as this resulted in significant changes in the coagulation profile without affecting liver enzymes. This evaluation included comparison of the dose ethinylestradiol with a similar dose of the natural occurring 17ß-estradiol (subcutaneous injection) in ovariectomized mice, which resulted in comparable effects on uterus weight and plasma coagulation (data not shown). Furthermore, oral treatment of non-ovariectomized female mice with 1 µg EE/mouse/day caused comparable effects on plasma coagulation as observed under ovariectomized conditions, although these were less pronounced in general, and a 50-day treatment regime (1 µg EE/mouse/day) did also not yield essentially different results as compared to the 10-day treatment period (data not shown).

Estrogen receptor antagonist

We subsequently determined whether the effects on the plasma coagulation profile and transcript levels observed under multiple doses of oral ethinylestradiol were mediated by estrogen receptors α and/or β, by additionally treating mice daily with the non-specific estrogen receptor antagonist ICI182780 (n=15 mice per group). This compound was also active in the mouse, because the uterus wet weight of mice treated with both ICI182780 and EE reverted to that of vehicle-treated animals (10.6±0.5 mg vs. 9.5±0.6 mg for the vehicle treatment), whereas EE alone induced a significant increase in the uterus wet weight (107.8±3.9 mg; $p<0.0001$). ICI182780 alone had no effect (9.7±0.6 mg).

Plasma coagulation factor activity levels of FVIII, FIX, FXII and combined FII/VII/X and antithrombin, as well as the antithrombin antigen levels were significantly altered due to estrogen administration (fig. 2A), which was consistent with the results of the dose-finding. Co-administration with ICI182780 counteracted the ethinylestradiol-induced changes in plasma, whereas ICI182780 treatment alone had no effect on the plasma coagulation profile.

Figure 2B shows that the strong estrogen-induced up-regulation of Gpx3 and Mrc1 transcript levels was completely antagonized by ICI182780 co-administration. Estrogen alone caused a significant down-regulation in mRNA levels of fibrinogen-γ, FII, FV, FVII, FX, FXII and antithrombin, whereas factor XI transcript levels were significantly increased, which was again comparable to the changes previously observed in the dose-finding study. The panel of coagulation factors was extended and revealed significant reductions following EE administration in mRNA levels of plasminogen, protein C, protein Z, protein Z inhibitor and heparin cofactor II. Again,

ICI182780 co-administration counteracted the EE-induced changes in hepatic transcript levels, resulting in mRNA levels comparable to vehicle-treated animals (fig. 2B) and ICI182780 treatment alone had no effect on hepatic transcript levels. Protein C inhibitor and α2-macroglobulin mRNA levels were also measured but were present in too low amounts.

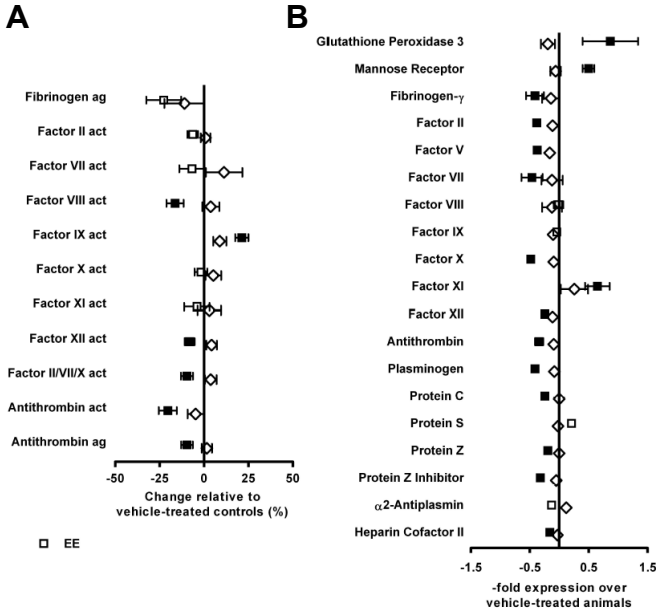

Figure 2: Hormone-induced effects on the coagulation profile of ovariectomized female mice. Differences between mice treated with 1µg ethinylestradiol (EE) alone as compared to vehicle-treated mice (□), or mice treated with 1µg EE and 100µg ICI182780 as compared to vehicle-treated animals◊), on the plasma coagulation profile (A; N=15 per group) and hepatic transcript levels (B; N=10 per group). Original data presented in supplemental tables 4 and 5. Data are presented as difference ± standard error of the difference; black symbols p<0.05 as compared to vehicle-treated controls (0µg EE / 0µg ICI182780). Ag: antigen level; act: activity level.

Recombinant mice

As the effects on the coagulation profile were sensitive to oral estrogen administration and an estrogen receptor antagonist was able to counteract these effects, experiments were repeated in ovariectomized mice lacking either estrogen receptor α (*Esr1*$^{-/-}$) or β (*Esr2*$^{-/-}$) to determine the contribution of the individual estrogen receptor subtypes in the EE-mediated changes of the coagulation profile. As expected, 10-day oral EE administration did not cause a significant increase in the uterus wet weight of *Esr1*$^{-/-}$ mice (uterus: 16.7±1.2 mg for the vehicle treatment vs. 16.3±1.4 mg for EE treatment), whereas it did in the *Esr1*$^{+/+}$ littermate controls (15.9±1.8 mg vs. 116.5±1.5 mg, p<0.0001). Upon EE administration, *Esr1*$^{-/-}$ mice displayed no effects on plasma coagulation factor levels, whereas the littermate *Esr1*$^{+/+}$ controls responded to EE with significant reduced FXI and increased FIX

activity levels (fig. 3A). Although the plasma coagulation profile of *Esr1*$^{+/+}$ mice was only modestly affected by ethinylestradiol treatment, evaluation of hepatic mRNA levels showed significant up-regulation of the estrogen-responsive GpX3 and Mrc1 transcripts (fig. 3B). In addition, hepatic mRNA levels of factor XI and protein S were also significantly higher in EE-treated *Esr1*$^{+/+}$ mice as compared to vehicle-treated animals of the same genotype. Fibrinogen-γ, factor V, VII and X, plasminogen, protein C, protein Z and α2-antiplasmin transcript levels were significantly down-regulated following EE treatment, thereby mimicking the observations in EE-treated wild-type C57Bl/6J mice. In contrast, for the *Esr1*$^{-/-}$ mice, no significant alterations were observed when comparing vehicle-treated to EE-treated mice (fig. 3B).

In the *Esr2*$^{-/-}$ mice, EE-induced effects on the uterus wet weight were comparable to those observed in the *Esr2*$^{+/+}$ mice (20.1±1.1 mg vs. 116.0±5.0 mg for *Esr2*$^{-/-}$ and 16.3±1.0 mg vs. 114.2±5.1 mg for *Esr2*$^{+/+}$, both p-values <0.0001). Figure 4A shows that oral ethinylestradiol administration resulted in comparable effects on the plasma coagulation profile for both genotypes and also with respect to hepatic transcript levels, *Esr2*$^{-/-}$ mice responded similarly to ethinylestradiol administration as their wild-type littermate controls (fig. 4B).

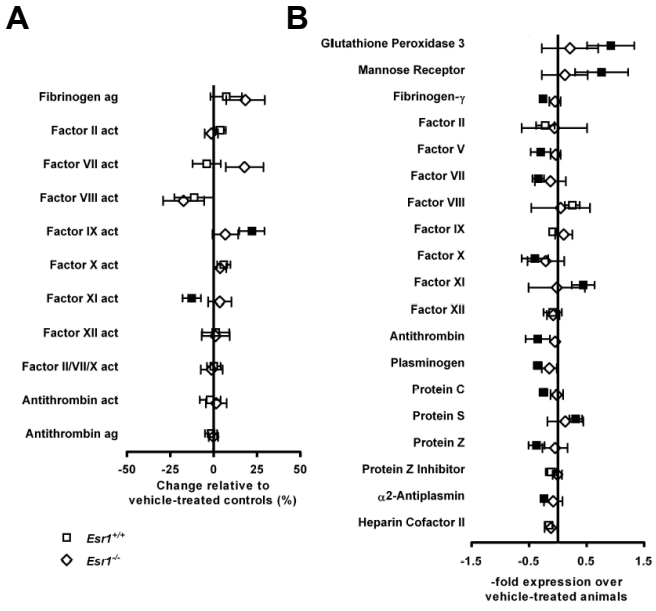

Figure 3: Ethinylestradiol-induced changes in the coagulation profile of ovariectomized female *Esr1*$^{+/+}$ and *Esr1*$^{-/-}$ mice.
Differences between *Esr1*$^{+/+}$ mice treated with 1µg ethinylestradiol (EE) as compared to vehicle-treated mice (□), or ovariectomized *Esr1*$^{-/-}$ mice treated with 1µg EE as compared to vehicle-treated animals (◊), on the plasma coagulation profile (A; N=12-15 per group) and hepatic transcript levels (B; N=10 per group). Original data presented in supplemental tables 6 and 7. Data are presented as difference ± standard error of the difference; black symbols p<0.05 as compared to vehicle-treated controls of the same genotype. Ag: antigen level; act: activity level.

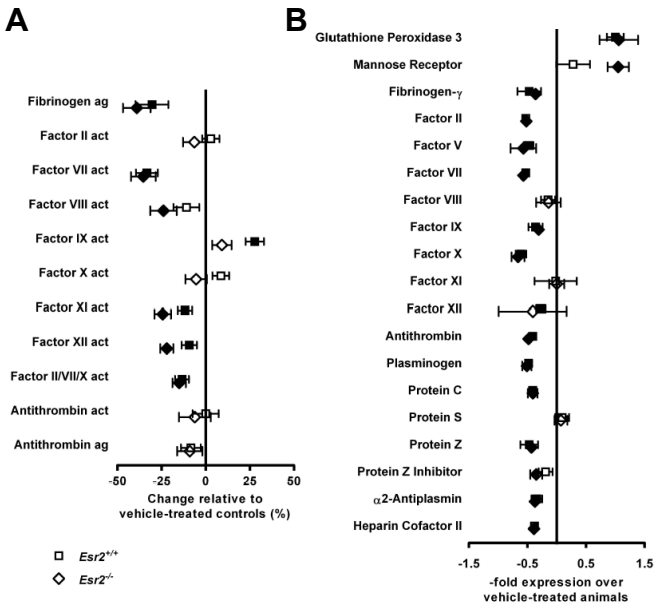

Figure 4: Ethinylestradiol-induced changes in the coagulation profile of ovariectomized female *Esr2*$^{+/+}$ and *Esr2*$^{-/-}$ mice.
Differences between ovariectomized *Esr2*$^{+/+}$ mice treated with 1µg ethinylestradiol (EE) as compared to vehicle-treated mice (□), or ovariectomized *Esr2*$^{-/-}$ mice treated with 1µg EE as compared to vehicle-treated animals (◊), on the plasma coagulation profile (A; N=13-15 per group) and hepatic transcript levels (B; N=10 per group). Original data presented in supplemental tables 8 and 9. Data are presented as difference ± standard error of the difference; black symbols $p<0.05$ as compared to vehicle-treated controls of the same genotype. Ag: antigen level; act: activity level.

Single EE dose

To extend the understanding of 17α-ethinylestradiol modulation of transcription of coagulation genes in the liver, we determined the immediate effects of EE on transcription in mice orally treated with a single dose of EE, alone or in combination with a subcutaneous ICI182780 injection. Hepatic transcript levels were determined 2.5 and 5 hours after administration and already 2.5 hours after the estrogen administration significant EE-induced changes were observed for fibrinogen-γ, FII, FIX, FX, antithrombin, plasminogen, protein C and protein S (fig. 5A). Evaluation of the hepatic transcript levels 5 hours after the EE treatment showed more pronounced effects of EE and ICI182780 for fibrinogen-γ, factor II, plasminogen and protein S when compared to the 2.5 hour time-point, and an additional down-regulation of FV, FVII, FXII, protein Z, protein Z inhibitor and α2-antiplasmin was observed, whereas factor XI mRNA levels were up-regulated (fig. 5B). All EE-induced effects were counteracted in the EE/ICI182780 treated animals. Comparing these results with the results after a 10-day treatment period demonstrates that only protein S acts differently, as transcript levels are reduced after a single dose, but increase after multiple EE doses.

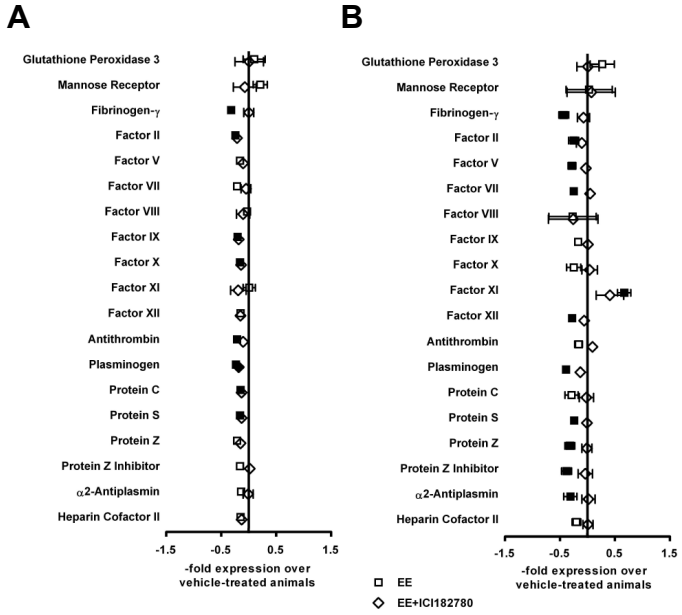

Figure 5: Short term hormone-induced effects on the coagulation profile of ovariectomized female mice.
Differences between ovariectomized mice treated with 1µg ethinylestradiol (EE) alone as compared to vehicle-treated mice (□), or mice treated with 1µg EE and 100µg ICI182780 as compared to vehicle-treated animals (◊), on hepatic transcript levels 2.5 hours after administration (A; N=6 per group) or 5 hours after administration (B; N=6 per group). Original data presented in supplemental table 10. Data are presented as difference ± standard error of the difference; black symbols $p<0.05$ as compared to vehicle-treated controls (0µg EE / 0µg ICI182780).

Discussion

In the present study, we demonstrate that oral administration of the synthetic estrogen 17α-ethinylestradiol to ovariectomized mice has a broad impact on the hepatic transcript levels of both pro- and anticoagulant genes. The 17α-ethinylestradiol effect on transcript levels of the coagulation genes was mostly down-regulating and, as analyzed for a limited set of factors, largely coincides with lowered plasma activity levels. Furthermore, the effects of 17α-ethinylestradiol on both the plasma and hepatic transcript levels of coagulation factors were dose-dependent, counteracted by the estrogen receptor antagonist ICI182780, absent in estrogen receptor α deficient mice, and already present shortly after oral administration. We conclude that in mice, 17α-ethinylestradiol has widespread and rapid effects on coagulation which requires the involvement of estrogen receptor α.
The results of the present study are in contrast with the study performed by Movérare et al. in which microarray analyses of liver samples from mice treated with 17β-estradiol did not yield altered hepatic coagulation gene transcript levels as compared to vehicle-treated mice.[11] We have no explanation for the difference in findings other than a difference in treatment protocol, i.e. subcutaneous injection of 2.3 µg 17β-estradiol benzoate/mouse/day for 5 days per week for 3 weeks versus

our daily oral administration of 1 µg 17α-ethinylestradiol for 10 days, or the fact that the cut-off value of at least 1.6-fold of the microarray data analyses might be too high to detect the subtle changes in coagulation gene transcript levels. In contrast, two recent genome-wide screens identified high-affinity estrogen receptor binding sites in the mouse liver for many genes belonging to both the pro- and anticoagulant pathway, including fibrinogen, factor II, V, VII, X, XI, antithrombin, plasminogen, protein C, protein S, protein Z, α2-antiplasmin and heparin cofactor II.[9,10] Thus, these genome-wide studies predict that estrogens can potentially modulate transcription of a large number of coagulation genes expressed in the liver through a direct interaction with estrogen receptors. Our study demonstrates that the transcription of these genes is indeed estrogen-responsive and controlled by ERα. However, our study does not allow the conclusion that the observed responses upon EE are the result of a direct interaction between ERα and the gene of interest, as predicted by the genome wide studies.

In humans, oral contraceptive use causes changes in the plasma coagulation profile, resulting in a prothrombotic shift of the hemostatic balance and hence an increased venous thrombotic risk. In mice however, the effects of oral ethinylestradiol on plasma and/or hepatic mRNA levels of procoagulant factors were unexpectedly mostly down-regulatory, with the exception of factor IX and XI. Therefore, we considered the possibility that our EE dose was outside the therapeutically relevant window, and the results being in part an artefact or side effect of a relatively high oral estrogen dose. However, several observations argue against such an artefact: although the liver weight of mice treated with 1 µg EE was increased as compared to the vehicle-treated mice, no effects on liver enzymes were observed and histological analyses of haematoxylin/eosin-stained liver sections did not yield morphological abnormalities like hepatocyte degeneration or infiltration of inflammatory cells which are typical for EE-induced hepatotoxicity.[18] As the decreasing effects are already present with low doses EE administered (from 0.1 µg EE/mouse/day onwards), this implies a specific effect of the estrogen treatment. The observed effects may be related to the synthetic nature of the estrogen, however subcutaneous injections with 17ß-estradiol resulted in comparable effects on both plasma and transcript levels, indicating that there is no difference in response to either 17α-ethinylestradiol or 17ß-estradiol. Furthermore, non-ovariectomized female mice also showed comparable (decreasing) effects on coagulation due to EE treatment, indicating that there is no additional effect of endogenous estrogen levels. Besides, with respect to plasma analyses, our

observations are in line with data from Wong et al. who showed that procoagulant factor activity levels are lower in female mice than in male mice. This may, at least in part, be due to the higher estrogen levels in females.[19] Taken together, we conclude that the observed EE-induced effects on both mouse plasma and hepatic transcript levels are within a biologically and pharmacologically relevant range.

The question remains how 17α-ethinylestradiol induces a fast ER-mediated decrease in transcript levels for a large number of coagulation genes. Estrogen generally regulates gene expression by transcriptional activation. However, an increasing number of studies demonstrate that ligand-bound estrogen receptors may also mediate transcriptional repression. A recent genome-wide gene expression profiling study of 47,000 murine transcripts showed that of the 78 genes regulated by EE in the liver for which the change was at least 1.5-fold, 17 genes were down-regulated.[20] As this cut-off of >1.5-fold change is higher than the fold changes observed in coagulation factors, this might not only explain why coagulation genes were not found in this genome-wide study, but it might also imply that many more genes are affected by EE administration, either in a positive or a negative fashion.

An other possible explanation for the reduced transcript levels is that estrogen induces or represses expression of a transcription factor important in the control of gene expression. For example, Gao et al. has shown that STAT3 expression levels in estrogen-treated obese mice are up-regulated, resulting in reduced transcript levels of genes involved in hepatic lipid biosynthesis.[21] With respect to coagulation genes, the hepatocyte nuclear factor 4α (HNF4α) could be such a transcription factor as it is known that it can regulate transcription of several coagulation factors and can interfere with estrogen signaling.[22,23] However, in our study, the rapidly EE-induced effects on coagulation factor mRNA levels where independent of significant changes in hepatic HNF4α transcript levels (data not shown). Furthermore, it has become clear that activating protein-1 (AP-1) proteins play a role in gene repression,[24] and in this respect it is striking that AP-1 elements are often found together with EREs,[10] which also might provide an explanation for the reduced transcript levels observed in the present study. Besides altering transcriptional activity and thereby lowering mRNA levels, EE could also affect the mRNA stability itself. However, for now this is a subject for speculation and further research is needed to clarify this overall decreasing mechanism.

In summary, our data demonstrate that oral ethinylestradiol treatment can have a major and rapid impact on mouse coagulation. Future studies should identify the

sequence of molecular steps through which the estrogen receptor α evokes the overall decreased transcription levels of coagulation factor levels. While this question for now remains unanswered, we believe that this work describes novel and important insights in the sequence of events contributing to the sex hormone-induced changes in coagulation.

Acknowledgements

We thank Dr. H.L. Vos for critical reading of the manuscript. This study was supported by grant 2006B045 from the Netherlands Heart Foundation.

References

1 Rosendaal FR. Venous thrombosis: the role of genes, environment, and behavior. Hematology Am Soc Hematol Educ Program 2005;1-12.
2 Middeldorp S. Oral contraceptives and the risk of venous thromboembolism. Gend Med 2005;2 Suppl A:S3-S9.
3 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.
4 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.
5 Jick S, Kaye JA, Li L, et al. Further results on the risk of nonfatal venous thromboembolism in users of the contraceptive transdermal patch compared to users of oral contraceptives containing norgestimate and 35 microg of ethinyl estradiol. Contraception 2007;76:4-7.
6 Johnson JV, Lowell J, Badger GJ, et al. Effects of oral and transdermal hormonal contraception on vascular risk markers: a randomized controlled trial. Obstet Gynecol 2008;111:278-84.
7 Farsetti A, Misiti S, Citarella F, et al. Molecular basis of estrogen regulation of Hageman factor XII gene expression. Endocrinology 1995;136:5076-83.
8 Di Bitondo R, Hall AJ, Peake IR, et al. Oestrogenic repression of human coagulation factor VII expression mediated through an oestrogen response element sequence motif in the promoter region. Hum Mol Genet 2002;11:723-31.
9 Bourdeau V, Deschenes J, Metivier R, et al. Genome-wide identification of high-affinity estrogen response elements in human and mouse. Mol Endocrinol 2004;18:1411-27.
10 Gao H, Falt S, Sandelin A, et al. Genome-wide identification of estrogen receptor alpha-binding sites in mouse liver. Mol Endocrinol 2008;22:10-22.
11 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.
12 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.

13 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.
14 de Visser YP, Walther FJ, Laghmani eH, et al. Sildenafil attenuates pulmonary inflammation and fibrin deposition, mortality and right ventricular hypertrophy in neonatal hyperoxic lung injury. Respir Res 2009;10:30.
15 Cleuren AC, Van Hoef B, Hoylaerts MF, et al. Short-term ethinylestradiol treatment suppresses inferior caval vein thrombosis in obese mice. Thromb Haemost 2009;102:993-1000.
16 Lansink M, Jong M, Bijsterbosch M, et al. Increased clearance explains lower plasma levels of tissue-type plasminogen activator by estradiol: evidence for potently enhanced mannose receptor expression in mice. Blood 1999;94:1330-6.
17 Lindberg MK, Moverare S, Skrtic S, et al. Estrogen receptor (ER)-beta reduces ERalpha-regulated gene transcription, supporting a "ying yang" relationship between ERalpha and ERbeta in mice. Mol Endocrinol 2003;17:203-8.
18 Yamamoto Y, Moore R, Hess HA, et al. Estrogen receptor alpha mediates 17alpha-ethynylestradiol causing hepatotoxicity. J Biol Chem 2006;281:16625-31.
19 Wong JH, Dukes J, Levy RE, et al. Sex differences in thrombosis in mice are mediated by sex-specific growth hormone secretion patterns. J Clin Invest 2008;118:2969-78.
20 Ahlbory-Dieker DL, Stride BD, Leder G, et al. DNA binding by estrogen receptor-alpha is essential for the transcriptional response to estrogen in the liver and the uterus. Mol Endocrinol 2009;23:1544-55.
21 Gao H, Bryzgalova G, Hedman E, et al. Long-term administration of estradiol decreases expression of hepatic lipogenic genes and improves insulin sensitivity in ob/ob mice: a possible mechanism is through direct regulation of signal transducer and activator of transcription 3. Mol Endocrinol 2006;20:1287-99.
22 Farsetti A, Moretti F, Narducci M, et al. Orphan receptor hepatocyte nuclear factor-4 antagonizes estrogen receptor alpha-mediated induction of human coagulation factor XII gene. Endocrinology 1998;139:4581-9.
23 Stauffer DR, Chukwumezie BN, Wilberding JA, et al. Characterization of transcriptional regulatory elements in the promoter region of the murine blood coagulation factor VII gene. J Biol Chem 1998;273:2277-87.
24 Carroll JS, Meyer CA, Song J et al. Genome-wide analysis of estrogen receptor binding sites. Nat Genet 2006;38:1289-97.

Table 1 : QPCR primer sequences

Gene	Forward primer (5'- 3')	Reverse primer (5'- 3')
ß-Actin	AGGTCATCACTATTGGCAACGA	CCAAGAAGGAAGGCTGGAAAA
Glutathione Peroxidase 3	ACTGAGGTCTGACAGACCAATACCT	CCCAGAATGACCAAGCCAAAT
Mannose Receptor	GATAGGCATGTTCCGAAATGTTG	GTTTTCCAGTTGACAAAGGAGACA
Fibrinogen-γ	TGCTGCCTGCTTTTACTGTTCTC	TCTAGGATGCAACAGTTATCTCTGGTA
Factor II	GGACGCTGAGAAGGGTATCG	CCCCACACAGCAGCTCTTG
Factor V	CATGGAAACCTTACCGACAGAAA	CATGTGCCCCTTGGTATTGC
Factor VII	CGTCTGCTTCTGCCTAGA	ATTTGCACAGATCAGCTGCTCAT
Factor VIII	CTTCACCTCCAGGGAAGGACTA	TCCACTTGCAACCATTGTTTTG
Factor IX	GCAAAACCGGGTCAAATCC	ACCTCCACAGAATGCCTCAATT
Factor X	GTGGCCGGGAATGCAA	AACCCTTCATTGTCTTCGTTAATGA
Factor XI	GAAGGATACGTGCAAGGGAGATT	CAAGTGCCAGACCCCATTGT
Factor XII	GGGCTTCTCCTCCATCACCTA	GCAACTGTTGGTTTTGCTTTCC
Antithrombin	TGGGCCTCATTGATCTCTTCA	CCTGCCTCCAGCAACGAT
Plasminogen	TGACATTGCCCTGCTGAAAC	CAGACAAGCTGGAATGACTTTATCC
Protein C	GCGTGGAGGGCACCAA	CCCTGCGTCGCAGATCAT
Protein S	GGTGGCATCCCAGATATTTCC	CACTTCCATGCAGCCACTGT
Protein Z	GCAGCCAGAGTCAGCCTAGCT	CACGCCGGCACAGAAGTC
Protein Z Inhibitor	TGGCCCTGGAGGACTACTTG	CCATTTTCCTGGTTTTCATATTCTG
α_2-Antiplasmin	TTCTCCTCAACGCCATCCA	GGTGAGGCTCGGGTCAAAC
Heparin Cofactor II	GAATGGCAATATGTCAGGCATCT	CACTGTGATGGTACTTTGGTGCTT

Table 2: plasma coagulation factor activity (act) and antigen (ag) levels of ovariectomized mice treated with increasing doses of oral ethinylestradiol (EE) for 10 consecutive days.

	0 µg EE	0.03 µg EE	0.1 µg EE	0.3 µg EE	1 µg EE	3 µg EE	10 µg EE
Factor VIII act (%)	100±8.4	112.7±16.9	108.3±6.0	101.3±5.2	91.3±4.9	81.0±6.8	72.8±7.6
Factor IX act (%)	100±2.8	97.9±4.2	113.6±5.4	121.7±4.6†	120.8±4.5†	121.6±4.2†	121.1±4.3*
Factor XI act (%)	100±5.2	95.7±7.4	95.0±9.0	111.1±3.6	112.6±6.7	98.3±3.0	87.7±6.7
Factor XII act (%)	100±4.1	96.7±13.2	89.0±4.6	85.9±2.7	92.1±6.6	82.2±2.7	80.1±1.1
Factor II/VII/X act (%)	100±3.8	92.8±2.7	89.0±3.2*	89.7±1.5‡	83.0±2.5†	86.7±2.5*	84.4±2.2
Antithrombin act (%)	100±1.9	88.8±10.0	95.2±4.2	87.2±4.2	82.9±3.7	90.1±1.8	88.0±2.7
Antithrombin ag (%)	100±2.0	102.9±5.3	94.8±1.4	93.2±3.6	87.4±3.9	86.7±2.5*	89.2±3.6

Data are presented as mean±SEM with the vehicle-treated , group set as a reference. *p<0.05, †p<0.01 and ‡p<0.001 versus vehicle-treatment.

Table 3: hepatic transcript levels of ovariectomized mice treated with increasing doses of ethinylestradiol (EE) for 10 consecutive days.

	0 µg EE	0.03 µg EE	0.1 µg EE	0.3 µg EE	1 µg EE	3 µg EE	10 µg EE
Glutathione Peroxidase 3	1±0.11	1.06±0.53	1.57±0.46	3.05±0.73‡	3.60±1.43‡	3.58±1.05‡	4.54±0.33‡
Mannose Receptor	1±0.12	1.12±0.15	1.17±0.15	0.92±0.21	1.30±0.12	1.15±0.12	1.40±0.11*
Factor II	1±0.18	1.10±0.09	0.97±0.17	0.87±0.09	0.79±0.12	0.75±0.08‡	0.60±0.18†
Factor V	1±0.14	1.27±0.13*	1.14±0.16	0.96±0.08	0.77±0.10*	0.74±0.07†	0.45±0.09‡
Factor VII	1±0.09	1.04±0.22	1.00±0.08	0.86±0.09	0.75±0.07*	0.58±0.13‡	0.52±0.16‡
Factor VIII	1±0.14	0.99±0.11	1.02±0.12	0.77±0.16	1.04±0.14	0.56±0.47	0.71±0.10
Factor IX	1±0.12	1.15±0.09	1.05±0.11	1.13±0.13	1.03±0.14	0.92±0.12	0.82±0.18
Factor X	1±0.12	0.99±0.12	0.75±0.07*	0.69±0.08†	0.74±0.11*	0.54±0.11‡	0.43±0.14‡
Factor XI	1±0.17	1.30±0.21	1.37±0.39	1.56±0.17†	1.54±0.20†	1.33±0.20	1.54±0.15*
Antithrombin	1±0.13	0.97±0.11	0.93±0.15	0.82±0.16	0.73±0.11*	0.65±0.14†	0.61±0.12†

Relative expression levels were compared using the comparative threshold cycle method with β-actin as internal control. Data are represented as mean±SEM and the vehicle-treated group is set as a reference. *p<0.05, †p<0.01 and ‡p<0.001 versus vehicle treatment.

Table 4: plasma coagulation factor activity (act) and antigen (ag) levels of ovariectomized mice treated with 1 µg oral ethinylestradiol (EE) and / or 100 µg subcutaneous ICI182780 injections for 10 consecutive days.

	Control	EE	EE + ICI182780	ICI182780
Fibrinogen ag (%)	100±8.6	77.3±5.0	89.0±7.3	98.6±5.8
Factor II act (%)	100±2.0	93.6±2.2	100.9±1.8	95.5±2.1
Factor VII act (%)	100±5.5	93.2±4.5	111.3±8.5	102.4±6.3
Factor VIII act (%)	100±3.5	83.7±3.3†	103.8±3.3	104.4±2.3
Factor IX act (%)	100±2.2	121.3±2.9‡	108.9±2.9	102.0±3.0
Factor X act (%)	100±2.6	98.3±2.5	105.3±3.6	101.9±3.2
Factor XI act (%)	100±5.5	96.0±4.6	103.0±3.8	97.7±3.6
Factor XII act (%)	100±2.0	91.9±2.0*	104.3±2.2	99.4±1.8
Factor II/VII/X act (%)	100±2.3	90.4±2.4*	103.8±2.4	99.0±2.4
Antithrombin act (%)	100±3.7	79.6±3.5‡	95.3±2.3	97.1±2.1
Antithrombin ag (%)	100±2.0	90.4±2.6*	101.6±2.0	99.7±2.0

Data are represented as mean±SEM and the vehicle-treated group is set as a reference. *$p<0.05$, $^{\dagger}p<0.01$ and $^{\ddagger}p<0.001$ versus vehicle treatment.

Table 5: hepatic transcript levels of ovariectomized mice treated with 1 µg oral ethinylestradiol (EE) and / or 100 µg subcutaneous ICI182780 injections.

	Control	EE	EE + ICI182780	ICI182780
GpX3	1±0.14	1.87±0.38‡	0.81±0.18	0.78±0.1
Mannose Receptor	1±0.17	1.50±0.14†	0.94±0.11	1.00±0.23
Fibrinogen-γ	1±0.21	0.59±0.07‡	0.86±0.10	0.98±0.19
Factor II	1±0.10	0.62±0.07‡	0.89±0.10	0.99±0.07
Factor V	1±0.10	0.63±0.04‡	0.84±0.07	0.96±0.11
Factor VII	1±0.24	0.55±0.03‡	0.88±0.09	0.97±0.08
Factor VIII	1±0.15	0.99±0.09	0.88±0.22	0.89±0.22
Factor IX	1±0.07	0.96±0.10	0.99±0.15	1.15±0.11
Factor X	1±0.11	0.52±0.05‡	0.91±0.06	1.00±0.13
Factor XI	1±0.14	1.65±0.25†	1.26±0.27	1.06±0.27
Factor XII	1±0.08	0.76±0.07†	0.89±0.12	0.92±0.08
Antithrombin	1±0.15	0.66±0.06‡	0.91±0.07	1.04±0.13
Plasminogen	1±0.06	0.59±0.04‡	0.92±0.14	1.05±0.13
Protein C	1±0.08	0.76±0.11*	1.00±0.13	1.04±0.12
Protein S	1±0.09	1.21±0.12	0.98±0.13	0.98±0.12
Protein Z	1±0.07	0.81±0.08*	1.00±0.12	1.11±0.11
Protein Z Inhibitor	1±0.11	0.68±0.09‡	0.95±0.11	1.06±0.15

α2-Antiplasmin	1±0.06	0.87±0.10	1.12±0.14	1.01±0.46
Heparin Cofactor II	1±0.05	0.84±0.05*	0.97±0.11	1.03±0.11

Data are represented as mean±SEM and normalized for β-actin with the vehicle-treated group set as reference. *p<0.05, †p<0.01 and ‡p<0.001 versus vehicle treatment.

Table 6: plasma coagulation factor activity (act) and antigen (ag) levels of ovariectomized *Esr1*$^{+/+}$ and *Esr1*$^{-/-}$ mice orally treated with 1 µg ethinylestradiol (EE).

	Esr1$^{+/+}$		*Esr1*$^{-/-}$	
	Control	EE	Control	EE
Fibrinogen ag (%)	100±4.6	107.3±8.4	115.4±6.3	136.7±13.0
Factor II act (%)	100±2.5	103.9±1.9	105.7±2.3	107.7±4.6
Factor VII act (%)	100±4.4	96.0±6.9	100.1±4.5	118.0±11.6
Factor VIII act (%)	100±7.2	88.8±9.0	118.5±8.3	104.8±12.4
Factor IX act (%)	100±3.4	122.1±6.6†	86.1±4.6	96.2±5.4
Factor X act (%)	100±2.6	105.9±2.6	104.0±2.2	108.4±2.7
Factor XI act (%)	100±2.9	87.4±4.5*	84.3±2.5	90.2±5.9
Factor XII act (%)	100±5.2	101.2±5.8	95.1±4.9	97.6±5.5
Factor II/VII/X act (%)	100±2.8	100.1±2.7	104.5±3.9	106.9±6.0
Antithrombin act (%)	100±4.9	98.0±3.3	104.1±4.6	109.9±5.4
Antithrombin ag (%)	100±2.3	98.5±2.7	103.3±2.1	103.8±1.2

Data are represented as mean±SEM and the wild-type vehicle-treated group is set as a reference. *p<0.05 and †p<0.01 versus vehicle treatment of the same genotype.

Table 7: hepatic transcript levels of *Esr1*$^{+/+}$ and *Esr1*$^{-/-}$ mice orally treated with ethinylestradiol (EE).

	Esr1$^{+/+}$		*Esr1*$^{-/-}$	
	Control	EE	Control	EE
GpX3	1±0.20	1.92±0.36‡	1.04±0.24	1.26±0.41
Mannose Receptor	1±0.19	1.76±0.37†	0.79±0.20	0.88±0.26
Fibrinogen-γ	1±0.09	0.75±0.13*	1.00±0.15	0.95±0.16
Factor II	1±0.22	0.78±0.11	0.83±0.21	0.78±0.35
Factor V	1±0.23	0.70±0.07*	0.95±0.13	0.91±0.13
Factor VII	1±0.17	0.66±0.09†	0.94±0.11	0.81±0.28
Factor VIII	1±0.19	1.25±0.15	0.86±0.14	0.90±0.35
Factor IX	1±0.08	0.91±0.14	0.83±.011	0.92±0.18
Factor X	1±0.27	0.60±0.11†	0.87±0.22	0.68±0.24
Factor XI	1±0.23	1.44±0.17*	0.81±0.14	0.79±0.32
Factor XII	1±0.23	0.91±0.05	1.00±0.16	0.92±0.14

Antithrombin	1±0.22	0.65±0.21*	1.06±0.15	1.01±0.11
Plasminogen	1±0.12	0.65±0.13†	0.97±0.15	0.82±0.19
Protein C	1±0.14	0.75±0.11*	0.85±0.10	0.83±0.16
Protein S	1±0.16	1.31±0.15*	0.97±0.18	1.10±0.30
Protein Z	1±0.21	0.63±0.12†	0.71±0.10	0.67±0.19
Protein Z Inhibitor	1±0.15	0.88±0.13	0.95±0.13	1.33±0.64
α2-Antiplasmin	1±0.08	0.76±0.11†	1.00±0.13	0.93±0.23
Heparin Cofactor II	1±0.14	0.84±0.11	0.91±0.09	0.80±0.17

Data are represented as mean±SEM and normalized for β-actin with the wild-type vehicle-treated group set as reference. *p<0.05, †p<0.01 and ‡p<0.001 versus vehicle treatment of the same genotype.

Table 8: plasma activity (act) and antigen (ag) levels of *Esr2*$^{+/+}$ and *Esr2*$^{-/-}$ mice treated with oral ethinylestradiol (EE).

	Esr2$^{+/+}$		*Esr2*$^{-/-}$	
	Control	EE	Control	EE
Fibrinogen ag (%)	100±8.0	69.6±4.5†	105.8±7.7	64.6±4.0‡
Factor II act (%)	100±3.8	102.9±2.9	104.2±5.9	97.6±3.6
Factor VII act (%)	100±4.6	66.7±4.2‡	112.9±4.7	73.1±6.0‡
Factor VIII act (%)	100±5.4	89.1±4.9	100.9±5.5	76.8±5.2†
Factor IX act (%)	100±4.1	127.8±3.2‡	116.6±4.2	127.3±4.7
Factor X act (%)	100±3.5	108.6±3.0	107.6±5.6	101.8±3.8
Factor XI act (%)	100±2.5	88.3±3.2†	106.4±3.3	80.5±3.6‡
Factor XII act (%)	100±3.7	90.7±1.9*	109.0±3.5	85.0±2.3‡
Factor II/VII/X act (%)	100±3.0	86.8±2.4†	101.3±2.9	86.1±2.5‡
Antithrombin act (%)	100±4.1	99.3±5.4	114.9±7.6	107.2±8.6
Antithrombin ag (%)	100±4.9	91.6±2.6	102.7±3.8	93.4±5.9

Data are represented as mean±SEM and the wild-type vehicle-treated group is set as a reference. *p<0.05, †p<0.01 and ‡p<0.001 versus vehicle treatment of the same genotype.

Table 9: hepatic transcript levels of *Esr2*$^{+/+}$ and *Esr2*$^{-/-}$ mice orally treated with ethinylestradiol (EE).

	Esr2$^{+/+}$		*Esr2*$^{-/-}$	
	Control	EE	Control	EE
GpX3	1±0.18	2.34±0.72‡	1.04±0.16	2.14±0.33‡
Mannose Receptor	1±0.23	1.28±0.27	0.81±0.15	1.66±0.17‡
Fibrinogen-γ	1±0.18	0.53±0.23*	0.89±0.12	0.57±0.07‡
Factor II	1±0.10	0.47±0.09‡	0.92±0.13	0.44±0.05‡
Factor V	1±0.16	0.52±0.08‡	0.90±0.24	0.39±0.05‡
Factor VII	1±0.10	0.47±0.09‡	0.99±0.15	0.43±0.06‡

Factor VIII	1±0.15	0.85±0.18	0.86±0.22	0.74±0.13
Factor IX	1±0.12	0.64±0.18*	0.92±0.10	0.63±0.06‡
Factor X	1±0.17	0.39±0.09‡	1.00±0.19	0.34±0.07‡
Factor XI	1±0.29	0.98±0.28	1.07±0.19	1.08±0.17
Factor XII	1±0.15	0.73±0.12*	0.82±0.29	0.48±0.29
Antithrombin	1±0.12	0.59±0.09‡	0.98±0.13	0.51±0.05‡
Plasminogen	1±0.12	0.52±0.09‡	0.92±0.15	0.45±0.06‡
Protein C	1±0.13	0.59±0.09‡	0.92±0.14	0.54±0.06‡
Protein S	1±0.09	1.09±0.19	0.95±0.16	1.02±0.14
Protein Z	1±0.13	0.53±0.21†	0.99±0.14	0.56±0.06‡
Protein Z Inhibitor	1±0.17	0.81±0.15	1.00±0.17	0.65±0.06‡
α2-Antiplasmin	1±0.15	0.66±0.14†	0.98±0.14	0.62±0.08‡
Heparin Cofactor II	1±0.12	0.62±0.11‡	0.94±0.11	0.57±0.06‡

Data are represented as mean±SEM and normalized for β-actin with the vehicle-treated group per genotype set as reference. *$p<0.05$, †$p<0.01$ and ‡$p<0.001$ versus vehicle treatment of the same genotype.

Chapter 4

Progestins possess poor anti-estrogenic activity on murine hepatic coagulation gene transcription despite evident anti-estrogenic activity on uterine tissue

Audrey CA Cleuren, Iris Postmus, Hans L Vos,
Pieter H Reitsma, Bart JM van Vlijmen

Thromb Res. 2011 Aug;128(2):200-201

Dear editors,

Oral contraceptive (OC) use affects the plasma levels of several pro- and anticoagulant as well as fibrinolytic factors, overall leading to an increased risk of venous thrombosis. The estrogen in OCs is considered to be the main prothrombotic constituent, however, the introduction of the third generation contraceptives containing desogestrel instead of levonorgestrel, revealed a modulating anti-estrogenic effect of progestins on estrogen-induced alterations in the coagulation profile and thrombotic risk.[1] Such anti-estrogenic effects of progestins are also established for murine uterine gene transcription.[2]

Here, we examined the anti-estrogenic effects of the second-generation progestin levonorgestrel (LNG) and third-generation desogestrel (DSG) on coagulation gene transcription in the liver, i.e. the major source of plasma coagulation factor synthesis. As a model we use mice orally treated with the estrogen 17α-ethinylestradiol (EE) for which we previously demonstrated rapid estrogen receptor (ER) α-dependent changes in hepatic transcription of coagulation genes.[3] As changes could be fully counteracted by the ER antagonist ICI182780, this model is suitable for detecting anti-estrogenic effects on coagulation factor levels (table 1, reference [3]).

Ovariectomized C57Black/6J mice (Charles River, Maastricht, the Netherlands) were treated with either vehicle (100 µl arachid oil/day), 3 or 10 µg LNG/day, or 3 or 10 µg DSG/day, both in the absence and presence of 1 µg EE/day (Sigma, Steinheim, Germany and Duchefa Farma, Haarlem, the Netherlands; n=12 per group). This EE dose was based on our previous experiments,[3] whilst the progestin doses were chosen to reflect the 5-fold (w/w) ratio with EE in OCs. RNA isolation and quantitative real-time PCR of individual liver samples, as well as plasma analyses were performed as previously described.[3]

To establish the biological effect of ethinylestradiol and the progestins, haematoxylin/eosin stained vaginal smears were evaluated, showing predominantly leukocytes in smears of vehicle-treated mice and mainly cornified cells in smears of EE-treated mice. Mice treated with both EE and a progestin (either LNG or DSG) showed nucleated epithelial and cornified cells with some leukocytes. In addition, gross inspection of the uterus (haematoxylin/eosin stained sections) showed that EE-treated mice had increased myometrium, stromal and endometrial epithelium thickness, resulting in an increased uterus wet weight as compared to vehicle-treated mice (16.3±0.1 mg vs. 123.1±7.6 mg, $p<0.0001$). In

contrast, mice treated with both EE and a progestin showed a significant dose-dependent reduction of the uterus weight when compared to EE-only treated animals, with uterus weights of 53.2±2.9 mg and 46.3±3.8 mg for EE/10 µg LNG and EE/10 µg DSG respectively ($p<0.01$). LNG or DSG alone had no effect on vaginal smears or uterus phenotype. Based on these results, we conclude that both progestins have similar biological activity in the mouse, and are able to modulate the estrogen-induced effects on the uterus.

Table 1 shows significant increases in hepatic transcript levels of glutathione peroxidase 3 (GpX3), coagulation factor (F) XI and protein S levels, and significant decreases in FV, FVII, FX, FXII and antithrombin transcript levels upon EE treatment as expected,[3] while fibrinogen-γ and FII levels were not affected (data not shown). Importantly, addition of either LNG or DSG to EE treatment did not result in statistically significant modulating effects on transcript levels as compared to EE treatment alone (table 1).

Table 1: Summary significantly affected relative transcript levels of mice treated with 17α-ethinylestradiol (EE), levonorgestrel (LNG), desogestrel (DSG), or the estrogen receptor antagonist ICI182780.

	Vehicle	EE	EE+ 10µg LNG	EE+ 10µg DSG	EE+ ICI182780[a]
GpX3	1±0.20	2.57±0.67*	2.40±0.35*	1.92±0.71*	0.81±0.18[#]
Factor V	1±0.16	0.65±0.08*	0.67±0.08*	0.63±0.09*	0.84±0.07[#]
Factor VII	1±0.16	0.69±0.12*	0.70±0.11*	0.62±0.08*	0.88±0.09[#]
Factor X	1±0.19	0.51±0.12*	0.61±0.10*	0.56±0.14*	0.91±0.06
Factor XI	1±0.10	1.50±0.17*	1.39±0.23*	1.26±0.25	1.26±0.27
Factor XII	1±0.11	0.75±0.12*	0.87±0.11	0.76±0.09*	0.89±0.12
Antithrombin	1±0.11	0.71±0.10*	0.75±0.09*	0.59±0.08*	0.91±0.07[#]
Protein S	1±0.10	1.25±0.12*	1.11±0.18	1.16±0.08	0.98±0.13

GpX3: glutathione peroxidase 3.

Relative expression levels were compared using the comparative threshold cycle method with β-actin as internal control. Data are presented as mean±standard error of the mean of n=12 mice per group and statistical differences between groups were evaluated using a one-way analysis of variances (ANOVA) with a Bonferroni post-hoc test. *$p<0.05$ compared to vehicle treatment, [#]p-value <0.05 compared to EE treatment.

[a]: Combined EE/ICI182780 treatment was performed in a separate experiment [3] and data are compared to vehicle- and EE-treated controls of that experiment with results of EE treatment being essentially the same as observed in the present experiments.

In addition to mRNA levels, we also determined the plasma levels of a limited set of coagulation factors and observed an increase in FIX activity (+12%), and decreases in combined factor II/VII/X (-7%) and antithrombin (-10%) activity levels upon EE treatment, which were comparable to previous observations.[3] Neither LNG or DSG alone, nor in combination with EE had an effect on these plasma parameters (data not shown).

As DSG in combination with EE compared to EE treatment alone, showed a tendency to modulate GpX3 ($p=0.20$), factor XI ($p=0.13$), protein S ($p=0.22$) and antithrombin ($p=0.06$) transcript levels, we increased the DSG dose to the supratherapeutical range of 50 to 500 µg/mouse/day to further explore this potential (n=5/group). These high DSG doses did not cause liver toxicity, as evaluated by circulating liver enzyme levels, whereas the uterus phenotype was strongly affected, resulting in almost complete reversal of the EE-induced increase in uterus weight (122.7±4.6 mg for 1 µg EE vs. 34.5±1.9 mg for 1 µg EE/500 µg DSG, $p<0.001$). Only for the 500 µg DSG dose, a significant reversal of the EE-induced effects on hepatic transcript levels of GpX3, FX and antithrombin was observed (relative expression levels 1 µg EE vs. 1 µg EE/500 µg DSG: 3.44±0.62 vs. 1.79±0.52 for GpX3, 0.58±0.09 vs. 0.85±0.07 for FX and 0.69±0.13 vs. 0.97±0.09 for antithrombin).

In summary, the hepatic transcript levels of coagulation genes were only minimally affected by combined estrogen/progestin treatment, which is in contrast to the anti-estrogenic effects observed on the uterus. One possible explanation for this discrepancy in responses could be that progesterone receptor transcript levels are only 2% of the ERα levels in the liver, which may not be sufficient to exert the anti-estrogenic effects observed in the uterus.[2]

The mouse data presented here suggest that the anti-estrogenic effects of progestins on the thrombotic risk in humans lay outside transcriptional modulation of coagulation factors produced in the liver. However, as the liver also produces several fibrinolytic factors, we cannot exclude that progestins modulate estrogenic effects on fibrinolysis. We speculate that the effects of progestins on thrombosis are at the level of the vasculature, as blood vessels express both estrogen and progesterone receptors. This may involve modulation of expression of coagulation genes like tissue factor, tissue factor pathway inhibitor, von Willebrand factor, endothelial protein C receptor or thrombomodulin.

In conclusion, the present studies show that in the mouse the progestins levonorgestrel and desogestrel, while evidently and equally biologically active on

uterine tissue, possess poor anti-estrogenic activity regarding hepatic coagulation gene transcription.

Acknowledgements

This study was supported by grant 2006B045 from the Netherlands Heart Foundation.

References

1 Tchaikovski SN, Rosing J. Mechanisms of estrogen-induced venous thromboembolism. Thromb Res 2010;126:5-11.
2 Kraus WL, Katzenellenbogen BS. Regulation of progesterone receptor gene expression and growth in the rat uterus: modulation of estrogen actions by progesterone and sex steroid hormone antagonists. Endocrinology 1993;132:2371-9.
3 Cleuren AC, Van der Linden I, De Visser YP, et al. 17alpha-Ethinylestradiol rapidly alters transcript levels of murine coagulation genes via estrogen receptor alpha. J Thromb Haemost 2010;8:1838-46.

Chapter 5

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

Audrey CA Cleuren, Berthe van Hoef, Marc F Hoylaerts, Bart JM van Vlijmen, H Roger Lijnen

Thromb Haemost. 2009 Nov;102(5):993-1000

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 ΔC_t values of the individual samples were related to the mean ΔC_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.

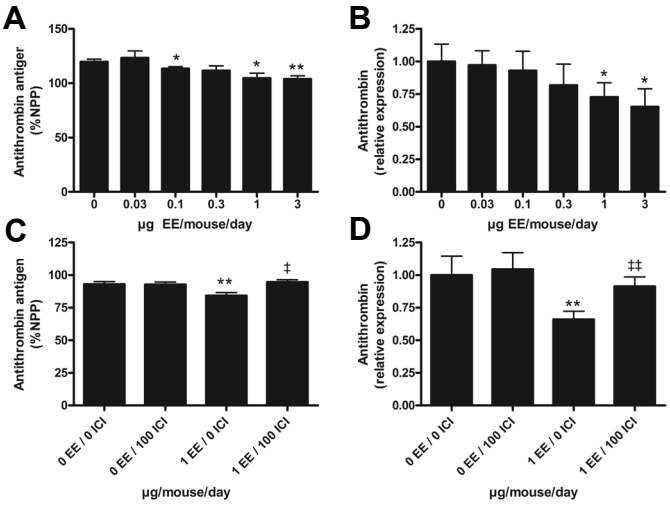

Figure1: Dose-dependent effects of oral ethinylestradiol (EE) on the plasma antithrombin antigen (panel a) and hepatic mRNA levels related to ß-actin (b). The effects of 1 µg EE per mouse per day on plasma and hepatic mRNA levels of antithrombin are normalized by additional treatment with 100 µg of the 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.

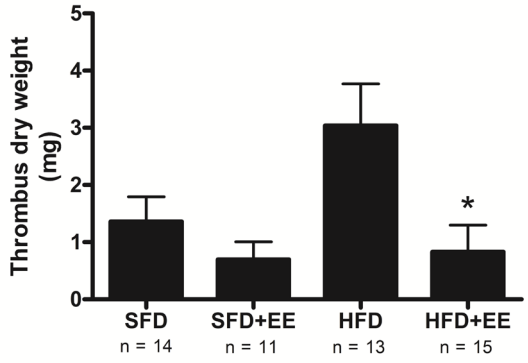

Figure 2: Thrombus weight for mice kept on a standard (SFD) or high fat (HFD) diet, treated with ethinylestradiol (EE+) or vehicle (EE-). 69 animals were subjected to the inferior caval vein thrombosis model (see Methods) of which 11 veins were embedded for histological analyses and 5 animals died during 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-γ 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.

Acknowledgements

Sources of Funding: This study was supported by the Leducq Foundation (Paris, France; LINAT project). The Center for Molecular and Vascular Biology is supported by the “Excellentiefinanciering KU Leuven” (EF/05/13). Participation of A. Cleuren was supported by a grant from the Netherlands Heart Foundation (2006B045).

Skilful technical assistance by S. Helsen, L. Frederix, I. Van der Linden and R. Dirven is gratefully acknowledged. G. Wagenaar and Y. De Visser are gratefully acknowledged for help and advice with the RNA analysis.

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.

Chapter 6

Long-term estrogen treatment of mice with a prothrombotic phenotype induces a sustained increase in thrombin generation without affecting tissue fibrin depositions

Audrey CA Cleuren, Rene van Oerle, Pieter H Reitsma,
Henri M Spronk, Bart JM van Vlijmen

Manuscript submitted for publication

Dear editors,

In thrombin generation assays, the resulting area under the curve (endogenous thrombin potential; ETP) is considered to have a predictive value. A low ETP value is associated with a bleeding tendency, whereas an increased value represents a hypercoagulable state. Regarding this latter category, it has been shown that women using oral contraceptives, which are associated with an increased risk for venous thrombosis, have higher ETP values than non-users.[1]

The estrogen in oral contraceptives, often 17α-ethinylestradiol, is considered to be the predominant thrombogenic component. We have previously shown that oral ethinylestradiol (EE) in mice has profound effects on the plasma levels of pro- and anticoagulant factors.[2] Now, we report the overall effect of these estrogen-induced alterations on the hemostatic balance by assessing thrombin generation and determine the net effect on a spontaneous thrombotic phenotype, i.e. fibrin depositions in lung tissues of the prothrombotic factor V Leiden ($FV^{Q/Q}$) and thrombomodulin proline mutant ($TM^{pro/pro}$) mice.

Hereto, ovariectomized $FV^{Q/Q}$ and $TM^{pro/pro}$, and wild-type mice as a reference, were orally treated with 1 µg EE/day or a vehicle for either 10 days or 10 weeks (n=12 mice per group). After the last EE or vehicle administration, mice were exsanguinated and platelet-poor plasma was obtained.[3] In order to determine the estrogen-induced effects on fibrin depositions, lungs of 10-week treated mice were isolated as previously described.[4,5]

To evaluate the effects on the overall plasma coagulability after 10 days of treatment, we assessed thrombin generation by means of the Calibrated Automated Thrombogram method using 1 pM tissue factor to trigger 1:6 diluted mouse plasma.[6] Oral EE treatment resulted in significantly increased ETP values in $FV^{Q/Q}$ (fig. 1A) and $TM^{pro/pro}$ mice (fig. 1B), which was comparable to the effects observed in wild-type animals (448±30 nM*min vs. 930±41 nM*min, $p<0.001$). The thrombin generation curves in figures 1A and 1B show that the inhibition of thrombin activity, i.e. the tail of the curve, is predominantly affected in EE-treated mice, which largely determines the differences in ETP values between the vehicle- and EE-treated groups. Since antithrombin is the main inhibitor of thrombin activity, we measured the antithrombin activity levels in plasma (Coamatic antithrombin kit, Chromogenix) and found that estrogen administration caused a significant 15% reduction in antithrombin activity levels, which negatively correlated with the ETP values (Pearson r=-0.51, p=0.002).

To determine the relation between thrombin generation and a spontaneous thrombotic phenotype, $FV^{Q/Q}$ and $TM^{pro/pro}$ mice were daily treated with vehicle or 1 µg EE/day for 10 weeks. During these 10 weeks, none of the mice died or showed signs of abnormalities. In addition, careful inspection of the mice upon sacrifying revealed no signs of macrovascular thrombosis. Thrombin generation curves and ETP values after 10 weeks of treatment showed a comparable pattern to the 10-day treatment, with ETP values of 446±40 nM*min vs. 746±98 nM*min ($p<0.01$) for $FV^{Q/Q}$ mice and 683±75 nM*min vs. 901±70 nM*min ($p<0.05$) for $TM^{pro/pro}$ animals, indicating a sustained increase in thrombin generation during the 10 weeks of oral ethinylestradiol treatment.

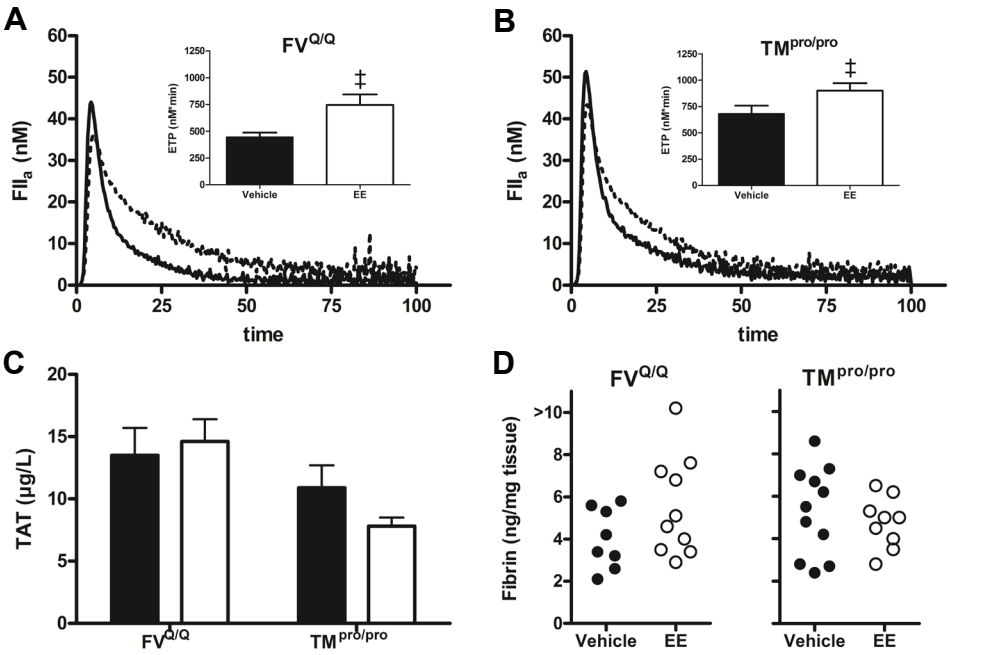

Figure 1: Factor V Leiden (A) and thrombomodulin proline mutant (B) mice were orally treated with 1 µg ethinylestradiol (EE; dashed line) or a vehicle (solid line) per day for 10 days after which thrombin generation was determined in 1:6 diluted plasma triggered with 1 pM tissue factor. The resulting endogenous thrombin potential (ETP) values are presented in the inserts. Plasma thrombin-antithrombin (TAT) complexes (C) and fibrin concentrations in lung homogenates of $FV^{Q/Q}$ and $TM^{pro/pro}$ (D) were determined after 10 weeks of treatment. Data are presented as mean ± standard error of the mean of n=12 mice per group (A-C) or as individual measurements (D). ‡$p<0.001$ as compared to vehicle-treated animals.

As a biochemical marker for a thrombotic phenotype, fibrin depositions in lung homogenates were determined via Western blotting.[5] Although fibrin depositions of $FV^{Q/Q}$ and $TM^{pro/pro}$ mice were in the lower range of detection (2-10 ng/mg tissue), they were higher than fibrin concentration present in wild-type mice, which did not exceed the detection threshold of 2 ng/mg. However, oral estrogen administration for 10 weeks did not result in significant increased fibrin depositions in either the $FV^{Q/Q}$ (4.0±0.5 ng/mg vs. 5.5±0.7 ng/mg) or $TM^{pro/pro}$ mice (5.3±0.6 ng/mg vs. 4.8±0.4 ng/mg; figure 1C). In addition, plasma thrombin-antithrombin complex analyses (Enzygnost TAT micro, Dade Behring) did also not differ between vehicle- and EE-treated animals (figure 1D), which supports the absence of an effect on fibrin depositions despite a sustained increased potential to generate thrombin upon estrogen treatment.

Previous studies have shown that the $FV^{Q/Q}$ status converts to a spontaneous perinatal lethal phenotype when combined with mice carrying gene deletions in anticoagulant genes that in itself are not lethal, like tissue factor pathway inhibitor and protein Z.[7,8] In addition, microvascular thrombosis in lungs of $TM^{pro/pro}$ mice proved highly responsive to hypoxia,[5] showing that these models are capable of displaying enhanced fibrin deposition upon risk factor exposure. On the other hand, with respect to oral ethinylestradiol administration, we have previously shown that short-term EE exposure was not able to aggravate the thrombogenicity in a stasis-induced thrombosis model,[9] which is in line with these current observations.

From the data presented here, we conclude that despite the fact that long-term oral ethinylestradiol administration can induce a sustained increase in thrombin generation in factor V Leiden and thrombomodulin proline mutant mice, this does not translate into a spontaneous macrovascular or microvascular thrombotic phenotype, which argues against the use of these mice in studying the effects of estrogens on thrombosis.

Acknowledgements

We thank Patricia Pluijmen and Diane Fens from the Cardiovascular Research Institute Maastricht for their technical assistance. This study was financially supported by the Netherlands Heart Foundation (grant 2006B045).

References

1 Tchaikovski SN, van Vliet HA, Thomassen MC, et al. Effect of oral contraceptives on thrombin generation measured via calibrated automated thrombography. Thromb Haemost 2007;98:1350-6.

2 Cleuren AC, Van der Linden IK, de Visser YP, et al. 17alpha-Ethinylestradiol rapidly alters transcript levels of murine coagulation genes via estrogen receptor alpha. J Thromb Haemost 2010;8:1838-46.
3 Sommeijer DW, van Oerle R, Reitsma PH, et al. Analysis of blood coagulation in mice: pre-analytical conditions and evaluation of a home-made assay for thrombin-antithrombin complexes. Thromb J 2005;3:12.
4 Cui J, Eitzman DT, Westrick RJ, et al. Spontaneous thrombosis in mice carrying the factor V Leiden mutation. Blood 2000;96:4222-6.
5 Weiler-Guettler H, Christie PD, Beeler DL, et al. A targeted point mutation in thrombomodulin generates viable mice with a prethrombotic state. J Clin Invest 1998;101:1983-91.
6 Dargaud Y, Spronk HM, Leenders P, et al. Monitoring platelet dependent thrombin generation in mice. Thromb Res 2010;126:436-41.
7 Eitzman DT, Westrick RJ, Bi X, et al. Lethal perinatal thrombosis in mice resulting from the interaction of tissue factor pathway inhibitor deficiency and factor V Leiden. Circulation 2002;105:2139-42.
8 Yin ZF, Huang ZF, Cui J, et al. Prothrombotic phenotype of protein Z deficiency. Proc Natl Acad Sci U S A 2000;97:6734-8.
9 Cleuren AC, Van Hoef B, Hoylaerts MF, et al. Short-term ethinyl estradiol treatment suppresses inferior caval vein thrombosis in obese mice. Thromb Haemost 2009;102:993-1000.

Chapter 7

Changes in dietary fat content rapidly alter the mouse plasma coagulation profile without affecting relative transcript levels of coagulation genes

Audrey CA Cleuren, Vicky T Blankevoort,
Janna A van Diepen, Peter J Voshol, Pieter H Reitsma,
Bart JM van Vlijmen

Manuscript in preparation

Summary

Background: Obesity is associated with a hypercoagulable state and increased risk for thrombotic cardiovascular events.

Objective: Establish the onset and reversibility of the hypercoagulable state during the development and regression of nutritionally-induced obesity in mice, and its relation to transcriptional changes and clearance rates of coagulation factors as well as its relation to changes in metabolic and inflammatory parameters.

Methods: Male C57Black/6J mice were fed a low fat (10% kcal fat; LFD) or high fat diet (45% kcal; HFD) for 2, 4, 8 or 16 weeks, and *in vivo* clearance rates of human factor (F) VII, FVIII and FIX proteins were determined after 2 weeks of HFD-feeding. To study the effects of weight loss, mice were fed the HFD for 16 weeks and switched to the LFD for 1, 2 or 4 weeks. For each time point plasma and hepatic mRNA analyses were performed after overnight fasting.

Results: HFD feeding gradually increased the body and liver weight, which was accompanied by a significant increase in plasma glucose levels from 8 weeks onwards, while insulin levels were affected after 16 weeks. Plasma levels of fibrinogen, FII, FVII, FVIII, FIX, FXI and FXII were significantly higher in mice on a HFD for 2 weeks, which in general persisted throughout the 16 weeks of HFD-feeding. Remarkably, the effects on plasma levels were in general not paralleled by changes in relative hepatic transcript levels and neither by decreased clearance rates. Switching from the HFD to the LFD reversed the HFD-induced procoagulant shift, but again this did not coincide with transcriptional modulation.

Conclusions: Changes in dietary fat content rapidly alter the mouse plasma coagulation profile, thereby preceding metabolic changes. In addition, these rapid changes could not be explained by changes in relative expression levels of coagulation genes or decreased clearance rates.

Introduction

The prevalence of obesity in the Western world is rising and forms an increasing public health problem since obesity affects, amongst others, the development of cardiovascular diseases through its influence on risk factors like hyperlipidemia, hypertension, glucose intolerance and inflammation. The risk for thrombotic cardiovascular events is even further enhanced by the hypercoagulable state that is associated with obesity, as obese subjects have increased plasma levels of procoagulant factor (F) VII, VIII, XII and fibrinogen, while fibrinolysis is decreased as reflected by increased levels of plasminogen activator inhibitor-1 (PAI-1).[1-3] On the other hand, levels of the anticoagulant factors protein C and protein S are higher, and tissue plasminogen activator (tPA) levels are lower under obese conditions, which might be considered to be a compensatory response to the hypercoagulable state.[4,5]

Previous studies evaluating the effect of weight loss on hemostatic parameters showed that levels of tissue factor, FVII, PAI-1 and tPA decreased upon weight loss, resulting in a decrease in thrombin generation.[6,7] In addition, it has been suggested that almost one-third of all thrombotic events could be prevented by weight loss.[8] Taken together, these data indicate that the plasma coagulation profile and the subsequent thrombotic risk may follow both the unfavorable and favorable changes in body weight gain and weight loss, respectively.

Using an experimental animal approach, we and others have previously shown that obesity in mice also results in a hypercoagulable state, which is characterized by increased plasma levels of procoagulant factors and decreased fibrinolysis.[9,10] These results were obtained in mice being on a high fat diet for 4 to 5 months, and during this time, many other metabolic changes may have occurred influencing the coagulation profile indirectly. Therefore, the aim of the present study was to identify whether changes in coagulation stand by itself or whether it is the result of other, earlier manifesting metabolic changes. Hereto, we used mice to establish the onset and reversibility of the hypercoagulable state during the development and regression of nutritionally-induced obesity, and determined its relation to changes in hepatic transcript levels and clearance rates of coagulation factors, as well as it relation to changes in metabolic and inflammatory parameters.

Materials and methods

Animals

Six week old male C57Black/6J mice (Charles River) were fed a diet with 10% kcal as fat (low fat diet; Research Diets) for 4 weeks as a run-in period, after which half of the group switched to an iso-caloric diet with 45% kcal as fat (high fat diet; Research Diets), while the other group remained on the low fat diet (LFD). After 2, 4, 8 or 16 weeks mice (n=15 per group) were fasted overnight and subsequently anesthetized with a mixture of ketamine, xylazine and atropine. The abdomen was opened and a blood sample on sodium citrate (final concentration of 0.32%) was directly drawn from the inferior caval vein. Platelet-poor plasma was obtained and stored at -80°C until use. In addition, part of the left liver lobule and lungs were snap-frozen for mRNA analyses.

In order to compare nutritionally-induced obesity with genetically-induced obesity, 6 weeks old *ob/ob* mice, and their lean wild-type littermate controls (Charles River) were fed the low fat diet for 4 weeks and plasma and tissue samples were obtained for analyses after overnight fasting.

Plasma clearance of the vitamin K-dependent coagulation factors VII and IX, and FVIII were determined in a separate experiment in which 2-week HFD-fed mice received a single intravenous injection (200 µl) of either the human prothrombinase complex (Cofact) or FVIII concentrate (Aafact, both kindly provided by Dr. K. Mertens, Sanquin). Clearance rates of the human plasma-derived factors from the mouse plasma were determined by successive blood sampling via the tail vein.

To study the effects of weight loss on the obese phenotype, mice received the HFD for 16 weeks, and while half of the group remained on the HFD (n=45), the other half switched to the low fat diet. After 1, 2 or 4 weeks, mice were sacrificed after overnight fasting for plasma and tissue mRNA analyses.

All experimental animal procedures were approved by the animal welfare committee of the Leiden University.

Plasma analyses

Plasma triglyceride and insulin levels were measured using commercially available kits from Roche Diagnostics and Crystal Chem Inc., and glucose levels were determined according to the hexokinase method (Instruchemie). Plasma levels of multiple cytokines were evaluated simultaneously by using pre-coated multisport plates in an ELISA-based electrochemiluminescence assay (Meso Scale Discovery).

Coagulation factor levels were measured as previously described and pooled mouse plasma was used to generate standard curves.[11] The *in vivo* clearance of

human coagulation factors VII, VIII and IX was analyzed with home-made ELISAs specific for human proteins that were proven not to cross-react with mouse plasma proteins. Standard curves were generated by adding Cofact or Aafact to pooled mouse plasma (final concentration 20%) to calculate human antigen levels, and the level measured directly after injection (1 minute) was set as a reference (100%).

RNA isolation and real-time RT-PCR

Individual liver samples (15-20 mg) of 10 animals per group were homogenized in RNAzol (Tel-Test), and RNA isolation and cDNA synthesis was executed as previously reported.[11] Quantitative real-time PCR was performed using SybrGreen (Applied Biosystems) and gene-specific primers[11] and the comparative threshold cycle method with ß-actin as internal control was used for quantification and normalization. To evaluate the effects of weight gain on transcript levels, LFD-fed mice were set as a reference, whereas the HFD-fed mice were set as a reference to determine the effects of weight loss. The ΔC_t values of individual samples were related to the mean ΔC_t of the reference group.

Statistical analyses

Data are expressed as mean ± standard error of the mean (SEM) or as the median and range for cytokine levels. Gene expression data are presented as the mean including the minimum and maximum expression levels. Data were analyzed with the GraphPad Instat software and statistical differences between the LFD and HFD groups were evaluated using a Student's t-test, or a Mann-Whitney test in case of cytokine levels, to compare the LFD with HFD group. P-values <0.05 were considered to be statistically significant.

Results

Induction of obesity

Two weeks of HFD-feeding resulted in a significantly increased fasted body weight as compared to the LFD-fed mice (25.0±0.6 g vs. 22.3±0.3g, p<0.001), which gradually increased further over time (table 1). From 8 week onwards, liver weights of HFD-fed mice were also significantly higher than those of LFD-fed mice.

Plasma cytokine levels showed a transient rise in interleukin (IL) 1ß and keratinocyte chemoattractant (KC) levels after 2 weeks of HFD-feeding (3.2 (0.7-20.7) pg/mL vs. 7.0 (1.1-88.9) pg/mL for IL-1β, p<0.05; 34.6 (19.1-246.8) pg/mL vs. 55.9 (28.7-233.6) pg/mL for KC, p<0.05). The levels of IL-6, IL-10, IL-12, interferon-

γ (IFN-γ) and tumor necrosis factor α (TNFα) were not affected (data not shown). Triglyceride levels were also transiently increased at the 2 week time point in mice fed the HFD (0.70±0.04 mmol/L vs. 0.86±0.06 mmol/L, $p<0.05$), and again after 16 weeks of HFD feeding (table 1). Plasma glucose levels were higher due to the high fat diet from 8 week onwards (5.7±0.2 mmol/L vs. 7.7±0.4 mmol/L, $p<0.001$), whereas insulin levels were only increased after 16 weeks (table 1).

The high fat feeding-related changes in metabolic parameters after 16 weeks largely resembled the observations in the LFD-fed *ob/ob* mice, which had a comparable weight and enlarged liver (table 1). In addition, the insulin and glucose levels were also higher as compared to their wild-type littermates. KC and IFN-γ levels were increased in *ob/ob* mice (KC 4.4 (16.5-229.1) pg/mL vs. 100.1 (17.6-268.9) pg/mL, $p<0.05$; IFN- γ 7.3 (2.0-53.9) pg/mL vs. 45.9 (1.2-125.0) pg/mL, $p<0.05$), but they displayed surprisingly lower fasted plasma triglyceride levels (table 1).

Table 1: Metabolic parameters of mice on a low fat diet (LFD) or high fat diet (HFD) for 16 weeks as compared to genetically obese *ob/ob* mice with their littermate wild-type controls after 4 weeks of LFD feeding.

	LFD (n=15)	HFD (n=15)	WT (n=15)	*ob/ob* (n=15)
Body weight (g)	27.4±0.5	41.6±0.9‡	22.1±0.6	40.3±0.6‡
Liver weight (g)	0.79±0.01	1.08±0.06‡	0.78±0.04	2.14±0.06‡
Triglycerides (mmol/L)	0.54±0.03	0.65±0.04*	0.62±0.05	0.39±0.02‡
Insulin (pg/mL)	97.4±1.0	105.0±2.8*	94.1±0.9	105.4±1.8‡
Glucose (mmol/L)	5.7±0.2	8.0±0.6‡	5.8±0.6	11.6±0.9‡

Data are expressed as mean±SEM. *$p<0.05$ and ‡$p<0.001$ as compared to LFD-fed mice or wild-type controls as appropriate.

Within 2 weeks, the high fat diet induced a clear procoagulant shift of the plasma coagulation profile, with significant increases in fibrinogen, FII, FVII, FVIII, FIX, FXI and FXII levels (fig. 1A). Continuation of the HFD resulted in sustained increased levels of fibrinogen, FII and FVII while the effects on FVIII, FIX, FXI and FXII levels became less pronounced and did not reach statistical significance after 16 weeks HFD-feeding. Factor X and antithrombin plasma levels were only significantly increased after 16 weeks of HFD-feeding (fig. 1B). Comparing the 16 week HFD-fed animals with *ob/ob* mice showed a similar procoagulant shift, although these

effects were more pronounced in *ob/ob* mice (fig. 1C). Remarkably, factor VII levels, like the triglyceride levels, were lower in *ob/ob* mice than in the wild-type controls, while plasma levels of fibrinogen and FXI were not significantly affected.

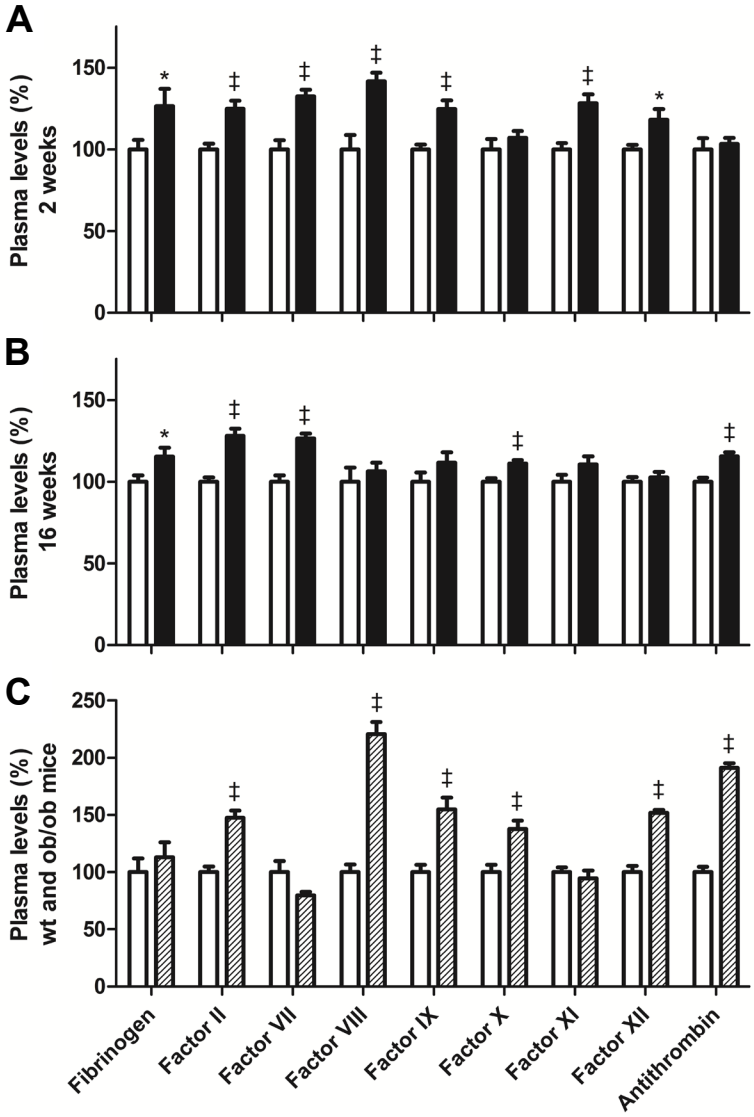

Figure 1: Effects on plasma coagulation parameters after 2 (panel A) and 16 (panel B) weeks of low fat diet (white) or high fat diet (black) feeding. Panel C shows the plasma coagulation profile of genetically obese *ob/ob* mice (striped) and their wild-type littermates (white) after 4 weeks on a low fat diet. Data are presented as mean±SEM. *$p<0.05$ and ‡$p<0.001$ as compared to the LFD-fed mice or wild-type controls as appropriate.

Since the liver is the main site of production of plasma coagulation factors, we determined whether the changes in the plasma coagulation profile due to the high fat diet were related to changes in hepatic transcript levels, as we have previously shown that changes in plasma levels can coincide with transcriptional effects.[11] Surprisingly, at the 2-week time point where the liver weight between diet treatment groups are comparable but clear increases in plasma levels are observed, relative mRNA levels of coagulation genes were not affected with the exception of FXI which was increased and FVIII which was decreased (table 2). Despite the differences in liver weight after 16 weeks of high fat-feeding, we evaluated whether prolonged exposure to dietary fat was able to affect transcription. However, HFD-feeding for 16 weeks was also not able to induce increases in relative mRNA levels of hepatically expressed coagulation factors (data not shown).

As the changes in the plasma coagulation profile were not paralleled by changes in transcript levels, we determined whether HFD-feeding for 2 weeks affected plasma protein turnover, i.e. decreased the clearance rate. A bolus injection of either the human FVIII concentrate or prothrombin complex concentrate resulted in both the HFD and LFD group in single-phase clearance curves with comparable half-lives between LFD-fed and HFD-fed mice (FVIII 18.6±1.8 min vs. 15.3±1.7 min, FVII 112.6±7.1 min vs. 99.4±5.9 min and FIX 79.0±9.5 vs. 76.5±5.7 min).

Table 2 Hepatic mRNA levels of coagulation genes of mice on a low fat diet (LFD) or high fat diet (HFD) for 2 weeks.

	LFD (n=10)	HFD (n=10)
Fibrinogen	1 (0.93-1.08)	0.87 (0.83-0.90)
Factor II	1 (0.93-1.08)	1.05 (0.97-1.13)
Factor VII	1 (0.95-1.05)	1.07 (1.01-1.13)
Factor VIII	1 (0.94-1.06)	0.64 (0.54-0.77)*
Factor IX	1 (0.95-1.05)	1.08 (1.03-1.13)
Factor X	1 (0.96-1.04)	1.01 (0.96-1.07)
Factor XI	1 (0.92-1.09)	1.51 (1.44-1.58)‡
Factor XII	1 (0.95-1.05)	1.06 (1.01-1.12)
Antithrombin	1 (0.95-1.05)	1.05 (1.00-1.10)

Data are expressed as mean (minimum-maximum expression level). *p<0.05 and ‡p<0.001 as compared to LFD mice.

Regression of obesity

Since the dietary fat intake resulted in a rapid procoagulant shift of the plasma coagulation profile, we determined whether regression of the nutritionally-induced obesity also resulted in an altered coagulation profile. Therefore, part of the mice receiving the HFD for 16 weeks switched to the low fat diet (n=45) while the remaining mice continued the HFD (n=45). Switching to the LFD resulted in an immediate decrease in body weight, which was already significant after 1 week (37.3±1.5 g vs. 42.9±1.1 g, $p<0.01$), while effects on the liver weight were apparent after 2 weeks (1.05±0.06 g vs. 1.22±0.08 g, $p<0.05$). Fasted plasma glucose levels were also rapidly affected (6.4±0.4 mmol/L vs. 8.7±0.7 mmol/L, $p<0.01$), whereas insulin levels only differed at 4 weeks after switching to the LFD (103.5±1.0 pg/mL for the mice switched to LFD vs. 109.3±2.4 pg/mL for the HFD mice, $p<0.05$) and triglyceride levels were not affected due to the change in dietary fat content. Furthermore, KC levels were significantly increased after the first week of switching to the LFD (58.2 (36.1-168.2) pg/mL for HFD vs. 90.8 (36.3-542.6) pg/mL, $p<0.05$).

The plasma coagulation profile showed a remarkably rapid shift after switching to a low fat diet, with significantly reduced activity levels of FII, FVII, FIX, FX and FXI after only 1 week (figure 2a), and these changes persisted throughout the remaining for 4 weeks. In addition, factor VIII and antithrombin levels were altered after 2 weeks of switching diets (100±9.8% vs. 72.4±5.0%, $p<0.01$ and 100±2.3% vs. 86.6±4.4, $p<0.05$ respectively), whereas FXII levels were lower after 4 weeks (100±1.5% vs. 92.1±1.5, $p<0.01$).

As one week after switching diets was able to alter the plasma coagulation profile, while liver weights were not significantly affected, hepatic mRNA analyses were performed to determine whether transcript levels of coagulation genes were modulated by the diet switch. The non-significant decrease in plasma fibrinogen levels coincided with a significant reduction in transcript levels, and also for FXI the decrease in plasma was paralleled by a decrease in relative mRNA levels. However, for all other coagulation factors measured in plasma, no effects on hepatic mRNA levels were observed (figure 2).

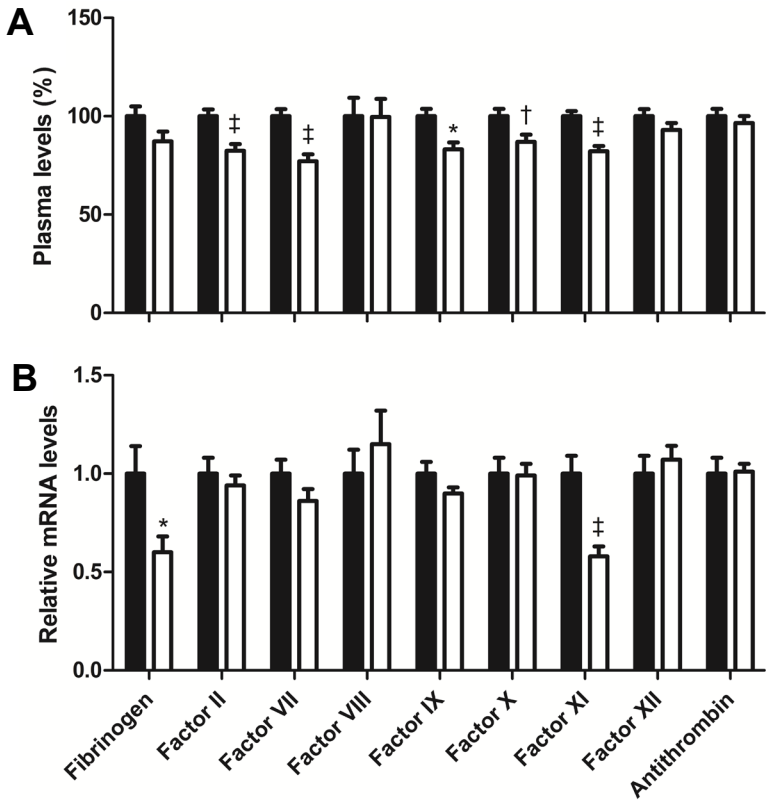

Figure 2: Plasma (A) and relative transcript levels (B) of mice on a high fat diet for 17 weeks (black) and mice that were switched after 16 weeks of HFD-feeding to the LFD for 1 week (white). Data are presented as mean±SEM for the plasma data and as mean with the error bar representing the calculated maximum expression level of n=10 mice per group for the expression levels. Relative expression levels were compared using the comparative threshold cycle method with ß-actin as internal control and the HFD-fed mice were set as a reference. *$p<0.05$, †$p<0.01$ and ‡$p<0.001$ as compared to the LFD-fed mice or wild-type controls as appropriate.

Discussion

Obesity is becoming an increasing public health problem and it is associated, amongst others, with a hypercoagulable state. In order to gain more insight in this relation between obesity and hypercoagulability we used an *in vivo* approach to study the onset and potential reversibility of the hypercoagulable state during the development and regression of nutritionally-induced obesity. In addition, we determined the mechanisms leading to this hypercoagulable state by evaluating transcription and clearance of coagulation factors, as well as determining metabolic and inflammatory parameters since they may aggravate the hypercoagulable state associated with obesity.

We have shown that that nutritionally-induced obesity coincides with an early-onset procoagulant shift of the plasma coagulation profile, which was already apparent within 2 weeks after the start of the HFD. Furthermore, these changes largely persisted during the continuation of the HFD for 16 weeks, thereby preceding the effects in metabolic parameters like glucose and insulin levels since these were only affected in overt obese mice. Switching from a high fat to a low fat diet to induce weight loss resulted in a rapid reversal of the HFD-induced procoagulant shift of the plasma coagulation profile, as evaluated 1 week after switching diets. Surprisingly, these effects on the plasma coagulation profile after switching dietary fat intake appeared to be independent of changes in relative transcript levels and clearance rates of coagulation factors.

A remarkable observation in this study is that the changes observed in plasma activity levels were not paralleled by changes in relative transcript levels of hepatically expressed coagulation genes. In addition, clearance studies, although performed with human proteins, could also not explain the increased plasma levels in HFD-fed mice. This difference between mRNA levels and clearance rates on the one hand, and plasma levels on the other, may have several reasons. Regarding the clearance studies, human coagulation factors may be differently cleared from mouse plasma than murine factors. Secondly, since high fat feeding can affect liver physiology, we were interested whether the RNA recovery in liver samples of LFD and HFD mice differed. Although the liver weights after 2 weeks of HFD feeding were comparable, the amount of RNA per mg liver weight was approximately 20% higher as compared to the RNA recovery from LFD-fed mice. One can speculate that this increased recovery might result in an overall increase in absolute transcript levels, while the relative mRNA levels are similar between diets, and therefore contribute to the increased plasma activity levels. Finally, although we have previously shown that transcript levels correlate well to plasma activity levels,[9] post-transcriptional or post-translational mechanisms may affect the activity of the resulting protein. A comparable situation, in which protein activity is increased while transcription is down-regulated under nutritionally-induced obesity, has also been reported for phosphoenolpyruvate carboxykinase (PEPCK), an enzyme associated with hepatic glycogen storage.[12]

The fact that there is a transient rise in IL-1β and KC levels when mice switch diet types, suggests that the system has to adapt to the new diet in order to maintain homeostasis. These data are in concordance with the metabolic stress response that occurs during short-term high fat feeding.[13] Furthermore, a recent genome-

wide mRNA expression study which focused on changes in hepatic gene expression during high fat feeding, also showed that exposure to dietary fat first results in inflammation which under long-term high fat feeding causes a switch to a steatotic transcriptional program.[14] Besides their roles in coagulation, fibrinogen and factor VIII also play a role in inflammation as acute phase proteins and their transcription can be induced by nuclear factor (NF)-κB, which transcriptional activity is increased under inflammatory conditions.[15,16] However, although both fibrinogen and FVIII levels in plasma are increased after 2 weeks of HFD-feeding, their transcript levels are decreased, making an increased transcriptional activity of NF-κB resulting in increased plasma coagulation factor levels less likely.

By studying coagulation during the development and regression of nutritionally-induced obesity, we were able to show that the dietary fat content plays and important role in affecting the plasma coagulation profile. We also evaluated genetically obese *ob/ob* mice, mainly as a control for determining the effects of HFD on metabolic parameters like insulin resistance. The *ob/ob* mice have been predominantly used to study metabolic disorders leading to type 2 diabetes, and although they have been used in studies focusing on tissue factor and PAI-1, in general little is known about their overall plasma coagulation profile.[17,18] Here we show that *ob/ob* mice have more pronounced increases in plasma procoagulant factor levels as compared to mice on a HFD for 16 weeks, with the exception of FVII levels since they are decreased in *ob/ob* mice. As these mice display metabolic abnormalities, this may aggravate the hypercoagulable state, for example via an increased transcriptional activity of nuclear factor (NF)-κB which in turn can induce expression of coagulation genes as previously mentioned. Because of the underlying pathologies that can potentially affect coagulation, the *ob/ob* mouse seems to be less suitable to study obesity with respect to coagulation and a (short-term) nutritionally-induced obesity model seems warranted.

In summary, this *in vivo* study shows that the plasma coagulation profile is able to rapidly respond to changes in dietary fat content, both in weight gain which is associated with a procoagulant shift, and in subsequent weight loss resulting in a reversal of the HFD-induced hypercoagulability. These changes in the plasma coagulation profile seem to be independent of changes in relative transcript levels of coagulation genes and changes in protein clearance rates. In addition, the effects on coagulation precede alterations in metabolic parameters like insulin and glucose levels. The fact that weight loss is associated with rapid beneficial effects

on coagulation, and late effects of metabolic parameters, may eventually translate in a risk reduction for thrombotic cardiovascular events.

Acknowledgements

This study was supported by the Leducq Foundation (Paris, France; LINAT project) and grant 2006B045 from the Netherlands Heart Foundation.

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 Mertens I, Van Gaal LF. Obesity, haemostasis and the fibrinolytic system. Obes Rev 2002;3:85-101.
3 Rosito GA, D'Agostino RB, Massaro J, L et al. Association between obesity and a prothrombotic state: the Framingham Offspring Study. Thromb Haemost 2004;91:683-9.
4 Godsland IF, Crook D, Proudler AJ, et al. Hemostatic risk factors and insulin sensitivity, regional body fat distribution, and the metabolic syndrome. J Clin Endocrinol Metab 2005;90:190-7.
5 Sola E, Navarro S, Medina P, et al. Activated protein C levels in obesity and weight loss influence. Thromb Res 2009;123:697-700.
6 Ay L, Kopp HP, Brix JM, et al. Thrombin generation in morbid obesity: significant reduction after weight loss. J Thromb Haemost 2010;8:759-65.
7 Folsom AR, Qamhieh HT, Wing RR, et al. Impact of weight loss on plasminogen activator inhibitor (PAI-1), factor VII, and other hemostatic factors in moderately overweight adults. Arterioscler Thromb 1993;13:162-9.
8 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.
9 Cleuren AC, Van Hoef B, Hoylaerts MF, et al. Short-term ethinyl estradiol treatment suppresses inferior caval vein thrombosis in obese mice. Thromb Haemost 2009;102:993-1000.
10 Nagai N, Hoylaerts MF, Cleuren AC, et al. Obesity promotes injury induced femoral artery thrombosis in mice. Thromb Res 2008;122:549-55.
11 Cleuren AC, Van der Linden IK, de Visser YP, et al. 17alpha-Ethinylestradiol rapidly alters transcript levels of murine coagulation genes via estrogen receptor alpha. J Thromb Haemost 2010;8:1838-46.
12 Do GM, Oh HY, Kwon EY, et al. Long-term adaptation of global transcription and metabolism in the liver of high-fat diet-fed C57BL/6J mice. Mol Nutr Food Res 2011;55:S173-S185.
13 Muller M, Kersten S. Nutrigenomics: goals and strategies. Nat Rev Genet 2003;4:315-22.
14 Radonjic M, de Haan JR, van Erk MJ, et al. Genome-wide mRNA expression analysis of hepatic adaptation to high-fat diets reveals switch from an inflammatory to steatotic transcriptional program. PLoS One 2009;4:e6646.

15 Iwasaki Y, Nishiyama M, Taguchi T, et al. Insulin exhibits short-term anti-inflammatory but long-term proinflammatory effects in vitro. Mol Cell Endocrinol 2009;298:25-32.
16 Liu SF, Malik AB. NF-kappa B activation as a pathological mechanism of septic shock and inflammation. Am J Physiol Lung Cell Mol Physiol 2006;290:L622-L645.
17 Oishi K, Ohkura N, Wakabayashi M, et al. CLOCK is involved in obesity-induced disordered fibrinolysis in ob/ob mice by regulating PAI-1 gene expression. J Thromb Haemost 2006;4:1774-80.
18 Samad F, Loskutoff DJ. Hemostatic gene expression and vascular disease in obesity: insights from studies of genetically obese mice. Thromb Haemost 1999;82:742-7.

Chapter 8

Thyroid hormone modulates murine coagulation through immediate and late effects on hepatic and endothelium-associated coagulation gene transcription

Audrey CA Cleuren, Anita Boelen, Pieter H Reitsma,
Bart JM van Vlijmen

Manuscript submitted for publication

Summary

Background: Thyroid dysfunction is associated with changes in coagulation.

Objective: To gain more insight in the role of thyroid hormone in coagulation control.

Methods: C57Black/6J mice received a low-iodine diet and drinking water supplemented with perchlorate to suppress endogenous triiodothyronine (T_3) and tetraiodothyronine (T_4) production. Under these conditions, the impact of exogenous T_3 on plasma coagulation, and hepatic and endothelium-associated coagulation gene transcription was studied.

Results: A single T_3 injection (0.5µg/mouse) increased hepatic transcript levels of the T_3-responsive genes deiodinase type 1 and Spot14 within 4 hours. This coincided with significantly reduced mRNA levels of fibrinogen-γ, antithrombin, protein C, protein Z and protein Z-related protease inhibitor (ZPI), and the reduction of the latter three persisted upon daily treatment with T_3 for 14 days. Prolonged T_3 treatment also induced a significant down-regulation in factor (F) II, IX and X transcript levels, while FXI and FXII levels increased. Activity levels in plasma largely paralleled these mRNA changes. Thrombomodulin transcript levels in the lung were significantly up-regulated after a single T_3 injection, which persisted upon prolonged T_3 exposure. Two-week T_3 administration also resulted in increased von Willebrand factor (VWF) and tissue factor pathway inhibitor (TFPI) mRNA levels, whereas tissue factor levels decreased.

Conclusions: Our mouse data show that T_3 has profound effects on coagulation, with fibrinogen-γ, antithrombin, protein C, protein Z, ZPI and thrombomodulin responding rapidly, making these likely direct thyroid hormone receptor targets. FII, FIX, FX, FXI, FXII, VWF, TF and TFPI are late responding genes and therefore probably indirectly regulated by T_3.

Introduction

Abnormalities of blood coagulation are common in patients with thyroid dysfunctions. In general, hyperthyroidism is associated with a hypercoagulable state and increased risk for venous thrombosis.[1] In hypothyroidism, the severity of the disorder determines whether the coagulation profile is shifted towards an anticoagulant or procoagulant state as subclinical hypothyroidism is associated with hypercoagulation whereas patients with overt hypothyroidism have a bleeding tendency.[2,3]

The mechanism of action of thyroid hormones and thyroid hormone receptors has been well established in a number of metabolic processes, including lipoprotein homeostasis, cell proliferation and mitochondrial respiration.[4-6] Experimental evidence regarding the mechanisms by which thyroid hormones modulate blood coagulation is more limited, and consists mainly of *in vitro* data showing that T_3 is able to modulate transcript levels of fibrinogen, factor (F) II, FX and antithrombin.[7-9] *In vivo* studies with thyroid hormone receptor knock-out mice identified fibrinogen-γ to be modulated by T_3, whereas T_3 administration in thyroidectomized rats was able to increase fibrinogen-α, FII and FX mRNA levels.[5,9]

Here, we used an *in vivo* approach to study the modulatory role of thyroid hormone on plasma coagulation and transcript levels of coagulation genes, by treating mice with the active thyroid hormone, i.e. triiodothyronine (T_3), under conditions of a suppressed endogenous thyroid hormone production. Our mouse data demonstrate that under these conditions, T_3 is able to modulate coagulation by controlling transcription of coagulation genes, and that these effects can be both immediate and late.

Material and Methods

Animal experiments

Eight week old C57Bl/6J mice were purchased from Charles River (Maastricht, the Netherlands) and fed a low iodine diet (ICN Biomedicals, Inc.) while drinking water was supplemented with 1% (wt/vol) potassium perchlorate (Sigma Aldrich) to suppress endogenous thyroid hormone production. 3,3′,5-Triiodo-L-thyronine sodium salt (T_3; Sigma Aldrich) stocks of 1 mg/ml were prepared in 4 mM sodium hydroxide and stored at 4°C until use. Before injections, the T_3 stock was diluted (0-10 µg T_3 /200 µl) in phosphate buffered saline (PBS) supplemented with 0.02% bovine serum albumin with a final concentration of 0.2 mM sodium hydroxide. To determine the effects of a prolonged T_3 exposure on transcription as well as plasma

levels of coagulation factors, mice received a daily intraperitoneal injection of 200 µl T_3 for 14 days. To determine which factors are rapidly modulated by T_3, a single dose of either 0.5 µg T_3/mouse or vehicle (PBS supplemented with 0.02% bovine serum albumin with a final concentration of 0.2 mM sodium hydroxide) was administered. After the last administration, mice were anesthetized by an intraperitoneal injection with a mixture of ketamine (100 mg/kg), xylazine (12.5 mg/kg) and atropine (125 µg/kg) after which the abdomen was opened by a midline incision and a blood sample on sodium citrate (final concentration 0.32%) was drawn from the inferior caval vein. Platelet-poor plasma was obtained and stored at -80°C until use. The liver was isolated and weighed, and part of the left liver lobule and the lungs were snap-frozen for mRNA analyses. All experimental procedures were approved by the animal welfare committee of the Leiden University.

Plasma analyses

Plasma triiodothyronine (T_3) and tetraiodothyronine (T_4) levels were measured with in-house radioimmunoassays as previously described.[10] Plasma alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) levels were determined using routine clinical chemistry assays. Plasma FII and FX activity were analyzed by means of chromogenic substrate conversion, factor VII activity was evaluated using the commercially available Biophen FVII kit (Hyphen Biomed) and activity levels of factor VIII, IX, XI, XII were measured in APTT based assays.[11] Plasma fibrinogen and protein C antigen levels were assessed with a commercial murine ELISA kit from Affinity Biologicals and an in-house ELISA using antibodies from Haematologic Technologies Inc, respectively. Antithrombin activity was measured by means of the Coamatic Antithrombin kit (Chromogenix). For all plasma assays of individual coagulation factors, pooled normal mouse plasma was used to generate standard curves and the vehicle-treated group was subsequently set as a reference (100%).

Global coagulability of the plasma was determined by measuring the activated partial thromboplastin time (APTT) using the STA Neoplastine Plus reagent on the STart 4 analyzer (Diagnostica Stago). The prothrombin time was determined with the Simple Simon PT system (Zafena) and thrombin generation was assessed by means of the Calibrated Automated Thrombogram, using 5 pM tissue factor (Thrombinoscope) to trigger 1:6 diluted mouse plasma. Thrombin generation was measured on the Fluoroskan Ascent reader (Thermo Scientific) and the curves and

area under the curve (endogenous thrombin potential; ETP) were calculated using the Thrombinoscope software.

RNA isolation and real-time RT-PCR

Individual liver (20-30 mg) and lung samples (40-50 mg), as a substitute for the endothelium, were homogenized in RNAzol (Tel-Test) and RNA isolation and cDNA synthesis was performed as previously described.[11] Quantitative real-time PCR using SybrGreen (Applied Biosystems) and gene-specific primers (supplemental table S1) was performed on the ABI Prism 7900 HT Fast Real-Time PCR System from Applied Biosystems. Data were analyzed using the accompanying Sequence Detection System software. The comparative threshold cycle method with β-actin as internal control was used for quantification and normalization. Vehicle-treated animals were set as a reference and the ΔC_t values of the individual samples were related to the mean ΔC_t of the reference group.

Statistical analyses

Data are analyzed with the GraphPad Instat software and statistical differences between groups of the dose-finding were evaluated using a one-way analysis of variance (ANOVA) with a Dunnett post-hoc test. For studies using only one dose of thyroid hormone, a Student's t-test was used to compare the thyroid hormone- and vehicle-treated groups. A p-value of <0.05 was considered to be statistically significant.

Results

Dose-finding study

A dose-finding study was performed in which the effects of exogenous T_3 in a range from 0-10 µg/mouse/day (n=6 per group) was tested in order to determine specific T_3-induced effects on a representative panel of coagulation factors, and to establish an optimal T_3 dose to use for further evaluation.

Increasing amounts of exogenous T_3 in hypothyroid mice, i.e. having a suppressed endogenous thyroid hormone production, resulted in a dose-dependent increase in body weight up to a dose of 5 µg/day (weight gain during 14 days: 0.18±0.26 g vs. 2.26±0.36 g, $p<0.01$), whereas the liver weight dose-dependently decreased (1.51±0.07 g vs. 1.09±0.01 g, $p<0.01$; table S2). As expected, plasma T_3 levels increased dose-dependently from 0.31±0.01 nmol/L for vehicle-treated hypothyroid mice to a maximum of 51.2±5.8 nmol/L for mice treated with 10 µg T_3/day ($p<0.01$).

T_4 levels were low (range 8.8±0.6 – 14.0±1.0 nmol/L) and did not differ between treatment groups (table S2). Plasma T_3 and T_4 levels of regular C57Bl/6J mice were measured for reference values, being 1.22±0.04 nmol/L and 60.7±4.2 nmol/L respectively, indicating that the endogenous thyroid hormone production in our experimental set-up was successfully suppressed.

Circulating liver enzymes were determined as a marker of possible liver damage due to T_3 administration and showed that ALT and AST levels were not affected in T_3-treated mice as compared to vehicle treatment. However, administration of increasing amounts of T_3 (from 0.5 µg/day upwards) resulted in relatively modest increased ALP levels that differed significantly from vehicle-treated mice (61.3±1.0 U/L vs. 140.0±15.1 U/L for 0 and 0.5 µg T_3/mouse/day respectively, $p<0.01$; table S2).

Plasma levels of a limited but representative panel of coagulation factors were measured and revealed for fibrinogen antigen and factor II, X and antithrombin activity levels dose-dependent decreases (maximal effects -60%, -50%, -30% and -30% respectively for the 10 µg T_3/day dose), whereas FXII activity was increased (+30%), and as compared to the vehicle treatment these effects were significantly different from a dose of 0.5 µg upwards (table S2). FIX was not significantly affected and FVIII levels showed no unambiguous dose-dependent increase or decrease as compared to vehicle-treated mice.

As doses of 5 and 10 µg T_3/day had no additional effects on plasma coagulation factor levels, these doses were excluded from subsequent mRNA analyses. In the range from 0–1 µg T_3/mouse/day, hepatic transcript levels of deiodinase type 1 (D1), a well-established T_3 response gene,[12] was dose-dependently up-regulated as expected (table S3). Dose-dependent decreases in plasma fibrinogen and FII levels coincided with reduced transcript levels, which were significantly different in mice treated with 0.5 µg T_3/day as compared to vehicle-treated animals (-50% for fibrinogen and -35% for FII), whereas the transcript levels of FXII and antithrombin were not significantly affected (table S3). Transcript levels of the endothelium-associated coagulation factors were determined in the lung as a substitute for the endothelium since this organ is highly vascularized. Increasing T_3 doses resulted in a dose-dependent decrease of tissue factor mRNA levels (-30%) and a rise in thrombomodulin levels (+55%). Transcript levels of von Willebrand factor (VWF) and the endothelial protein C receptor (EPCR) were not affected (table S3).

Based on these data, a daily dose of 0.5 µg T_3/mouse/day was chosen for further studies, as this caused higher plasma T_3 levels as compared to regular C57Bl/6J

mice and was the lowest dose able to induce nearly maximal changes in both plasma and transcript levels of coagulation factors.

Prolonged T_3 treatment

To evaluate the effects of T_3 on coagulation in more detail, 12 mice with a suppressed thyroid hormone production per group were treated with either the vehicle (0 µg T_3/mouse/day) or a dose of 0.5 µg T_3/mouse/day for 2 weeks. This treatment regime again resulted in an increase in body weight (weight gain: 0.24±0.15 g vs. 2.12±0.20 g, $p<0.001$) and a reduction in liver weight (0.84±0.02 g vs. 0.74±0.02 g, $p<0.001$). As expected, T_3 levels were significantly higher in T_3-treated mice (5.64±0.21 nmol/L vs. 0.33±0.01 nmol/L, $p<0.001$), whereas T_4 levels were low and did not differ between treatment groups (7.2±0.2 nmol/L vs. 8.7±1.0 nmol/L).

Figure 1A shows that, in line with the dose-finding study, plasma FII and FX activity levels decreased upon T_3 treatment, while FXII levels increased and FVIII and FIX levels were not significantly affected. In addition, FVII levels did also not differ between vehicle- and T_3-treated animals, whereas FXI activity levels increased due to T_3 administration. Plasma antithrombin and protein C levels were both significantly lower in T_3-treated mice (100±1.6% vs. 92.6±1.2%, $p<0.001$ for antithrombin; 100±4.0% vs. 85.9±3.1%, $p=0.009$ for protein C).

Consistent with the lower FII and FX levels upon T_3 treatment, the prothrombin time was longer in T_3-treated animals and the increased FXI and FXII levels resulted in a shorter APTT (fig. 2). To assess the overall plasma coagulability, thrombin generation was measured, showing a lower endogenous thrombin potential in mice treated with 0.5 µg T_3/day as compared to vehicle-treated mice, which was mainly due to a lower peak height and an earlier onset of the inhibition of thrombin activity (fig. 2C).

With the exception of FIX levels, the T_3-induced effects on plasma proteins were completely paralleled by changes in hepatic transcript levels (fig. 1A and 1B). The transcript levels of additional anticoagulant genes and the fibrinolytic factors plasminogen and α_2-antiplasmin were determined and presented in figure 3A. Strikingly, T_3 administration caused only significant decreases, no increases, in mRNA levels of anticoagulant factors which included protein C, protein S, protein Z, protein Z-related protease inhibitor (PZI) and plasminogen. Antithrombin, heparin cofactor II and α_2-antiplasmin levels were not affected.

In line with the dose-finding study, tissue factor transcript levels in the lung were reduced and thrombomodulin levels were up-regulated upon two-week T_3 exposure (fig. 4A). In addition, VWF levels now showed a significant increase, as well as the mRNA levels of tissue factor pathway inhibitor (TFPI), whereas EPCR levels remained unaffected by T_3 treatment.

These data show that prolonged T_3 exposure is able to modulate the plasma coagulation profile by controlling hepatic transcript levels of coagulation genes. In addition, transcription of endothelium-associated coagulation genes, measured at the level of the lung, can also be modulated by T_3 administration.

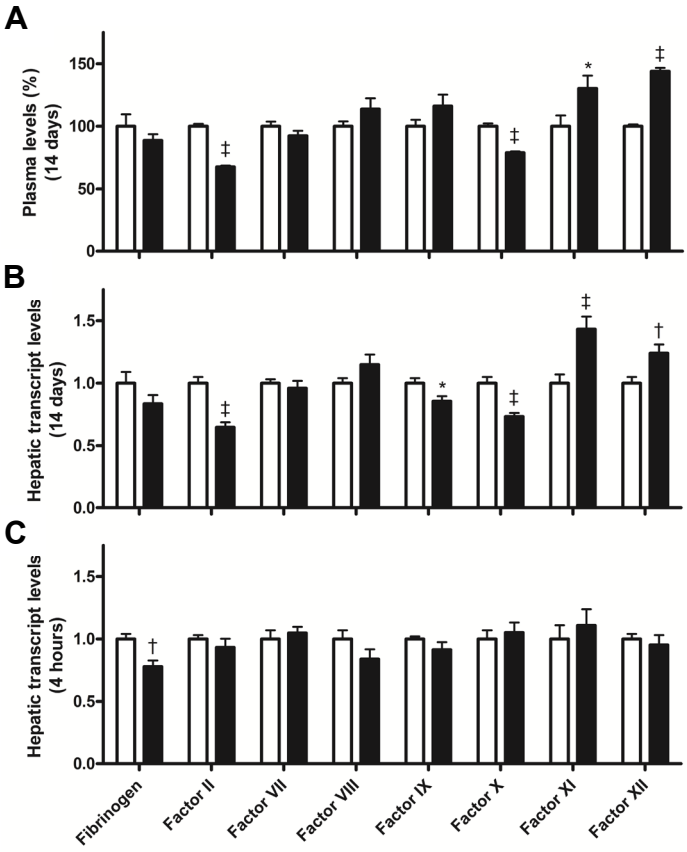

Figure 1: Plasma (A) and hepatic transcript levels (B and C) of procoagulant coagulation factors in mice treated with 0 µg T_3 (white bars) or 0.5 µg T_3 (black bars) for 14 days (panels A and B). Panel C shows the T_3-induced changes in hepatic transcript levels 4 hours after a single T_3 injection. Data are presented as mean±standard error of the mean (panel A) or mean with the error bar representing the calculated maximum expression level (panels B and C) of n=12 mice per group, with the vehicle-treated group set as a reference. Relative expression levels (B and C) were compared using the comparative threshold cycle method with ß-actin as internal control. *$p<0.05$, $^{\dagger}p<0.01$ and $^{\ddagger}p<0.001$ as compared to vehicle-treated mice.

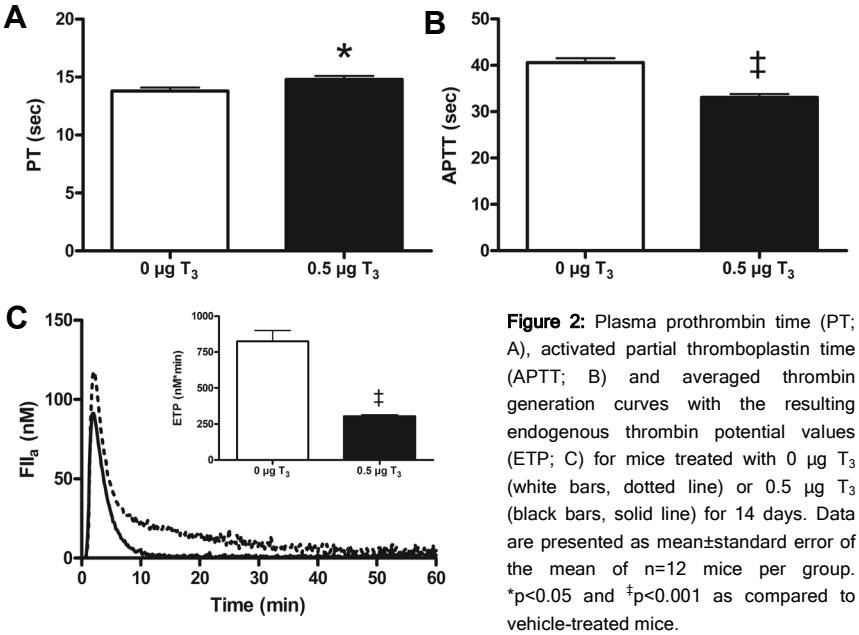

Figure 2: Plasma prothrombin time (PT; A), activated partial thromboplastin time (APTT; B) and averaged thrombin generation curves with the resulting endogenous thrombin potential values (ETP; C) for mice treated with 0 µg T_3 (white bars, dotted line) or 0.5 µg T_3 (black bars, solid line) for 14 days. Data are presented as mean±standard error of the mean of n=12 mice per group. *$p<0.05$ and ‡$p<0.001$ as compared to vehicle-treated mice.

Single T_3 dose

In order to determine whether T_3 is able to directly affect transcription of coagulation genes, i.e. via a direct interaction between the ligand-bound thyroid hormone receptor and the promoter region of coagulation genes, we examined the mRNA levels 4 hours after a single T_3 injection (0.5 µg/mouse).

Already within these 4 hours, the mRNA levels of the canonical T_3-responsive genes D1 and Spot14 were increased (1±0.18 vs. 136.4±29.3 for D1 and 1±0.36 vs. 5.80±1.26 for Spot14). Under these conditions, hepatic transcript levels of fibrinogen-γ and antithrombin were significantly reduced (fig. 1C and 3B), whereas these effects were not apparent after prolonged T_3 exposure. Although the immediate decrease of protein S mRNA levels was comparable to the reduction observed after a prolonged T_3 treatment, this did not reach statistical significance. Protein C, protein Z and PZI transcript levels were significantly affected after both the prolonged T_3 administration and a single dose of T_3. In contrast to the two-week T_3 treatment, a single T_3 dose was not able to induce significant changes in FII, FX, FXI, FXII and plasminogen transcript levels (fig. 3B). Transcript levels of most endothelium-associated clotting factors in the lung remained unaffected, however, thrombomodulin transcript levels increased markedly after a single T_3 injection (fig. 4B).

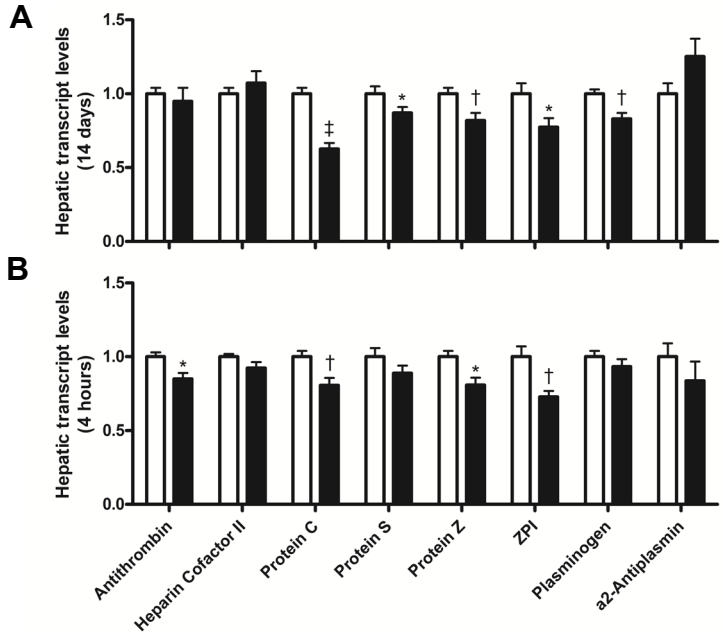

Figure 3: Hepatic transcript levels of anticoagulant and fibrinolytic factors in mice treated with 0 µg T_3 (white bars) or 0.5 µg T_3 (black bars) for 14 days (A) or given a single dose (B). Data are presented as mean with the error bar representing the calculated maximum expression level of n=12 mice per group and the vehicle-treated group set as a reference. Relative expression levels were compared using the comparative threshold cycle method with ß-actin as internal control. *p<0.05, †p<0.01 and ‡p<0.001 as compared to vehicle-treated mice.

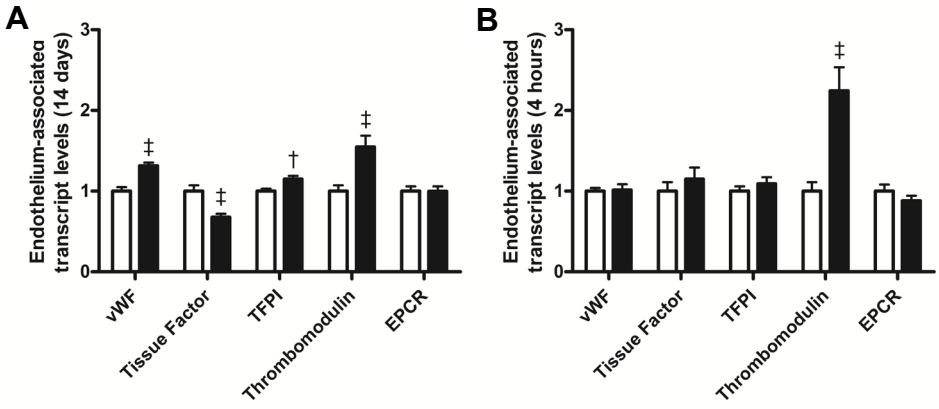

Figure 4: Transcript levels of endothelium-associated coagulation factors measured in mice treated with 0 µg T_3 (white bars) or 0.5 µg T_3 (black bars) for 14 days (A) or given a single dose (B). Data are presented as mean with the error bar representing the calculated maximum expression level of n=12 mice per group and the vehicle-treated group set as a reference. Relative expression levels were compared using the comparative threshold cycle method with ß-actin as internal control. *p<0.05, †p<0.01 and ‡p<0.001 as compared to vehicle-treated mice.

Discussion

Evidence is accumulating that overt hypothyroidism and hyperthyroidism are associated with changes in the hemostatic balance, which translates to either a bleeding tendency or an increased thrombosis risk.[1-3] However, the underlying mechanism how thyroid hormone can modulate coagulation is largely unknown. In the present study, we demonstrate that intraperitoneal administration of the thyroid hormone triiodothyronine (T_3) to mice with a suppressed endogenous thyroid hormone production is able to modulate transcription of both hepatic and endothelium-associated coagulation factors. Fibrinogen-γ, antithrombin, protein C, protein Z, protein Z-related protease inhibitor and thrombomodulin responded rapidly upon a single T_3 injection. On the other hand, factors II, IX, X, XI, XII, von Willebrand factor, tissue factor and tissue factor pathway inhibitor were only modulated after a prolonged T_3 exposure, i.e. 14 days. Although analyzed for a limited set of liver-derived coagulation factors, the changes in transcript levels were largely paralleled by changes in plasma levels. Based on these observations, we conclude that thyroid hormone can have widespread effects coagulation in mice.

Our data are in line with observations by Flores-Morales et al., who showed that T_3 can have both immediate and late effects on mouse hepatic gene transcription, and that these effects can be either up- or down-regulating.[5] The coagulation factors that responded within 4 hours after injection are likely to be directly regulated by thyroid hormone, via interaction with the thyroid hormone receptor and specific response elements in the promoter region of coagulation genes. Surprisingly, most of these genes are regulated in a negative fashion, with the exception of thrombomodulin. Although it is known that transcriptional suppression is a common action of thyroid hormones,[4-6] the mechanisms underlying this negative regulation are poorly understood and may involve binding of co-repressors like NCOR1 or post-transcriptional microRNA binding, while chromatin remodelling and DNA methylation may also play a role in transcriptional suppression.[13,14]

For the genes that require a prolonged T_3 exposure to evoke a clear transcriptional response, an indirect modulation is more likely which can involve intermediate transcription factors additional to the thyroid hormone receptor. Despite the fact that there are many intermediates possible, we hypothesized that the hepatic nuclear factor 4α (HNF4α) would be a good candidate since it is known that thyroid hormone can up-regulate HNF4 expression,[15] and the well-established HNF4α targets coagulation factor XI and XII[16] are clearly up-regulated upon prolonged T_3

exposure. However, the candidature of HNF4α was not supported by the data as we observed a 20% decrease in hepatic mRNA levels in livers of T_3 mice.

Our observations in lung samples as a substitute for the vasculature clearly indicate that also the (micro)vasculature with its endothelium-associated coagulation factors is responsive to T_3, as the levels of most factors analyzed were affected after prolonged T_3 administration. Interestingly, thrombomodulin appeared to be an immediate responder and could be induced by a T_3 dose as low as 0.05 µg/mouse/day, indicating that thrombomodulin transcription is highly sensitive to variation in T_3. Although we were not able to determine whether the changes in thrombomodulin transcript levels translated into altered protein levels, it has been reported that patients with hyperthyroidism have increased levels of circulating soluble thrombomodulin.[17]

In humans, hyperthyroidism is associated with an increased risk for thrombosis, while we showed that thyroid hormone administration in mice results in an increase in prothrombin time and a decrease in the endogenous thrombin potential, which point towards a bleeding tendency instead of a thrombotic tendency. These results can at least be partially explained by the observed decreases in plasma FII and FX levels. On the other hand, the APTT was shorter due to the increased FXI and FXII levels, suggesting a thrombosis-prone condition, which is more in line with what would be expected based on human observations. Altogether, these findings indicate that the use of mice in studying thyroid disorders in human-like coagulopathies, i.e. bleeding or thrombosis, faces limitations.

In conclusion, our mouse study demonstrates that T_3 administration to mice with suppressed endogenous thyroid hormone production has widespread effects on transcription of hepatic and endothelium-associated coagulation genes, as measured at the level of the lung. Furthermore, we identified both immediate and late responding coagulation genes, suggesting that T_3 can either directly or indirectly control transcription. In addition, the transcriptional changes resulted in altered plasma levels as analyzed for a panel of coagulation factors. We believe that this mouse study contributes to a better understanding of the relation between thyroid dysfunctions and coagulation disorders in human.

Acknowledgements

This study was supported by grant 2006B045 from the Netherlands Heart Foundation.

References

1 Van Zaane B, Squizzato A, Huijgen R, et al. Increasing levels of free thyroxine as a risk factor for a first venous thrombosis: a case-control study. Blood 2010;115:4344-9.

2 Erem C. Thyroid disorders and hypercoagulability. Semin Thromb Hemost 2011;37:17-26.

3 Franchini M, Montagnana M, Manzato F, et al. Thyroid dysfunction and hemostasis: an issue still unresolved. Semin Thromb Hemost 2009;35:288-94.

4 Feng X, Jiang Y, Meltzer P, et al. Thyroid hormone regulation of hepatic genes in vivo detected by complementary DNA microarray. Mol Endocrinol 2000;14:947-55.

5 Flores-Morales A, Gullberg H, Fernandez L, et al. Patterns of liver gene expression governed by TRbeta. Mol Endocrinol 2002;16:1257-68.

6 Yen PM, Feng X, Flamant F, et al. Effects of ligand and thyroid hormone receptor isoforms on hepatic gene expression profiles of thyroid hormone receptor knockout mice. EMBO Rep 2003;4:581-7.

7 Lin KH, Lee HY, Shih CH, et al. Plasma protein regulation by thyroid hormone. J Endocrinol 2003;179:367-77.

8 Niessen RW, Pfaffendorf BA, Sturk A, et al. The influence of insulin, beta-estradiol, dexamethasone and thyroid hormone on the secretion of coagulant and anticoagulant proteins by HepG2 cells. Thromb Haemost 1995;74:686-92.

9 Shih CH, Chen SL, Yen CC, et al. Thyroid hormone receptor-dependent transcriptional regulation of fibrinogen and coagulation proteins. Endocrinology 2004;145:2804-14.

10 Wiersinga WM, Chopra IJ. Radioimmunoassay of thyroxine (T4), 3,5,3'-triiodothyronine (T3), 3,3',5'-triiodothyronine (reverse T3, rT3), and 3,3'-diiodothyronine (T2). Methods Enzymol 1982;84:272-303.

11 Cleuren AC, Van der Linden IK, De Visser YP, et al. 17alpha-Ethinylestradiol rapidly alters transcript levels of murine coagulation genes via estrogen receptor alpha. J Thromb Haemost 2010;8:1838-46.

12 Amma LL, Campos-Barros A, Wang Z, et al. Distinct tissue-specific roles for thyroid hormone receptors beta and alpha1 in regulation of type 1 deiodinase expression. Mol Endocrinol 2001;15:467-75.

13 Takeuchi Y, Murata Y, Sadow P, et al. Steroid receptor coactivator-1 deficiency causes variable alterations in the modulation of T(3)-regulated transcription of genes in vivo. Endocrinology 2002;143:1346-52.

14 Yen PM. Physiological and molecular basis of thyroid hormone action. Physiol Rev 2001;81:1097-142.

15 Selva DM, Hammond GL. Thyroid hormones act indirectly to increase sex hormone-binding globulin production by liver via hepatocyte nuclear factor-4alpha. J Mol Endocrinol 2009;43:19-27.

16 Inoue Y, Peters LL, Yim SH, et al. Role of hepatocyte nuclear factor 4alpha in control of blood coagulation factor gene expression. J Mol Med 2006;84:334-44.

17 Burggraaf J, Lalezari S, Emeis JJ, et al. Endothelial function in patients with hyperthyroidism before and after treatment with propranolol and thiamazol. Thyroid 2001;11:153-60.

Table 1: QPCR primer sequences

Gene	Forward primer (5'- 3')	Reverse primer (5'- 3')
β-Actin	AGGTCATCACTATTGGCAACGA	CCAAGAAGGAAGGCTGGAAAA
Deiodinase type 1	TTGCCTCCACAGCCGATT	TCTTAAAAGCCCAGCCATCTG
Spot14	AAGGTGGCTGGCAACGAA	TCGGCCTCCGTTTC
Fibrinogen-γ	TGCTGCCTGCTTTTACTGTTCTC	TCTAGGATGCAACAGTTATCTCTGGTA
Factor II	GGACGCTGAGAAGGGTATCG	CCCCACACAGCAGCTCTTG
Factor V	CATGGAAACCTTACCGACAGAAA	CATGTGCCCCTTGGTATTGC
Factor VII	CGTCTGCTTCTGCCTAGA	ATTTGCACAGATCAGCTGCTCAT
Factor VIII	CTTCACCTCCAGGGAAGGACTA	TCCACTTGCAACCATTGTTTTG
Factor IX	GCAAAACCGGGTCAAATCC	ACCTCCACAGAATGCCTCAATT
Factor X	GTGGCCGGGAATGCAA	AACCCTTCATTGTCTTCGTTAATGA
Factor XI	GAAGGATACGTGCAAGGGAGATT	CAAGTGCCAGACCCCATTGT
Factor XII	GGGCTTCTCCTCCATCACCTA	GCAACTGTTGGTTTTGCTTTCC

Table 1: QPCR primer sequences (continued)

Gene	Forward primer (5'- 3')	Reverse primer (5'- 3')
Antithrombin	TGGGCCTCATTGATCTCTTCA	CCTGCCTCCAGCAACGAT
Heparin Cofactor II	GAATGGCAATATGTCAGGCATCT	CACTGTGATGGTACTTTGGTGCTT
Protein C	GCGTGGAGGGCACCAA	CCCTGCGTCGCAGATCAT
Protein S	GGTGGCATCCCAGATATTTCC	CACTTCCATGCAGCCACTGT
Protein Z	GCAGCCAGAGTCAGCCTAGCT	CACGCCGGCACAGAAGTC
PZI	TGGCCCTGGAGGACTACTTG	CCATTTTCCTGGTTTTCATATTCTG
Plasminogen	TGACATTGCCCTGCTGAAAC	CAGACAAGCTGGAATGACTTTATCC
α_2-Antiplasmin	TTCTCCTCAACGCCATCCA	GGTGAGGCTCGGGTCAAAC
Von Willebrand factor	AGCTGTCAGCCAGGTTTTTCTT	GCAGAGGGCAGGCACCTT
Tissue Factor	GGAGGAGCCGCCATTTACA	CTGGCTGTCCAAGGTTTGTGT
TFPI	CCCAGGCGTCGGGATTA	AATCCACTGTCTGCTGGTTGAA
Thrombomodulin	GCGAAATGTTCTGCAATGAAAC	GGCATTCACAAACAGTAGGAGAGTT
EPCR	AACCACATCACCACGCAAAA	CCCAGGACCAGTGATGTGTAAG

PZI: protein Z-related protease inhibitor; TFPI: tissue factor pathway inhibitor; EPCR: endothelial protein C receptor

Table 2: General and plasma coagulation parameters upon increasing doses of T_3

T_3 (µg/mouse/day)	0	0.05	0.1	0.5	1	5	10	p-value
General parameters								
Δ Body weight (g)[a]	0.18±0.26	0.11±0.15	0.50±0.23	1.47±0.20†	1.92±0.15‡	2.26±0.36‡	1.77±0.33‡	<0.001
Liver weight (g)	1.51±0.07	1.30±0.06*	1.28±0.09*	1.24±0.04*	1.21±0.03†	1.09±0.01‡	1.10±0.02‡	<0.001
T_3 (nmol/L)	0.31±0.01	1.46±0.10	2.09±0.12	4.99±0.50	8.78±1.84	26.21±3.18‡	51.20±5.84‡	<0.001
T_4 (nmol/L)	12.0±1.5	9.3±0.8	8.8±0.6	9.6±1.7	11.8±0.7	14.0±1.0	10.0±0.7	0.016
ALT (U/L)	37.5±9.6	30.8±6.3	24.2±3.3	20.1±0.1	30.0±7.1	55.0±7.6	70.0±14.9	0.003
AST (U/L)	98.7±21.9	113.3±7.2	71.7±8.0	71.0±3.4	93.3±13.1	134.2±18.4	156.0±24.0	0.007
ALP (U/L)	61.3±1.0	72.5±3.4	81.7±6.0	140.0±15.1†	166.7±12.3‡	282.5±23.1‡	313.0±11.3‡	<0.001
Plasma coagulation parameters								
Fibrinogen (%)	100±5.2	97.6±3.2	91.7±15.8	59.3±7.3†	50.2±4.5‡	48.7±2.2‡	40.0±0.8‡	<0.001
Factor II (%)	100±1.6	73.3±2.8‡	74.1±2.0‡	59.4±3.5‡	50.5±3.1‡	48.1±2.8‡	50.4±2.3‡	<0.001
Factor VIII (%)	100±7.8	113.9±7.4	125.1±10.4	104.7±6.3	89.5±5.9	86.2±7.9	86.6±7.5	0.008
Factor IX (%)	100±2.9	91.6±3.8	99.2±3.2	92.2±3.5	95.5±3.7	87.7±3.8	91.8±4.2	NS
Factor X (%)	100±2.1	83.5±1.8†	88.1±2.7	79.0±3.2‡	71.3±7.2‡	65.8±2.0‡	68.4±3.4‡	<0.001
Factor XII (%)	100±3.1	117.4±3.2*	122.6±3.6‡	126.9±3.1‡	125.3±6.1‡	118.8±4.4†	128.6±2.9‡	<0.001
Antithrombin (%)	100±2.2	77.4±3.1‡	83.0±3.3‡	65.6±3.4‡	73.3±1.9‡	70.2±1.7‡	66.6±2.9‡	<0.001

[a] Difference in body weight between end and start of T3 treatment. ALT: alanine aminotransferase; AST: aspartate aminotransferase; ALP: alkaline phosphatase.
Data are presented as mean±standard error of the mean with the vehicle-treated group set as a reference in coagulation factor analyses. Overall p-values are calculated with an analysis of variance (ANOVA); *p<0.05, †p<0.01, ‡p<0.001 compared to vehicle-treated mice as calculated with a Dunnett post-hoc test.

Table 3: Transcript levels of coagulation factors upon increasing doses of T_3

T_3 (µg/mouse/day)	0	0.05	0.1	0.5	1	p-value
Hepatic transcript levels						
Deiodinase 1	1 (0.83-1.20)	24.2 (19.3-30.3)‡	76.1 (58.4-88.9)‡	181 (148-222)‡	191 (157-232)‡	<0.001
Fibrinogen-γ	1 (0.96-1.04)	1.03 (0.88-1.20)	1.07 (0.97-1.19)	0.56 (0.49-0.63)†	0.47 (0.42-0.53)‡	<0.001
Factor II	1 (0.93-1.07)	0.70 (0.60-0.82)	0.88 (0.84-0.92)	0.62 (0.56-0.68)†	0.64 (0.57-0.71)*	0.009
Factor XII	1 (0.90-1.11)	1.14 (1.04-1.25)	1.20 (1.06-1.35)	1.23 (1.14-1.32)	1.01 (0.89-1.15)	NS
Antithrombin	1 (0.97-1.04)	0.86 (0.79-0.94)	0.97 (0.95-1.00)	0.79 (0.76-0.82)	0.84 (0.75-0.95)	NS
Protein C	1 (0.95-1.05)	0.82 (0.76-0.88)	0.82 (0.78-0.86)	0.57 (0.53-0.62)‡	0.56 (0.51-0.61)‡	<0.001
Protein S	1 (0.94-1.06)	0.91 (0.85-0.98)	1.05 (0.99-1.11)	0.84 (0.81-0.87)	0.82 (0.74-0.91)	NS
Endothelium-associated transcript levels						
VWF	1 (0.95-1.05)	1.01 (0.97-1.05)	1.00 (0.94-1.05)	0.97 (0.92-1.03)	0.98 (0.93-1.03)	NS
Tissue factor	1 (0.94-1.07)	0.79 (0.75-0.83)	0.72 (0.67-0.77)*	0.66 (0.59-0.73)†	0.68 (0.61-0.76)*	0.015
Thrombomodulin	1 (0.93-1.07)	1.64 (1.54-1.73)‡	1.54 (1.43-1.66)†	1.61 (1.45-1.78)‡	1.54 (1.43-1.66)†	<0.001
EPCR	1 (0.94-1.07)	0.86 (0.76-0.97)	0.75 (0.69-0.83)	0.81 (0.71-0.91)	0.87 (0.80-0.96)	NS

VWF: Von Willebrand factor; EPCR: endothelial protein C receptor.

Data are presented as mean (range) with the vehicle-treated group set as a reference in coagulation factor analyses. Overall p-values are calculated with an analysis of variance (ANOVA); *p<0.05, †p<0.01, ‡p<0.001 compared to vehicle-treated mice as calculated with a Dunnett post-hoc test.

Chapter 9

Early death of factor V Leiden hemizygous mice: a fatal absence of wild-type factor V anticoagulant function

Audrey CA Cleuren, Hongmin Sun, Guojing Zhu,
Bart JM van Vlijmen, Pieter H Reitsma, David Ginsburg,
Randal J Westrick

Manuscript in preparation

Summary

Background: Factor V (FV) has a dual role in coagulation by expressing both a procoagulant and an anticoagulant function. In factor V Leiden (FVL) hemizygosity, i.e. the co-inheritance of FVL and a quantitative FV deficiency, defects of both FV functions are combined. Surprisingly, this combination confers a thrombotic risk, suggesting a complex role for FV in (anti)coagulation.

Methods and results: To gain further insight in FV function, FVL ($FV^{Q/Q}$) and $FV^{+/-}$ mice were crossed to generate FVL hemizygous mice. An underrepresentation of $FV^{Q/-}$ mice as compared to $FV^{Q/+}$ mice was observed at the time of weaning. Timed matings showed that $FV^{Q/-}$ animals progressed normally to term, but half of the $FV^{Q/-}$ mice died in the immediate postnatal period coinciding with features of thrombosis. On a tissue factor pathway inhibitor heterozygosity background, the $FV^{Q/-}$ genotype was fully fatal confirming that FVL hemizygosity is associated with a thrombotic and not a bleeding phenotype. Analyses of the plasma coagulation profiles of $FV^{Q/+}$, $FV^{Q/Q}$ and viable $FV^{Q/-}$ mice showed a reduced overall procoagulability in $FV^{Q/-}$ as compared to $FV^{Q/+}$ and $FV^{Q/Q}$ mice with decreased thrombin generation and longer prothrombin times. Activated protein C resistance and FV-mediated $FVIII_a$ inactivation was decreased in $FV^{Q/-}$ mice as compared to $FV^{Q/+}$ animals, but comparable to that of $FV^{Q/Q}$ mice.

Conclusion: This mouse study demonstrates the physiological importance of the anticoagulant function of normal FV, of which a critical fraction is retained in factor V Leiden.

Introduction

Coagulation factor V (FV) is a central regulator of the coagulation cascade. It serves as a non-enzymatic cofactor for activated FX (FX_a) in the prothrombinase complex, enabling rapid thrombin formation. Thrombin bound to endothelial thrombomodulin can activate protein C, which subsequently inactivates FV_a and $FVIII_a$, thereby providing a natural feedback loop that down-regulates thrombin formation. To inactivate FV_a, activated protein C (APC) together with its cofactor protein S, cleaves FV_a at arginine 506, 306 and 679.[1,2] Although the Arg^{506} is the kinetically favored cleavage site, approximately 40% activity of FV_a remains and an additional cleavage at Arg^{306} is necessary for complete inactivation.[1]

Factor VIII is highly homologous to FV, not only in domain structure, but also in function as it acts as the non-enzymatic cofactor for FIX_a in the tenase complex. Activated FVIII is also inactivated by APC, which cleaves $FVIII_a$ at arginine 336 and 562. Although the cleavage at Arg^{336} is the fastest, this results in only partial inactivation, whereas the slower Arg^{562} cleavage completely inactivates $FVIII_a$.[2] To perform this function, APC needs protein S and factor V as cofactors,[3-5] indicating an additional anticoagulant function for FV.

The factor V Leiden mutation (FVL) leads to an arginine to glutamine substitution at position 506 of factor V and therefore the FVL protein confers a partial resistance to the inactivation by activated protein C. Additional to this reduced susceptibility to inactivation, it also possesses little cofactor activity in the inactivation of activated FVIII, as FV first has to be cleaved at Arg^{506} to express this cofactor function.[6,7] These two functional defects result in an increased risk for venous thrombosis, which is 7-fold increased in FVL heterozygotes and 80-fold increased in homozygotes.[8]

Factor V Leiden hemizygosity, also known as pseudo-homozygosity for APC resistance, is the co-inheritance of the FVL mutation and a quantitative FV deficiency on different alleles,[9] and thus combines the factor V Leiden-related increased venous thrombotic risk and the deficiency-related bleeding tendency. With the prevalence of parahemophilia (FV deficiency with levels <1%) being $1:10^6$ and the prevalence of factor V Leiden being 1:20 in the Caucasian population, it is estimated that 1:1000 FVL carriers is actually hemizygous and with approximately 26 million FVL carriers in Europe alone, this would make up for a substantial number of hemizygotes.[10] However, most knowledge on this condition comes from case reports and small patient studies. Thus far, it has been shown that FVL hemizygosity results in an increased venous thrombotic risk comparable to, or even

higher than, the risk in FVL homozygotes.[11-13] This unexpected finding suggests a more complex role and contribution for FV in coagulation. To gain further insight into the complex role of factor V in (anti)coagulation, we used an *in vivo* approach in which we crossed mice carrying the factor V Leiden knock-in allele with factor V heterozygous mice to generate FVL hemizygous animals.

Materials and Methods

Mice

Generation of mice carrying the R504Q knock-in mutation, which is orthologous to the human FVL mutation, was previously described as was the generation of $FV^{+/-}$ mice.[14,15] Tissue factor pathway inhibitor (TFPI) deficient mice were a kind gift of Dr. George Broze.[16] All mice used in this study were backcrossed to C57BL/6J mice for at least 20 generations before intercrossing. Genotyping for FV, FVL and TFPI was performed by PCR analysis of tail DNA using primers as previously reported.[14-16] Timed matings were performed to analyze in utero progeny from gestational day 18.5.[17] Mice were housed and cared for at the University of Michigan, under compliance with the University of Michigan Committee on the Use and Care of Animals.

Histology

Newborn pups were zinc formalin fixed and sagittally sectioned. Whole-mount paraffin sections were routinely stained with hematoxylin and eosin. Fibrin(ogen) staining was performed as previously described.[16]

Plasma analyses

Mice were anesthetized and blood samples on sodium citrate (final concentration 0.32%) were drawn directly from the inferior caval vein. Blood was centrifuged twice to obtain platelet free plasma which was immediately frozen at -80°C until analysis.

Plasma activity levels of factors II, VIII, IX, X, XI and XII were determined as described elsewhere.[18] The activity of FV was assessed in a 2-step procedure, in which FV is activated with thrombin and subsequently the FV_a activity is quantified by determining the rate of FX_a-catalyzed prothrombin action. Plasma FVII, antithrombin and TFPI activity levels were analyzed by means of commercially available kits from respectively Hyphen Biomed, Chromogenix and American Diagnostica. Fibrinogen antigen levels were measured with a commercial murine

ELISA kit (Affinity Biologicals), and protein C levels were determined with an in-house ELISA using antibodies from Haematologic Inc. Pooled mouse plasma from wild-type mice was used to generate standard curves from which the plasma activity and antigen levels of individual mouse samples could be calculated.
To evaluate overall coagulability of mouse plasmas, the activated partial thromboplastin time (aPTT-SP reagent, Instrumentation Laboratories) and the prothrombin time (STA neoplastin plus reagent, Roche) were measured on the STart 4 analyzer (Diagnostica Stago). Thrombin generation was assessed by means of the Calibrated Automated Thrombogram, using 1 pM tissue factor (PPP-reagent Low, Thrombinoscope) to trigger 1:6 diluted mouse plasma. Thrombin generation was measured on the Fluoroskan Ascent reader (Thermo Scientific) and the curves and area under the curve (endogenous thrombin potential; ETP) were calculated using the Thrombinoscope software. APC resistance was determined by quantifying the effect of added APC on the ETP.[19] The cofactor activity of FV on $FVIII_a$ inactivation was evaluated with the Immunochrom APC Response assay (Progen Biotechnik), according to the protocol described by Govers-Riemslag et al.[20] with the last incubation step being reduced to 2 minutes.

Statistical analyses
Plasma data is presented as mean ± standard error of the mean (SEM) and were analyzed with the Graphpad Instat software package. Survival data was evaluated using a Chi-square test and statistical differences between genotypes were evaluated using a one-way analysis of variance (ANOVA) with a Bonferroni post-hoc test to compare FVL hetero- and homozygous mice with FVL hemizygous mice. A p-value <0.05 was considered to be statistically significant.

Results

Effect of FVL hemizygosity on survival
To generate FVL hemizygous ($FV^{Q/-}$) mice, $FV^{+/-}$ mice were crossed with $FV^{Q/Q}$ mice. Although equal numbers of $FV^{Q/+}$ and $FV^{Q/-}$ progeny were expected, only 23 of 91 mice carried the $FV^{Q/-}$ genotype at the time of weaning ($p<0.001$; table 1). To address the effect of FVL dosage on viability, $FV^{Q/Q}$ and $FV^{Q/-}$ mice were mated and again less $FV^{Q/-}$ mice were present at the time of weaning (53 $FV^{Q/-}$ vs. 131 $FV^{Q/Q}$, $p<0.001$), indicating that decreasing the dose of FVL in the absence of wild-type FV has adverse effects on viability. $FV^{Q/+}$ and $FV^{Q/-}$ mice were crossed to provide a direct comparison of $FV^{Q/+}$, $FV^{Q/Q}$ and $FV^{Q/-}$ in an identical genetic

context, which resulted in a significant reduction of $FV^{Q/-}$ progeny as compared to both $FV^{Q/+}$ and $FV^{Q/Q}$ ($p<0.001$ and $p<0.01$ respectively; table 1).
Timed matings were performed to analyze progeny from gestational day 18.5, just prior to birth. Genotypes for 171 mice from a $FV^{Q/+}$ x $FV^{+/-}$ cross showed that the observed distribution did not differ from the expected distribution (table 1), indicating that the $FV^{Q/-}$ mice progress normally to term but succumb between birth and weaning. A second series of timed matings between $FV^{+/-}$ and $FV^{Q/Q}$ mice were performed, resulting in 52 mice of which 29 carried the $FV^{Q/+}$ and 23 the $FV^{Q/-}$ genotype. However, 20 mice died within 24 hours after birth, of which 15 were $FV^{Q/-}$ ($p=0.02$). These data show that the distribution of genotypes at birth is as expected, but that a substantial fraction of the FVL hemizygotes dies in the immediate postnatal period.

Table 1: Genotype distribution of weaning pups and embryos obtained from experiments designed to assess the lethality of the $FV^{Q/-}$ genotype compared to $FV^{Q/+}$ and $FV^{Q/Q}$.

Number of offspring/genotype		$FV^{+/+}$	$FV^{+/-}$	$FV^{Q/+}$	$FV^{Q/Q}$	$FV^{Q/-}$	Total number of pups analyzed
Parental genotypes							
$FV^{+/-}$	$FV^{Q/Q}$	-	-	68	-	23**	91
$FV^{Q/Q}$	$FV^{Q/-}$	-	-	-	131	53**	184
$FV^{Q/-}$	$FV^{Q/+}$	-	18	23	14	5*	60
Embryonic mating							
$FV^{Q/+}$	$FV^{+/-}$	36	49	50	-	36	171

* $p<0.01$, ** $p<0.001$ (Chi square analysis)

To establish the cause of death, newborn pups that died shortly after birth were collected, of which some showed macroscopic features of coagulopathy (figure 1A). Additional histological analyses of $FV^{Q/-}$ pups revealed signs of macrovascular thrombosis and in 2 out of 6 $FV^{Q/-}$ pups large thrombi within vessels were found which demonstrated the typical layering of fibrin as a hallmark of thrombosis, discriminating these from postmortem blood clots (figure 1). Furthermore, livers of 2 $FV^{Q/-}$ animals featured infarcted areas with necrosis and the presence of multicellular infiltrates. Analysis of serial sections did not show thrombi located outside blood vessels or the presence of bleeding. No histological abnormalities were found in the $FV^{Q/+}$ pups (n=5).

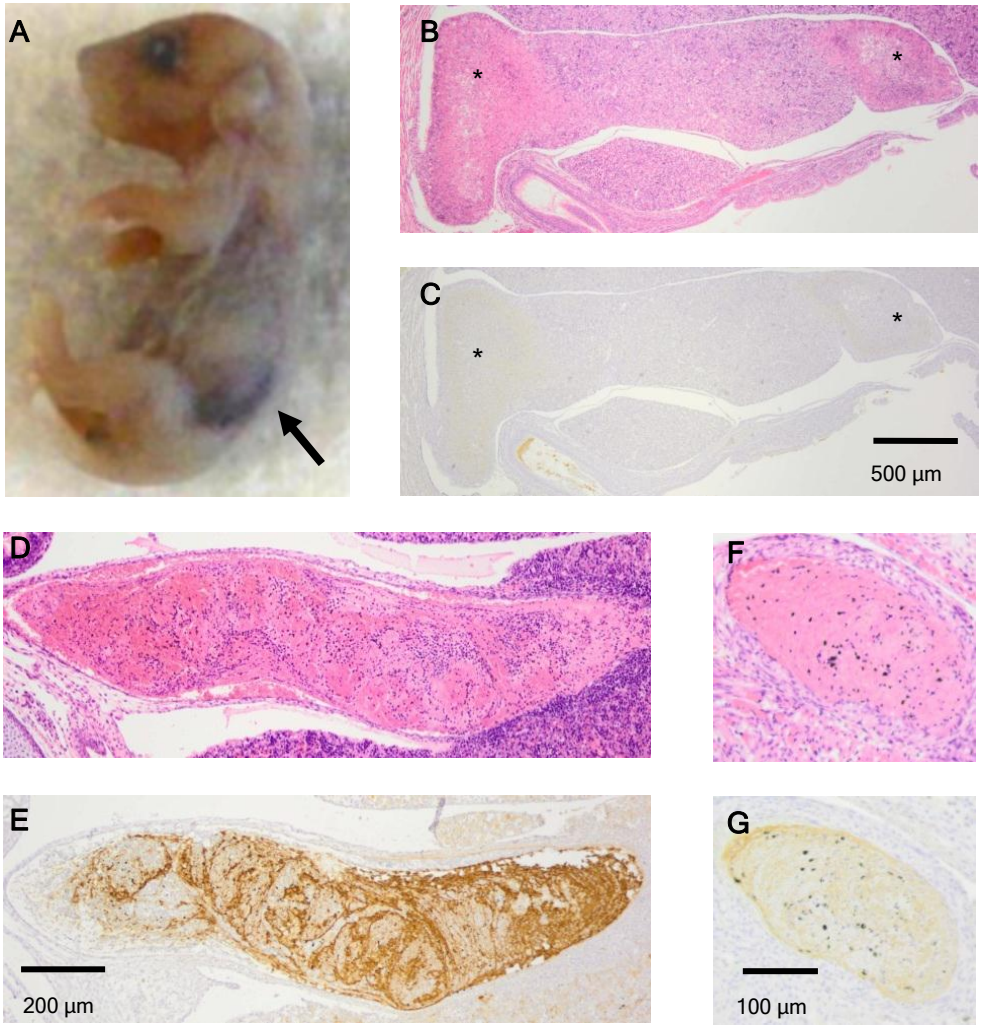

Figure 1: Macroscopic and microscopic examination of dead factor V Leiden hemizygous mice.
Macroscopic features of coagulopathy were observed in $FV^{Q/-}$ mice that succumbed within 24 hours after birth (A). Microscopic analyses of these $FV^{Q/-}$ animals (hematoxylin/eosin and fibrin(ogen) staining) showed infarcted areas in the liver (asterisks in panels B and C) and revealed macrovascular thrombi (panels D-G).

In order to confirm that the $FV^{Q/-}$ genotype predisposes to a thrombotic and not bleeding tendency, we combined these mice with mice lacking one allele of TFPI. Whereas either FVL homozygosity or TFPI heterozygosity does not result in early lethality, we previously demonstrated that a $TFPI^{+/-}$ background transforms homozygosity for factor V Leiden into nearly complete lethality in the immediate perinatal period due to widespread thrombosis.[21] Of the 203 progeny analyzed from a $FV^{Q/-}$,$TFPI^{+/+}$ x $FV^{Q/+}$,$TFPI^{+/-}$ cross, 27 mice were $FV^{Q/Q}$ $TFPI^{+/+}$ which corresponds with the expected 12.5% distribution. However, only 16 $FV^{Q/-}$ $TFPI^{+/+}$ mice were present at the time of weaning, confirming the decreased viability of the hemizygous mice on a wild-type background. On a $TFPI^{+/-}$ background, only 3 $FV^{Q/Q}$ mice and 1 $FV^{Q/-}$ mouse out of 203 were present. Thus, on a prothrombotic $TFPI^{+/-}$ background, the $FV^{Q/-}$ genotype was nearly fully fatal supporting the presence of a thrombotic and not a bleeding phenotype.

Plasma coagulation

The mating studies suggested that the reduced anticoagulant rather than the reduced procoagulant function of FV(L) contributed to the early lethality of the FVL hemizygous mice as compared to the FVL heterozygous mice. Therefore we studied the effect of hemizygosity on coagulation by comparing plasmas from viable $FV^{Q/-}$ mice and $FV^{Q/+}$ littermates. In addition, plasmas from $FV^{Q/Q}$ mice from a parallel cross were included in the comparison.

As expected, FV activity levels were decreased in $FV^{Q/-}$ mice as compared to both $FV^{Q/+}$ and $FV^{Q/Q}$ animals (table 2). In addition, FVIII levels were also lower whereas FII and FVII levels were higher in $FV^{Q/-}$ mice than in $FV^{Q/+}$ and $FV^{Q/Q}$ mice. To assess the impact of the differences in individual coagulation factors on the overall coagulability, thrombin generation assays were performed showing a significantly higher endogenous thrombin potential (ETP) in $FV^{Q/+}$ mice (272±5 nM*min) as compared to $FV^{Q/-}$ mice (213±13 nM*min, $p<0.01$), with an intermediate ETP value for $FV^{Q/Q}$ mice (232±10 nM*min, p=n.s.). Furthermore, the prothrombin time was longer in $FV^{Q/-}$ (11.9±0.1 sec) than in $FV^{Q/+}$ and $FV^{Q/Q}$ animals (both 11.3±0.1 sec, $p<0.05$). The activated partial thromboplastin time was also increased in $FV^{Q/-}$ mice, although this did not reach statistical significance ($FV^{Q/+}$ 29.3±0.6 sec, $FV^{Q/Q}$ 31.0±0.9 sec and $FV^{Q/-}$ 31.3±0.9 sec).

Table 2: Plasma levels of procoagulant factors from factor V Leiden heterozygous ($FV^{Q/+}$), homozygous ($FV^{Q/Q}$) and viable hemizygous mice ($FV^{Q/-}$).

	$FV^{Q/+}$	$FV^{Q/Q}$	$FV^{Q/-}$	p-value
Fibrinogen	121.8±5.0	112.5±6.0	106.7±8.5	n.s.
Factor II	126.2±3.9	121.4±2.5**	134.6±2.9	0.012
Factor V	101.8±4.7**	97.2±6.1**	56.5±3.8	<0.001
Factor VII	103.4±2.7*	103.6±3.0	115.9±5.1	0.034
Factor VIII	110.0±3.4*	101.9±6.8	88.5±6.3	0.020
Factor IX	96.3±1.8	98.7±3.7	100.6±3.9	n.s.
Factor X	99.6±3.1	101.6±2.9	109.9±4.1	n.s.
Factor XI	104.9±5.3	107.4±9.7	106.1±7.7	n.s.
Factor XII	105.3±2.0	98.8±2.3*	108.8±1.8	0.014

Data are expressed as mean percentage of pooled mouse plasma ± standard error of the mean of n=15 ($FV^{Q/+}$ and $FV^{Q/Q}$) or n=10 ($FV^{Q/-}$) mice. *p<0.05 and **p<0.01 as compared to $FV^{Q/-}$ mice by performing an analysis of variance (ANOVA) with an additional Bonferroni post-hoc test.

To evaluate the effects of FVL hemizygosity on anticoagulation, plasma levels of antithrombin, protein C and TFPI were measured. However, no differences between genotypes were present (data not shown). In addition, an ETP-based APC sensitivity ratio was determined. The APC resistance of $FV^{Q/-}$ mice was comparable to $FV^{Q/Q}$ (2.85±0.12 vs. 3.12± 0.31) but significantly higher than $FV^{Q/+}$ mice (1.78±0.11, p<0.01; figure 2). As the ETP-based APC sensitivity ratio does not distinguish between the 2 functional defects of factor V Leiden, i.e. the decreased susceptibility to be inactivated by APC and the reduced cofactor activity in $FVIII_a$ inactivation, we performed an APTT-based APC sensitivity test in which pooled mouse plasma from either wild-type or FVL homozygous mice were titrated in FV deficient plasma.(22) Figure 2b shows that in wild-type plasma, factor V has an obvious cofactor function, whereas this is less present in FVL plasma. Therefore, the specific FV-mediated $FVIII_a$ inactivation was measured, showing a significant decreased capability of $FVIII_a$ inactivation in $FV^{Q/-}$ mice (1.25±0.03) than in $FV^{Q/+}$ mice (1.59±0.07, p<0.01), with $FV^{Q/Q}$ having an intermediate phenotype (1.40±0.03, fig. 2c).

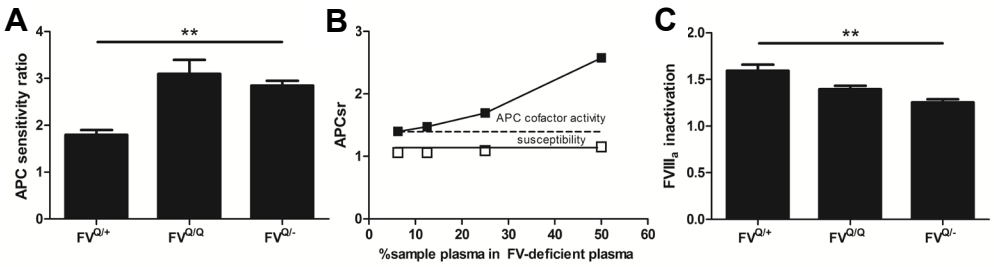

Figure 2: Overview of anticoagulant properties of factor V Leiden heterozygous ($FV^{Q/+}$), homozygous ($FV^{Q/Q}$) and viable hemizygous mice ($FV^{Q/-}$).

The activated protein C (APC) sensitivity ratio for individual mouse plasmas was based on the endogenous thrombin potential (panel A). The APC sensitivity ratio was also assessed in an APTT-based assay, in which pooled wild-type mouse plasma (black) or factor V Leiden mouse plasma (open) was titrated with human factor V deficient plasma, showing that murine FV acts as a cofactor in $FVIII_a$ inactivation (B). FV-mediated $FVIII_a$ inactivation per genotype is shown in panel C. Data are expressed as mean ± standard error of the mean of n=15 ($FV^{Q/+}$ and $FV^{Q/Q}$) or n=10 ($FV^{Q/-}$) mice. **p<0.01 as compared to $FV^{Q/-}$ mice by performing an analysis of variance (ANOVA) with an additional Bonferroni post-hoc test.

Discussion

In the present study we combined $FV^{Q/Q}$ and $FV^{+/-}$ mice to generate unique factor V Leiden hemizygous mice that allowed us to gain further insight into the complex role of factor V and factor V Leiden in (anti)coagulation. The data presented here demonstrate that the $FV^{Q/-}$ genotype induces an unexpected partial lethality in the immediate postnatal period that is related to macrovascular thrombosis; findings that were also confirmed on a $TFPI^{+/-}$ background. Compared to $FV^{Q/+}$ mice, viable $FV^{Q/-}$ mice had an expected overall reduced procoagulant plasma profile, with less thrombin generation and longer prothrombin times. Anticoagulability was also decreased with a more pronounced APC resistance and a diminished capability to inactivate activated FVIII in a FV-dependent manner. As $FV^{Q/-}$ mice die with features of thrombosis and not bleeding, we conclude that the impaired FV anticoagulant function in FVL hemizygosity underlies the fatal phenotype. In addition, comparison of plasma coagulation profiles of $FV^{Q/-}$ and $FV^{Q/Q}$ animals showed only subtle differences. However, as $FV^{Q/Q}$ mice do not display early lethality, this implies that factor V Leiden has maintained a vital and critical fraction of its anticoagulant activity. Overall, this mouse study demonstrates the physiological importance of the anticoagulant function of the factor V allele.

Our data regarding the reduced anticoagulability in surviving FVL hemizygous mice reproduce two important observations for their human counterpart, i.e. decreased FV-mediated $FVIII_a$ inactivation and pseudo-homozygosity for APC resistance.[11] However, the recently reported lower TFPI levels associated with FV deficiency and FVL hemizygosity that is thought to significantly contribute to increased coagulability[12] could not be reproduced in mice as we did not detect a lower plasma TFPI levels upon lack of one FV allele (9.4±0.3 U/mL vs. 10.0±0.2 U/mL for $FV^{Q/+}$ and $FV^{Q/-}$ mice, respectively). Both in mice and human, TFPI has 2 isoforms: TFPIα and TFPIß, which are different in protein structure. The predominant isoform in mouse plasma is TFPIß, whereas in human TFPIα is the main plasma isoform,[23] which may explain the absence of an association between FV and TFPI levels in mice. Besides the absence of a FV-TFPI association, we found an evident postnatal lethality associated with the FVL hemizygous state in mice. For humans, it is estimated that 1:1000 FVL carriers is actually hemizygous, and with approximately 26 million FVL carriers in Europe alone, this would make up for a considerable number of hemizygotes.[10] Whether reduced postnatal survival in humans contributes to the relatively limited reports on factor V Leiden hemizygotes is subject to speculation.

Viable $FV^{Q/-}$ animals had lower fibrinogen and FVIII plasma levels as compared to $FV^{Q/+}$ mice. Although we have no good explanation for these findings, they were confirmed in offspring from multiple matings with different parental genotypes. Low levels of FVIII and fibrinogen provide a reduced procoagulant state and may have enabled survival of those expressing the low levels through selection. On the other hand, the lower fibrinogen and FVIII levels may also be the result of consumptive coagulopathy and thus a secondary effect.[24] In this respect, it is interesting that $FV^{Q/Q}$ mice, which have increased tissue fibrin deposition as a marker of microvascular thrombosis, show intermediate levels of both fibrinogen and factor VIII. Whether $FV^{Q/-}$ mice have more microvascular thrombosis and therefore lower plasma levels of both factors as compared to $FV^{Q/Q}$ mice has not been determined. Since FV and FVIII are structurally related and share steps in posttranslational processing,[25] we considered that the lower FVIII levels result from the decreased FV levels as a significant correlation between these factors was found (Pearson $r=0.52$, $p<0.001$). However, in $FV^{+/-}$ mice, we did not observe lower FVIII levels, thus making a direct relation between FV and FVIII in these mice less likely.

Although the survival of $FV^{Q/Q}$ and viable $FV^{Q/-}$ mice differs markedly, we were unable to detect major differences in plasma coagulation profiles. This implies a

residual anticoagulant function for the factor V Leiden allele that reaches a functionally adequate level in the homozygous but not hemizygous state. Indeed, the level of $FVIII_a$ inactivation appeared to be somewhat higher in $FV^{Q/Q}$ mice as compared to $FV^{Q/-}$, which has also been observed in humans.[11] However, FV might also have an additional function besides it pro- and anticoagulant role in coagulation. For example, a role for FV in fibrinolysis has been revealed,[26-28] and a more impaired fibrinolysis in FVL hemizygosity as compared to FVL homozygotes may contribute to the observed thrombotic phenotype.

In conclusion, our data show partial lethality of factor V Leiden hemizygous mice in the immediate postnatal period due to macrovascular thrombosis. These observations indicate that the lack of the anticoagulant function rather than the lack of the procoagulant function of factor V is of vital importance. In addition, our data imply a residual anticoagulant function of the factor V Leiden allele which can rescue the lethal phenotype associated with factor V Leiden hemizygosity.

Acknowledgements

The authors would like to thank the Histology Core Facility of the University of Michigan for preparing the histological slides and Marion Gijbels from the University of Maastricht for her help with the histological analyses. This study was financially supported by the Netherlands Heart Foundation (grant 2006B045).

References

1 Nicolaes GA, Tans G, Thomassen MC, et al. Peptide bond cleavages and loss of functional activity during inactivation of factor Va and factor VaR506Q by activated protein C. J Biol Chem 1995;270:21158-66.

2 Cramer TJ, Gale AJ. Function of the activated protein C (APC) autolysis loop in activated FVIII inactivation. Br J Haematol 2011;153:644-54.

3 Gale AJ, Cramer TJ, Rozenshteyn D, et al. Detailed mechanisms of the inactivation of factor VIIIa by activated protein C in the presence of its cofactors, protein S and factor V. J Biol Chem 2008;283:16355-62.

4 Lu D, Kalafatis M, Mann KG, et al. Comparison of activated protein C/protein S-mediated inactivation of human factor VIII and factor V. Blood 1996;87:4708-17.

5 Shen L, Dahlback B. Factor V and protein S as synergistic cofactors to activated protein C in degradation of factor VIIIa. J Biol Chem 1994;269:18735-8.

6 Thorelli E, Kaufman RJ, Dahlback B. Cleavage of factor V at Arg 506 by activated protein C and the expression of anticoagulant activity of factor V. Blood 1999;93:2552-8.

7 Varadi K, Rosing J, Tans G, et al. Factor V enhances the cofactor function of protein S in the APC-mediated inactivation of factor VIII: influence of the factor VR506Q mutation. Thromb Haemost 1996;76:208-14.

8 Rosendaal FR, Koster T, Vandenbroucke JP, et al. High risk of thrombosis in patients homozygous for factor V Leiden (activated protein C resistance). Blood 1995;85:1504-8.
9 Simioni P, Scudeller A, Radossi P, et al. "Pseudo homozygous" activated protein C resistance due to double heterozygous factor V defects (factor V Leiden mutation and type I quantitative factor V defect) associated with thrombosis: report of two cases belonging to two unrelated kindreds. Thromb Haemost 1996;75:422-6.
10 Castoldi E, Rosing J. APC resistance: biological basis and acquired influences. J Thromb Haemost 2010;8:445-53.
11 Brugge JM, Simioni P, Bernardi F, et al. Expression of the normal factor V allele modulates the APC resistance phenotype in heterozygous carriers of the factor V Leiden mutation. J Thromb Haemost 2005;3:2695-702.
12 Duckers C, Simioni P, Tormene D, et al. Factor V Leiden pseudo-homozygotes have a more pronounced hypercoagulable state than factor V Leiden homozygotes. J Thromb Haemost 2011;9:864-7.
13 Simioni P, Castoldi E, Lunghi B, et al. An underestimated combination of opposites resulting in enhanced thrombotic tendency. Blood 2005;106:2363-5.
14 Cui J, O'Shea KS, Purkayastha A, et al. Fatal haemorrhage and incomplete block to embryogenesis in mice lacking coagulation factor V. Nature 1996;384:66-8.
15 Cui J, Eitzman DT, Westrick RJ, et al. Spontaneous thrombosis in mice carrying the factor V Leiden mutation. Blood 2000;96:4222-6.
16 Huang ZF, Higuchi D, Lasky N, et al. Tissue factor pathway inhibitor gene disruption produces intrauterine lethality in mice. Blood 1997;90:944-51.
17 Westrick RJ, Bodary PF, Xu Z, et al. Deficiency of tissue factor pathway inhibitor promotes atherosclerosis and thrombosis in mice. Circulation 2001;103:3044-6.
18 Cleuren AC, Van der Linden IK, de Visser YP, et al. 17alpha-Ethinylestradiol rapidly alters transcript levels of murine coagulation genes via estrogen receptor alpha. J Thromb Haemost 2010;8:1838-46.
19 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.
20 Govers-Riemslag JW, Castoldi E, Nicolaes GA, et al. Reduced factor V concentration and altered FV1/FV2 ratio do not fully explain R2-associated APC-resistance. Thromb Haemost 2002;88:444-9.
21 Eitzman DT, Westrick RJ, Bi X, et al. Lethal perinatal thrombosis in mice resulting from the interaction of tissue factor pathway inhibitor deficiency and factor V Leiden. Circulation 2002;105:2139-42.
22 Castoldi E, Brugge JM, Nicolaes GA, et al. Impaired APC cofactor activity of factor V plays a major role in the APC resistance associated with the factor V Leiden (R506Q) and R2 (H1299R) mutations. Blood 2004;103:4173-9.
23 Maroney SA, Ferrel JP, Pan S, et al. Temporal expression of alternatively spliced forms of tissue factor pathway inhibitor in mice. J Thromb Haemost 2009;7:1106-13.
24 Nalbandian RM, Henry RL, Mammen EF, et al. Consumption coagulopathy: practical principles of diagnosis and management. Mich Med 1971;70:793-804.
25 Zhang B, McGee B, Yamaoka JS, et al. Combined deficiency of factor V and factor VIII is due to mutations in either LMAN1 or MCFD2. Blood 2006;107:1903-7.

26 Bajzar L, Kalafatis M, Simioni P, et al. An antifibrinolytic mechanism describing the prothrombotic effect associated with factor V Leiden. J Biol Chem 1996;271:22949-52.
27 Maas C, Meijers JC, Marquart JA, et al. Activated factor V is a cofactor for the activation of factor XI by thrombin in plasma. Proc Natl Acad Sci U S A 2010;107:9083-7.
28 Parker AC, Mundada LV, Schmaier AH, et al. Factor V Leiden inhibits fibrinolysis in vivo. Circulation 2004;110:3594-8.

Chapter 10

General discussion

In this thesis, we aimed to identify the biological mechanisms by which acquired risk factors like female hormones, thyroid hormone and obesity result in a hypercoagulable state and increased risk for venous thrombosis, as these mechanisms are currently poorly understood. Since these risk factors are all, to a certain extent, able to interfere with liver metabolism we hypothesized that they modulate hepatic transcription of coagulation genes, either directly via nuclear hormone receptors and hormone response elements in target genes (female hormones and thyroid hormone), or indirectly as a result of altered liver homeostasis (obesity). To study these hypotheses, we used an *in vivo* approach, which provides a complete physiological system including intact mechanisms through which risk factors potentially impact transcriptional modulation of coagulation genes. In addition, this approach allows us to study the relation between transcriptional changes on the one hand and plasma protein levels and a thrombotic tendency on the other.

We show that estrogens and thyroid hormone are both able to modulate transcription of hepatically expressed coagulation genes, and that these effects can be immediate, i.e. within only a few hours after administration. These observations make it likely that these hormones modulate transcription via a direct interaction with their receptor and subsequent binding to hormone response elements in the promoter region of coagulation genes. In contrast, progestins were not able to modulate transcription, neither in the presence nor absence of estrogens. Dietary fat intake rather than body fat mass was found to be a strong modulator of the plasma coagulation profile albeit transcription levels of only a limited number of coagulation genes were affected.

In genetic mouse models of thrombosis that lack a vascular injury component, oral estrogen-induced transcriptional modulation of coagulation genes was not able to trigger a micro- or macrovascular thrombotic phenotype. This is in strong contrast to conditions where a disturbance of the coagulation balance is evoked by genetic means, as illustrated by the partial perinatal death of factor V Leiden hemizygous mice.

Taken together, our studies clearly demonstrate that modulation of hepatic coagulation gene transcription is a key mechanism by which acquired risk factors for venous thrombosis impact the hemostatic balance.

Transcriptional modulation of coagulation genes

Over the past years, mouse studies have demonstrated that estrogens, progestins, thyroid hormone and dietary fat intake are able to modify gene transcription of multiple pathways in multiple tissues. A large body of evidence has come from mouse studies employing microarrays as a key technology to identify pathways and networks of genes affected.[1-10] However, transcript levels of coagulation genes never appeared to be altered in microarray studies dedicated to the liver.[3,7,8] This seems to be rather counterintuitive, as female hormones, thyroid hormone and obesity are all associated with changes in the plasma coagulation profile and most of these plasma factors are synthesized in the liver.

In chapters 3, 4 and 8 we have demonstrated, by using quantitative PCR methods, that the transcript levels of coagulation genes are clearly modulated by the risk factors studied. The apparent discrepancy with previous studies might be explained by the different detection techniques used, i.e. quantitative PCR versus microarrays. Microarrays generate large amounts of data and give a relatively high background signal. Therefore, it is necessary to set a threshold value to determine truly induced effects, and these thresholds are often set so only changes over 1.5-fold are included in the analyses.[2,5,7,10] With our qPCR data, we have shown that the risk factor-induced effects on transcript levels of coagulation genes are relatively small, generally remaining within the 50% range, and therefore not exceed the threshold values set in microarray studies. Hence, microarray analysis should not be the method of choice for studying modulating effects on transcript levels of genes that, like coagulation genes, are regulated within a relatively narrow window.

In addition to microarrays*, in silico* and ChIP-on-chip analyses are valuable tools to predict how risk factors may impact the transcription of coagulation genes. Previous *in silico* and ChIP-on-chip studies have predicted and identified several estrogen receptor targets belonging to coagulation.[4,11,12] These observations were used as a starting point for the hypothesis that oral ethinylestradiol can directly modulate transcription via ligand-bound estrogen receptor interaction with estrogen response elements in coagulation genes. We have shown that transcript levels of nearly all coagulation genes could be modulated within 2.5 to 5 hours after oral ethinylestradiol administration, indicating immediate effects. Comparing our data with the *in silico* and ChIP-on-chip data showed that almost all genes we found were previously predicted to have either an estrogen response element, or are able

to bind to the estrogen receptor α *in vivo*.[4,11] In addition, we also found several factors that have not been identified previously, including factor V and factor IX.
Taken together, these data show that predicted estrogen response elements *in silico* will not necessarily bind estrogen receptors *in vivo*. On the other hand, estrogen receptor binding to coagulation genes will not automatically lead to transcriptional modulation. By determining transcript levels within 5 hours after estrogen administration, we were able to identify coagulation genes that can be rapidly modulated. Although we are aware of the fact that with this approach fast indirect effects on transcription cannot be excluded, it is highly likely that these effects are caused by direct interactions. However, the unequivocal evidence whether estrogen receptor binding to coagulation genes truly results in transcriptional modulation can only come from experiments in which ChIP is combined with a technique like RNA sequencing.

For oral estrogen administration, prolonged exposure hardly yielded additional coagulation gene targets, and thus all coagulation targets appear to be under direct control, which fitted our hypothesis. In contrast, for thyroid hormone prolonged exposure affected transcription of a large number of coagulation genes, but for most an immediate response (4 hours) was absent, suggesting that a large panel of coagulation genes is indirectly regulated by thyroid hormone. This indirect regulation may still involve a relatively short chain of events where thyroid hormone, via its receptor, interferes with the constitutive expression of coagulation genes. However, it may also involve a long chain of events in which thyroid hormone for example affects liver metabolism which subsequently via a multistep process leads to changes in coagulation gene transcript levels by affecting transcriptional rate or RNA stability. By nature it will be hard to unwind the intermediate steps involved in this process resulting in altered transcript levels. Nevertheless, we considered the first option of interference with constitutive expression the most likely and therefore evaluated two potential intermediate transcription factors: hepatic nuclear factor 4α (HNF4α) and the growth hormone receptor, in an attempt to unravel the key players in this multistep process.
HNF4α is an important transcription factor for the basal expression of a number of pro- and anticoagulant factors,[13,14] and thyroid hormone has been reported to increase HNF4α expression in mouse hepatocytes.[15] Following prolonged thyroid hormone exposure, a remarkable increase in transcript levels of coagulation factors XI and XII was observed (chapter 6), representing two typical HNF4α targets.[13]

Therefore, we determined HNF4α transcript and protein levels in livers of mice treated with thyroid hormone and found that in a setting where FXI and FXII were increased by 30-40%, HNF4α levels were reduced by 20%, thereby making HNF4α an unlikely intermediate in modulating the thyroid hormone-mediated effects on the transcription of coagulation genes.

Another potential intermediate transcription we considered is the growth hormone receptor, as a recent report by Wong and colleagues showed that gender-specific growth hormone secretion contributes to the gender differences in hepatic anticoagulation gene transcription and thrombosis susceptibility.[16] As thyroid hormone is a well-known modulator of growth hormone secretion[17] and growth hormone receptor levels are key in the transcriptional effects of growth hormone, we analyzed growth hormone receptor transcript levels in the livers of mice treated with thyroid hormone. However, we were unable to detect differences in transcript levels between vehicle- and thyroid hormone-treated mice, which makes the growth hormone pathway also an unlikely intermediate in modulating the late effects of thyroid hormone.

As shown, it will be difficult to delineate the multiple steps involved in the late modulating effects of thyroid hormone exposure on coagulation gene transcript levels. However, a genome wide approach that accurately records all late transcriptional changes that occur in parallel with changes in coagulation gene transcription may shine a light on the key players involved.

Plasma coagulation profile

We have shown that estrogen and thyroid hormone administration in mice can modulate transcription, both in a direct and indirect fashion, and in addition these transcriptional effects are often translated into altered protein (activity) levels in plasma (chapters 3, 5 and 8). Although this risk factor exposure in mice results in changes in the plasma coagulation profile as it does in humans, the net effect on individual plasma levels, i.e. whether plasma levels increase or decrease upon exposure, may differ between mice and humans. For example, antithrombin levels decrease in both mice and humans, whereas the decrease in levels of procoagulant factors in mice is in contrast to human observations.[18,19]

Thrombin generation assays represent the overall hemostatic balance in plasma and are therefore considered to have a predictive value regarding a bleeding or thrombotic phenotype.[20] The risk factors studied in this thesis are all associated with a hypercoagulable state that in humans corresponds to an increased

endogenous thrombin potential (ETP).[21,22] Surprisingly, oral ethinylestradiol administration in mice, while causing decreases in procoagulant factor levels, resulted in an increased ETP although this did not translate into an increased thrombotic tendency (chapters 5 and 6). On the other hand, nutritionally-induced obesity resulted in higher procoagulant levels in plasma and an increased thrombotic tendency while this not coincided with higher ETP values (chapters 5 and 7). These data indicate that the ETP in mice does not have a predictive value regarding the overall coagulability, like it has in humans.

In order to find an explanation for the species-specific differences in coagulation factor levels, we first considered the way mice were exposed to the risk factor as a possible determinant. To study the effects of (oral) estrogens on coagulation, mice were treated with the semi-synthetic compound ethinylestradiol, and this compound might be differently metabolized in mice and humans. Nevertheless, we have shown that in mice the effects of oral ethinylestradiol on coagulation are dose-dependent, which indicates a specific estrogen-induced response (chapter 3). In contrast, endogenously increased estrogen levels as observed during pregnancy, have shown to be associated with increased plasma levels of FII, FV and FX.[23] However, since pregnancy has more metabolic effects than only an increase in estrogen levels, it is difficult to conclude that these changes on coagulation are truly and only due to increased endogenous estrogen levels, especially since we have shown that injections with the naturally occurring 17ß-estradiol did not yield different results as compared to the oral ethinylestradiol-induced effects on coagulation.

In chapter 7 we determined the relation between coagulation and nutritionally-induced weight gain and subsequent weight loss, and also evaluated the genetically obese *ob/ob* mice as a potential model to study obesity in relation to coagulation. *Ob/ob* mice had more pronounced increases in plasma levels of procoagulant factors as compared to nutritionally-induced obese mice. In addition, they also display metabolic abnormalities like insulin resistance, which may aggravate the hypercoagulable state. As we show that the dietary fat intake rather than being obese rapidly affects coagulation, which thereby precedes metabolic changes, genetically obese mice may be less suitable for studying coagulation-related effects.

Taken together, exogenous exposure to risk factors does not seem to explain the species-specific responses on the coagulation profile, and therefore the explanation may be found in the different organization of the hemostatic balance

between mice and human. It is known that there is a higher activity of natural anticoagulants in mouse plasma,[24] which in the case of oral ethinylestradiol administration may at least partially explain the increase in ETP value, since estrogens also cause a significant decrease in antithrombin levels (chapters 3 and 6). This difference in the center of gravity between pro- and anticoagulant factors in the hemostatic balance questions whether mice are a suitable model organism to study quantitative effects of acquired risk factors on the overall coagulability and the thrombotic phenotype, although they have proven to be a valuable tool for mechanistic studies.

Thrombosis models

With respect to the effects of acquired risk factors on thrombosis as a read-out, the species-specific differences in the hemostatic balance have also shown to be challenging. In order to find the "holy grail" of a genetic model for spontaneous macrovascular thrombosis in adult mice we reviewed the available models in chapter 2. As factor V Leiden and thrombomodulin proline mutant mice display fibrin depositions as a marker for thrombosis and have the potential to develop macrovascular thrombosis,[25,26] these models were used in studies described in chapter 6. However, prolonged ethinylestradiol administration did not result in spontaneous macrovascular thrombosis in factor V Leiden or thrombomodulin mutant mice, and for factor V Leiden mice it has been shown that pregnancy and obesity, two conditions associated with a hypercoagulable state, were also not able to induce thrombosis. [23,27]

In contrast, an additional genetic defect is able to cause a severe macrovascular thrombotic phenotype which might even result in partial lethality, as we have shown in factor V Leiden hemizygous mice which have a combination of factor V Leiden and factor V deficiency (chapter 9). An even more severe phenotype, with near-complete lethality has also been shown for factor V Leiden homozygous mice that are also deficient in tissue factor pathway inhibitor or protein Z.[28,29] The dual phenotype of factor V Leiden hemizygous mice implies that they are more prone to develop thrombosis and although further evaluation is necessary, this observation suggests that these mice can be valuable in the development of a new genetic thrombosis model.

Due to the current lack of a good genetic model to study spontaneous thrombosis, experimental thrombosis models may provide a better setting to study acquired risk factors for thrombosis. By applying these experimental models, a thrombus or

reduced blood flow can be induced in virtually any mouse, including wild-type mice that have not been exposed to a certain risk factor (chapter 5). However, the underlying mechanisms are often not completely known and each model focuses on different aspects of thrombus formation.[30,31] Therefore, a combination of models will be necessary to evaluate all aspects of thrombus formation in order to get a complete insight of how risk factors affect the thrombotic phenotype.

Future perspectives

Although we have come a long way to identify the biological mechanisms underlying the risk factor-associated an increase in the venous thrombotic risk, there is still a lot to explore. As more risk factors for venous thrombosis are being identified, and the prevalence of common acquired risk factors increases, it becomes more important to gain insight in biological mechanisms, as this may lead to disease prevention and better risk assessments. Fortunately, new high-throughput technologies and the use of novel approaches in animal models may quickly expand our current knowledge and gain new fundamental insights in the pathophysiology of venous thrombosis.

As previously discussed, microarray studies have contributed to our knowledge of hormonal regulation of gene transcription, but as the effects have to be relatively large to be detected, this method is less suitable to study minor modulating effects on transcription, as are observed for coagulation genes. With the development of next-generation sequencing methods, this limitation can be overcome. For example, RNA-sequencing can determine transcript levels with a comparable precision as has been shown for qPCR data, including accurate transcript levels in the low ranges. In addition, because of the high-throughput sequencing it will not only become less time-consuming than the current methods, but will also allow an unbiased approach and may therefore reveal new networks important for the regulation of coagulation gene transcription.[32-34]

The use of genetic mouse models in hemostasis has been of great value for expanding our knowledge on the role of specific coagulation factors in the pathophysiology of venous thrombosis. However, several coagulation factors also play an important role in development, and therefore the complete knock-out of these factors results in non-viable mice (chapter 2). By using conditional knock-out techniques in which a gene can be knocked-out in a specific tissue at a specific time during life, this problem can be overcome.[35,36] However, making conditional knock-outs can be very laborious and time-consuming. The recent discovery and

development of synthetic short interference RNA (siRNA) makes it possible to temporarily knock-down a gene or set of genes, and it has been shown that particularly hepatically expressed genes can be relatively easy targeted.[37-39] Therefore, the use of siRNAs may finally give us the opportunity to determine the contribution of a specific coagulation factor, or set of factors, on the thrombotic risk.
Thrombotic disorders, including both arterial and venous thrombosis, are the second most common causes of mortality and morbidity in developed countries. The evidence that arterial and venous thrombosis are more alike than previously thought is increasing, and they share a common pathophysiologic background as well as several risk factors including age, dyslipidemia and obesity.[40] More importantly, it is now known that experiencing a venous thrombotic event does not only predispose to recurrent venous thrombosis, but also predisposes to arterial thrombosis.[41,42] This observation may lead to a reconsideration of the treatment for venous thrombosis, as it has been shown that anticoagulant therapy can also reduce the risk of arterial thrombosis and statins may have beneficial effects on both the arterial and venous thrombosis risk. In addition, it underlines the importance of elucidating the biological mechanisms by which acquired factors increase the risk for venous thrombosis as this may eventually not only result in reducing the venous thrombotic risk, but can also reduce the prevalence of vascular diseases in general.

References

1 Do GM, Oh HY, Kwon EY, et al. Long-term adaptation of global transcription and metabolism in the liver of high-fat diet-fed C57BL/6J mice. Mol Nutr Food Res 2011;55 Suppl 2:S173-S185.
2 Feng X, Jiang Y, Meltzer P, et al. Thyroid hormone regulation of hepatic genes in vivo detected by complementary DNA microarray. Mol Endocrinol 2000;14:947-55.
3 Flores-Morales A, Gullberg H, Fernandez L, et al. Patterns of liver gene expression governed by TRbeta. Mol Endocrinol 2002;16:1257-68.
4 Gao H, Falt S, Sandelin A, et al. Genome-wide identification of estrogen receptor alpha-binding sites in mouse liver. Mol Endocrinol 2008;22:10-22.
5 Lee KY, Kim SJ, Cha YS, et al. Effect of exercise on hepatic gene expression in an obese mouse model using cDNA microarrays. Obesity (Silver Spring) 2006;14:1294-302.
6 Lindberg MK, Moverare S, Skrtic S, et al. Estrogen receptor (ER)-beta reduces ERalpha-regulated gene transcription, supporting a "ying yang" relationship between ERalpha and ERbeta in mice. Mol Endocrinol 2003;17:203-8.
7 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.

8 Radonjic M, de Haan JR, van Erk MJ, et al. Genome-wide mRNA expression analysis of hepatic adaptation to high-fat diets reveals switch from an inflammatory to steatotic transcriptional program. PLoS One 2009;4:e6646.
9 Shih CH, Chen SL, Yen CC, et al. Thyroid hormone receptor-dependent transcriptional regulation of fibrinogen and coagulation proteins. Endocrinology 2004;145:2804-14.
10 Yen PM, Feng X, Flamant F, et al. Effects of ligand and thyroid hormone receptor isoforms on hepatic gene expression profiles of thyroid hormone receptor knockout mice. EMBO Rep 2003;4:581-7.
11 Bourdeau V, Deschenes J, Metivier R, et al. Genome-wide identification of high-affinity estrogen response elements in human and mouse. Mol Endocrinol 2004;18:1411-27.
12 Carroll JS, Meyer CA, Song J, et al. Genome-wide analysis of estrogen receptor binding sites. Nat Genet 2006;38:1289-97.
13 Inoue Y, Peters LL, Yim SH, et al. Role of hepatocyte nuclear factor 4alpha in control of blood coagulation factor gene expression. J Mol Med (Berl) 2006;84:334-44.
14 Safdar H, Inoue Y, van Puijvelde GH, et al. The role of hepatocyte nuclear factor 4alpha in regulating mouse hepatic anticoagulation and fibrinolysis gene transcript levels. J Thromb Haemost 2010;8:2839-41.
15 Selva DM, Hammond GL. Thyroid hormones act indirectly to increase sex hormone-binding globulin production by liver via hepatocyte nuclear factor-4alpha. J Mol Endocrinol 2009;43:19-27.
16 Wong JH, Dukes J, Levy RE, et al. Sex differences in thrombosis in mice are mediated by sex-specific growth hormone secretion patterns. J Clin Invest 2008;118:2969-78.
17 DiPippo VA, Lindsay R, Powers CA. Estradiol and tamoxifen interactions with thyroid hormone in the ovariectomized-thyroidectomized rat. Endocrinology 1995;136:1020-33.
18 Rosendaal FR, Helmerhorst FM, Vandenbroucke JP. Female hormones and thrombosis. Arterioscler Thromb Vasc Biol 2002;22:201-10.
19 Tchaikovski SN, Rosing J. Mechanisms of estrogen-induced venous thromboembolism. Thromb Res 2010;126:5-11.
20 Castoldi E, Rosing J. Thrombin generation tests. Thromb Res 2011;127 Suppl 3:S21-S25.
21 Ay L, Kopp HP, Brix JM, et al. Thrombin generation in morbid obesity: significant reduction after weight loss. J Thromb Haemost 2010;8:759-65.
22 Tchaikovski SN, van Vliet HA, Thomassen MC, et al. Effect of oral contraceptives on thrombin generation measured via calibrated automated thrombography. Thromb Haemost 2007;98:1350-6.
23 Tchaikovski SN, Van Vlijmen BJ, Cleuren AC, et al. Pregnancy-associated changes in the hemostatic system in wild-type and factor V Leiden mice. J Thromb Haemost 2009;7:312-8.
24 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.
25 Cui J, Eitzman DT, Westrick RJ, et al. Spontaneous thrombosis in mice carrying the factor V Leiden mutation. Blood 2000;96:4222-6.
26 Weiler-Guettler H, Christie PD, Beeler DL, et al. A targeted point mutation in thrombomodulin generates viable mice with a prethrombotic state. J Clin Invest 1998;101:1983-91.
27 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.

28 Eitzman DT, Westrick RJ, Bi X, et al. Lethal perinatal thrombosis in mice resulting from the interaction of tissue factor pathway inhibitor deficiency and factor V Leiden. Circulation 2002;105:2139-42.
29 Yin ZF, Huang ZF, Cui J, et al. Prothrombotic phenotype of protein Z deficiency. Proc Natl Acad Sci U S A 2000;97:6734-8.
30 Eckly A, Hechler B, Freund M, et al. Mechanisms underlying FeCl3-induced arterial thrombosis. J Thromb Haemost 2011;9:779-89.
31 Furie B, Furie BC. In vivo thrombus formation. J Thromb Haemost 2007;5 Suppl 1:12-7.
32 Hawkins RD, Hon GC, Ren B. Next-generation genomics: an integrative approach. Nat Rev Genet 2010;11:476-86.
33 Wang Z, Gerstein M, Snyder M. RNA-Seq: a revolutionary tool for transcriptomics. Nat Rev Genet 2009;10:57-63.
34 Werner T. Next generation sequencing in functional genomics. Brief Bioinform 2010;11:499-511.
35 Burstyn-Cohen T, Heeb MJ, Lemke G. Lack of protein S in mice causes embryonic lethal coagulopathy and vascular dysgenesis. J Clin Invest 2009;119:2942-53.
36 Mullins ES, Kombrinck KW, Talmage KE, et al. Genetic elimination of prothrombin in adult mice is not compatible with survival and results in spontaneous hemorrhagic events in both heart and brain. Blood 2009;113:696-704.
37 Akinc A, Goldberg M, Qin J, et al. Development of lipidoid-siRNA formulations for systemic delivery to the liver. Mol Ther 2009;17:872-9.
38 Love KT, Mahon KP, Levins CG, et al. Lipid-like materials for low-dose, in vivo gene silencing. Proc Natl Acad Sci U S A 2010;107:1864-9.
39 Mahon KP, Love KT, Whitehead KA, et al. Combinatorial approach to determine functional group effects on lipidoid-mediated siRNA delivery. Bioconjug Chem 2010;21:1448-54.
40 Prandoni P. Venous and arterial thrombosis: Two aspects of the same disease? Clin Epidemiol 2009;1:1-6.
41 Borissoff JI, Spronk HM, ten Cate H. The hemostatic system as a modulator of atherosclerosis. N Engl J Med 2011;364:1746-60.
42 Green D. Risk of future arterial cardiovascular events in patients with idiopathic venous thromboembolism. Hematology Am Soc Hematol Educ Program 2009;259-66.

Chapter 11

Summary

Summary in Dutch
(Nederlandse samenvatting)

Summary

Over the years, a number of acquired risk factors for venous thrombosis, either resulting in stasis or a hypercoagulable state, have been identified in large epidemiological studies. However, the exact mechanisms that lead to this hypercoagulable state and increased thrombosis risk are poorly understood. Therefore, this thesis focuses on identifying the biological mechanisms underlying acquired risk factors leading to venous thrombosis.

Chapter 1 provides a general background on risk factors for venous thrombosis and shows that the risk factors studied in this thesis all have in common that they lead to a hypercoagulable state and can interfere with liver metabolism. Since the liver is also the main organ involved in the synthesis of plasma coagulation factors, we hypothesize that the risk factors of interest, i.e. female hormones, thyroid hormone and obesity, can modulate transcription of coagulation genes. Several mechanisms by which transcription can be modulated are discussed, ranging from a direct modulation via nuclear receptors that bind to response elements in coagulation genes, to indirect modulation where transcription is affected as a bystander effect of changes in liver homeostasis. In addition, different experimental approaches to study transcriptional modulation are explained, as well as the rationale to use mice as a model organism for the studies described in this thesis.

Mice do not develop thrombosis spontaneously. To overcome this limitation, transgenic mice have been generated that carry defective or mutant alleles of genes that are involved in anticoagulation and therefore have the potential to develop a thrombotic phenotype. These models have been reviewed in chapter 2, in which it is shown that complete deficiencies of an anticoagulant factor often result in embryonic lethality due to hemodynamic dysfunction, while heterozygosity in general coincides with normal survival. However, models like the factor V Leiden and thrombomodulin proline mutant mouse display a spontaneous thrombotic phenotype characterized by tissue fibrin depositions as a sign of low-grade thrombosis, and are therefore considered to be valuable tools in thrombosis research.

Experiments described in chapter 3 were performed in order to gain more insight in the underlying mechanism of estrogen-induced changes in coagulation. Oral ethinylestradiol treatment of mice is able to cause dose-dependent effects on the

plasma coagulation profile, which coincides with dose-dependent changes in hepatic transcript levels of coagulation genes. In addition, these ethinylestradiol-induced effects were counteracted by additional estrogen receptor (ER) antagonist administration and studies performed in ERα and ERß knock-out mice demonstrated a crucial role for the ERα subtype in mediating the coagulation-related alterations. Moreover, the changes in hepatic transcript levels could be rapidly induced, within 2.5 to 5 hours after oral ethinylestradiol administration, thereby suggesting a direct interaction between the estrogen-bound estrogen receptor and coagulation genes in the liver.

There is a known difference in venous thrombosis risk associated with second and third generation oral contraceptives and it is believed that this is due to the anti-estrogenic properties of the progestin compound, i.e. levonorgestrel or desogestrel. In chapter 4, these anti-estrogenic effects of progestins on hepatic mRNA levels of coagulation genes were determined, together with the biological changes in uterus morphology. Despite a comparable and clear anti-estrogenic effect of both levonorgestrel and desogestrel on the uterus, neither progestin was able to modulate the oral ethinylestradiol-induced alterations in transcript levels of coagulation genes expressed in the liver.

Chapter 5 focused on the interaction between obesity and oral estrogen use on the thrombotic tendency by applying a stasis-induced thrombosis model in mice that were fed a high fat diet to induce obesity and / or were treated with oral ethinylestradiol. High fat feeding resulted in a procoagulant shift of the coagulation profile and a borderline significant increase in thrombus weight as compared to mice on a standard fat diet. Although oral ethinylestradiol administration did not affect the plasma coagulation profile, both transcript levels of coagulation factors as well as the thrombus weight were decreased, of which the latter was significant in obese mice. These data indicate that ethinylestradiol can counteract the prothrombotic phenotype of nutritionally-induced obesity in mice.

After determining the oral ethinylestradiol-induced effects on individual coagulation factor levels in plasma, chapter 6 evaluates the overall effects of these changes on the hemostatic balance by measuring thrombin generation and determining the net effect on a spontaneous thrombotic phenotype in prothrombotic factor V Leiden and thrombomodulin proline mutant mice. Oral estrogen administration caused a

sustained increase in thrombin generation, however, this did not translate into a spontaneous macrovascular or microvascular thrombotic phenotype as evaluated by fibrin depositions in the lung. Therefore, these data imply the lack of a relation between an increased potential to generate thrombin and an enhanced thrombotic phenotype in these mice.

Chapter 7 describes the onset and reversibility of the hypercoagulable state during the development and regression of nutritionally-induced obesity in mice, and its relation to changes in transcriptional and clearance rates of coagulation factors as well as its relation to changes in metabolic and inflammatory parameters. The plasma coagulation profile is able to rapidly respond to changes in dietary fat intake, both in weight gain which is associated with a procoagulant shift, and in subsequent weight loss resulting in a reversal of the high fat diet-induced hypercoagulability. These changes in the plasma coagulation profile did not coincide with changes in hepatic transcript levels or clearance rates of coagulation factors. In addition, effects on coagulation preceded the alterations in metabolic parameters, both in nutritionally-induced weight gain and weight loss. Levels of inflammatory cytokines were only transiently increased early after starting the high fat diet or switching back to the low fat diet. This study shows that changes in dietary fat intake can rapidly alter the plasma coagulation profile and this could not be explained by changes in relative transcript levels or clearance rates of coagulation factors.

In chapter 8, the role of thyroid hormone in modulating coagulation was studied by evaluating the impact of exogenous triiodothyronine (T_3) administration on transcript and plasma levels of coagulation factors in mice with a suppressed endogenous thyroid hormone production. Prolonged exposure to T_3 affected transcript levels of several hepatically expressed and endothelium-associated coagulation genes, and for the genes expressed in the liver these effects were largely paralleled by changes in plasma activity levels. In addition, most anticoagulant genes, but not procoagulant genes, could be rapidly modulated, suggesting that thyroid hormone can have both immediate and late effects on coagulation gene transcription.

Factor V has a dual role in coagulation since it can act as both a procoagulant and anticoagulant factor. Chapter 9 describes the generation of the factor V Leiden

hemizygous mouse, which combines the factor V Leiden-related increased venous thrombotic risk with the deficiency-related bleeding tendency. In mice, this combination of opposites results in partial lethality in the early postnatal period which coincides with features of macrovascular thrombosis, suggesting that it is the lack of the wild-type anticoagulant function that causes lethality. In addition, as factor V Leiden homozygosity is not lethal, these hemizygous data also implies that the factor V Leiden allele has retained a critical anticoagulant function which is sufficient to rescue the partial lethal phenotype of factor V Leiden hemizygous mice.

Chapter 10 discusses the studies described in this thesis and puts them into perspective with respect to the currently available literature. Although we believe that our studies contribute to a better understanding how certain risk factors can affect the venous thrombotic risk, we are convinced that there is more to explore. Therefore, the final part of the general discussion includes some future perspectives and describes how new techniques can be of great value in thrombosis research.

Nederlandse samenvatting

Epidemiologische studies hebben in de afgelopen jaren verscheidene verworven risicofactoren voor veneuze trombose geïndentificeerd die de bloedstroom kunnen beïnvloeden en / of tot veranderingen in het plasma stollingsprofiel kunnen leiden, wat resulteert in een protrombotische verschuiving van de hemostase balans. Echter, de exacte biologische mechanismen waardoor deze factoren tot een protrombotische verschuiving en een verhoogd tromboserisico leiden zijn niet bekend. Daarom richt dit proefschrift zich op het indentificeren van de biologische mechanismen die ten grondslag liggen aan het verhoogde tromboserisico veroorzaakt door verworven factoren.

Hoofdstuk 1 geeft achtergrondinformatie over risicofactoren voor trombose en veneuze trombose in het algemeen, en laat zien dat behalve een protrombotische verschuiving van het stollingsprofiel, de bestudeerde risicofactoren ook allemaal kunnen interfereren met het lever metabolisme. Omdat de lever van groot belang is voor de aanmaak van stollingsfactoren die zich in het plasma bevinden, hebben we de hypothese gepostuleerd dat de risicofactoren van interesse, dat wil zeggen het gebruik van oestrogenen en progestagenen, schilklierhormoon en overgewicht, in staat zijn om transcriptie van stollingsfactoren te moduleren. Enkele mogelijke mechanismen waarop transcriptie kan worden gemoduleerd worden beschreven, welke kunnen varieëren van een directe modulatie via nucleaire receptoren die binden aan specifieke respons elementen in stollingsgenen, tot een indirecte modulatie waarbij transcriptie is aangedaan als gevolg van een verandering in lever homeostase. Daarnaast worden verschillende experimentele modellen uitgelegd die gebruikt kunnen worden om transcriptionele modulatie te bestuderen, alsmede de reden om muizen als model organisme te gebruiken voor de studies beschreven in dit proefschrift.

Een belangrijke tekortkoming van het gebruik van muizen in tromboseonderzoek, is dat ze niet spontaan trombose ontwikkelen. Er zijn echter transgene muizen gegenereerd die defecte of gemuteerde allelen hebben van genen die betrokken zijn in antistolling en daardoor wel een trombotisch fenotype kunnen ontwikkelen. **Hoofdstuk 2** geeft een overzicht van deze modellen en laat zien dat een volledige deficiëntie van een specifieke antistollingsfactor vaak resulteert in embryonale lethaliteit terwijl heterozygotie vaak samengaat met een normale overleving. Modellen als de factor V Leiden en trombomoduline proline mutant muizen

vertonen een spontaan trombotisch fenotype, wat gekenmerkt wordt door fibrine deposities in weefsels. Dit maakt deze modellen interessant voor het gebruik in trombose-gerelateerd onderzoek.

Experimenten beschreven in **hoofdstuk 3** zijn ondernomen om meer inzicht te krijgen in het mechanisme dat ten grondslag ligt aan oestrogeen-geïnduceerde veranderingen in stolling. Orale ethinyloestradiol toediening in muizen is in staat om dosis-afhankelijke effecten op het plasma stollingsprofiel te veroorzaken welke samengaan met dosis-afhankelijke veranderingen in transcript niveaus in de lever. Daarnaast kunnen deze ethinyloestradiol-geïnduceerde effecten teniet worden gedaan door een oestrogenreceptor antagonist toe te voegen en studies uitgevoerd in oestrogeenreceptor-α en -ß knock-out muizen laten zien dat het α subtype van cruciaal belang is voor de stollingsgerelateerde veranderingen. Verder laten we zien dat de effecten op transcript niveaus van stollingsgenen snel kunnen worden geïnduceerd, binnen 2 ½ tot 5 uur na ethinyloestradiol toediening, wat suggereert dat de oestrogeen-gebonden oestrogeenreceptor een directe interactie heeft met de stollingsgenen in de lever en daardoor transcriptie beïnvloedt.

Het is bekend dat er een verschil in tromboserisico is tussen anticonceptiepillen van de tweede en derde generatie en men veronderstelt dat dit komt door de anti-oestrogene werking van de progestageen-component waarvan levonorgestrel en desogestrel vertegenwoordigers zijn van de verschillende generaties. In **hoofdstuk 4** hebben we de anti-oestrogene effecten van deze 2 progestagenen getest met betrekking tot zowel mRNA niveaus van stollingsgenen in de lever als de uterus morfologie. Ondanks het feit dat zowel levonorgestrel als desogestrel duidelijke anti-oestrogene effecten op de uterus hebben, was geen van beiden in staat de ethinyloestradiol-geïnduceerde veranderingen in transcript niveaus van stollingsgenen in de lever moduleren.

De focus van **hoofdstuk 5** ligt op de interactie tussen overgewicht en oraal oestrogeen gebruik met betrekking tot de trombose gevoeligheid. Dit laatste is bepaald door middel van een stase-geïnduceerd trombose model in muizen met voedingsgeïnduceerd overgewicht, al dan niet in combinatie met orale ethinyloestradiol toediening. Overgewicht leidde tot een protrombotische verschuiving van het plasma stollingsprofiel en een verhoogd trombus gewicht ten opzichte van muizen met een normaal lichaamsgewicht. Ondanks dat de

ethinyloestradiol behandeling in deze opzet geen effect had op het plasma stollingsprofiel, leidde dit wel tot verlagingen in transcript niveaus. Bovendien zorgde de oestrogeen behandeling voor een verlaging in het trombus gewicht, wat significant was in muizen met overgewicht. Dit toont aan dat ethinyloestradiol het protrombotische fenotype van voedingsgeïnduceerd overgewicht teniet kan doen in muizen.

Nadat we de ethinyloestradiol-geïnduceerde effecten op plasma waarden van individuele stollingsfactoren hebben aangetoond, evalueren we in **hoofdstuk 6** het netto effect van deze veranderingen op de hemostase balans door de trombinegeneratie in plasma te meten. Daarnaast stellen we de effecten op een spontaan trombotisch fenotype vast in factor V Leiden en trombomoduline proline mutant muizen door te kijken naar fibrine deposities in longen. Orale ethinyloestradiol toediening zorgde voor een aanhoudende verhoging in trombinegeneratie, maar dit werd niet vertaald in een spontaan macrovasculair of microvasculair trombotisch fenotype. Deze data impliceert dat er geen relatie tussen een verhoogde potentie tot trombinegeneratie en een trombotisch fenotype in deze muizen.

Hoofdstuk 7 beschrijft het ontstaan en de reversibiliteit van de protrombotische status gedurende de ontwikkeling en regressie van voedingsgeïnduceerd overgewicht in muizen. Daarnaast worden ook de relaties tot veranderingen in transcriptie en klaring van stollingsfactoren, en de relaties tot veranderingen in metabole en ontstekingsgerelateerde parameters bekeken. Het plasma stollingsprofiel kan snel reageren op veranderingen in vet inname, zowel in gewichtstoename wat geassocieerd is met een protrombotische verschuiving, als bij het daar op volgende gewichtsverlies wat resulteert in reversibiliteit van de hoog vet-geïnduceerde protrombotische verschuiving. Deze veranderingen in het plasma stollingsprofiel gaan echter niet samen met veranderingen in mRNA niveaus of klaring van stollingsfactoren. Daarnaast gaan de effecten op stolling vooraf aan veranderingen in metabole parameters, zowel bij gewichtstoename als bij gewichtsverlies. Plasma waarden van cytokines zijn slechts tijdelijk verhoogd direct na de start van het hoog vet dieet en na het wisselen van een hoog naar een laag vet dieet om gewichtsverlies te bewerkstelligen. Deze studie laat daarmee zien dat een verandering in vet inname kan leiden tot snelle effecten op het plasma

stollingsprofiel en dat dit niet kan worden verklaard door veranderingen in transcriptie of klaring van stollingsfactoren.
In **hoofdstuk 8** hebben we gekeken naar de modulerende rol van schildklierhormoon op stolling, door naar de effecten van exogeen toegediende triiodothyronine (T_3) op transcript en plasma waarden van stollingsfactoren te kijken in muizen met een onderdrukte endogene schildklierhormoon productie. T_3 blootstelling voor langere termijn beïnvloedt transcript niveaus van verschillende stollingsgenen die ofwel in de lever ofwel in endotheelcellen worden aangemaakt. Voor de genen die in de lever tot expressie komen zijn deze veranderingen in het algemeen ook terug te zien in veranderingen in plasma waarden. Daarnaast konden de transcript niveaus van de meeste antistollingsgenen, maar niet de prostollingsgenen, snel worden gemoduleerd wat suggereert dat schilklierhormoon zowel snelle als late effecten op expressie van stollingsgenen kan hebben.

Factor V heeft een tweezijdige rol in stolling aangezien het zowel als prostollings- als antistollingsfactor kan dienen. **Hoofdstuk 9** beschrijft de ontwikkeliing van de zogenaamde factor V Leiden hemizygoot muis, waarin het factor V Leiden gerelateerde tromboserisico en de factor V deficiëntie gerelateerde bloedingsneiging wordt gecombineerd. In muizen resulteert dit in een gedeeltelijke lethaliteit in de vroege postnatale periode wat samengaat met tekenen van macrovasculaire trombose, wat suggereert dat het verlies van de antistollende functie van factor V de lethaliteit veroorzaakt. Omdat factor V Leiden homozygotie niet lethaal is, impliceren deze data bovendien dat het factor V Leiden allel een kritisch deel van de antistollende functie heeft behouden en dat dit voldoende is om het gedeeltelijk lethale fenotype van de factor V Leiden hemizygoot muizen te redden.

Hoofdstuk 10 bediscussieert de studies die beschreven zijn in dit proefschrift en plaatst deze in een breder perspectief met betrekking tot de huidige literatuur. Ondanks dat we van mening zijn dat onze studies bijdragen aan een beter begrip van de mechanismen die ten grondslag liggen aan hoe bepaalde verworven factoren het tromboserisico kunnen beïnvloeden, zijn we er ook van overtuigd dat meer onderzoek noodzakelijk is. Daarom richt het laatste deel van de algemene discussie zich op de toekomst en beschrijft hoe enkele nieuwe technieken van grote waarde kunnen zijn in tromboseonderzoek.

Acknowledgements

Promoveren doe je uiteraard niet alleen en daarom zou ik graag van de gelegenheid gebruik maken om een aantal mensen te bedanken, zonder wiens hulp dit proefschrift nooit geschreven had kunnen worden.

Allereerst wil ik graag al mijn (oud)collega's van het Einthoven lab bedanken voor alle hulp en de gezelligheid op het lab en tijdens de pauzes, maar een aantal zou ik op deze manier ook graag nog even persoonlijk bedanken. Shirley, Yaël en Vic: uiteindelijk heeft jullie gezelligheid er mede voor gezorgd dat ik ben blijven 'plakken' als OIO en de gezamenlijke tripjes (al dan niet wetenschap-gerelateerd) zal ik zeker niet gauw vergeten!
Lisa en Yascha, we hebben uiteindelijk bijna 4 jaar in hetzelfde schuitje gezeten en we hebben allemaal wel zo onze ups and downs gehad. Heel erg bedankt dat jullie er altijd voor me waren wanneer het allemaal even niet zo mee zat. Behalve collega's zijn jullie ook hele dierbare vrienden geworden en ook onze weekendjes weg waren altijd weer een feestje, net zoals de borrels op vrijdagmiddag. Ik hoop ook zeker dat we nog een keer met z'n allen naar de Surinamer gaan voor een borrelgarnituur en een glaasje Djogo.
Lieve Irma en Richard: wat heb ik een hoop van jullie geleerd! Niet alleen op het gebied van praktische labvaardigheden (na 2 epi-stages was het benchwerk toch echt even wennen), maar ook van de wijze woorden tijdens de lunchpauzes. Jullie onvoorwaardelijk luisterend oor heeft er zeker voor gezorgd dat dit proefschrift er uiteindelijk toch gekomen is. Bedankt voor al het geduld dat jullie met me hebben gehad en ik ben heel blij dat jullie mijn paranimfen willen zijn, ik had me geen betere kunnen wensen!

Behalve alle collega's op het lab heb ik ook het genoegen gehad om met andere onderzoeksgroepen te werken, zowel binnen als buiten het LUMC. Gerry en Yvonne, zonder jullie was ik waarschijnlijk nooit in staat geweest om RNA te isoleren of fibrinedeposities op blot te krijgen. Gerry, heel erg bedankt voor deze prettige samenwerking en de zowel wetenschappelijke als minder wetenschappelijke discussies die we hebben gehad (soms goon dinger zjus gèt gemekeleker op z'n Mestreechs). Lieve Yvonne, we zijn elkaar min of meer per toeval tegen gekomen en daar ben ik maar wat blij mee! Hoewel het tegenwoordig niet mee zo nodig is als voorheen om af en toe wat af te reageren, mis ik onze pogingen tot squashen en de House-avonden. Hoop dat je snel een keertje naar Ann Arbor komt om de DVD boxen te bekijken ;-).

Zoals gezegd heb ik ook het voorrecht gehad om met verschillende onderzoeksgroepen buiten het LUMC samen te werken. Allereerst zou ik graag Marc Hoylaerts en Roger Lijnen van de Katholieke Universiteit Leuven willen bedanken voor hun waardevolle bijdrage aan de studies met betrekking tot obesitas. Daarnaast zou ik ook graag Svetlana Tchaikovski, Guido Tans, Jan Rosing, Elisabetta Castoldi, Henri Spronk en Rene van Oerle van de Universiteit Maastricht willen bedanken, niet alleen voor het bijbrengen van de fijne kneepjes van de trombine generatie assay, maar ook voor hun gastvrijheid; ik heb de dagen bij jullie in het lab altijd heel leerzaam en gezellig gevonden.

I would like to thank all members of the Ginsburg lab at the University of Michigan for making me feel so welcome, not only during the 3 months of my PhD project, but also when I came back as someone 'in between graduate student and post-doc' (fortunately for all of you, those days are history, no more complaining about thesis-stuff that needs to be taken care of). It is a privilige to work with all of you! A special thanks to Randy for all your help and enthousiasm for science, it's really contagious. David, thank you so much for having me back after my visit in 2010. I couldn't have wished for a better place to continu my scientific career.

Leef pap en mam, wee had dit oets gedach! Bedaank tot geer destieds de beslissing höb gemaak um naor Leiden te goon, wee wèt boe iech aanders terech waor gekoume! Bedaank tot geer miech altied gesteund höb, iech weet tot iech neet altied de gemekelekste bin (gewees).

Allerliefste Martijn, waarom zou je dingen makkelijk doen als ze ook moeilijk kunnen? Ik denk dat we ondertussen wel hebben bewezen dat zelfs de moeilijkste dingen kunnen lukken, zolang je er maar in gelooft. Ik ben heel erg blij dat je er bent, ook al zit er soms wat afstand tussen.

Curriculum Vitae

Publications

Audrey Cleuren was born on February 15 1984 in Maastricht, the Netherlands. After graduating high school in 2002 at the Visser 't Hooft Lyceum in Leiden, she started the Biomedical Sciences program at the University of Leiden were she received her Bachelor of Science degree in 2005. As part of her Masters program, she joined the Department of Clinical Epidemiology at the Leiden University Medical Center for an internship in which she evaluated the effect of serum ferritin on mortality and morbidity in dialysis patients, under supervision of Diana Grootendorst. Audrey did a second internship at the Department of Thrombosis and Hemostasis of the LUMC, supervised by Bart van Vlijmen, in which she performed the initial studies for what later turned out to be her PhD project.

In August 2007 she received the Master of Science degree cum laude and continued her work at the Department of Thrombosis and Hemostasis in the Einthoven Laboratory for Experimental Vascular Medicine, under the mentorship of Pieter Reitsma and Bart van Vlijmen. She started her PhD project on studying the biological mechanisms underlying acquired risk factors for venous thrombosis, of which the results are presented in this thesis. As part of this project, she collaborated with several national and international research groups, including groups from the University of Maastricht (the Netherlands) and the Katholieke Universiteit Leuven (Belgium), and she worked at the University of Michigan (USA) for 3 months.

Since October 2011, Audrey is a member of the laboratory of David Ginsburg at the Life Sciences Institute of the University of Michigan, were she continues her work in the field of thrombosis and hemostasis.

Publications

Cleuren AC, van Oerle R, Reitsma PH, Spronk HM, van Vlijmen BJ. Long-term estrogen treatment of mice with a prothrombotic phenotype induces a sustained increase in thrombin generation without affecting tissue fibrin depositions. *Submitted for publication*

Cleuren AC, Boelen A, Reitsma PH, van Vlijmen BJ. Thyroid hormone modulates murine coagulation through immediate and late effects on hepatic and endothelium-associated coagulation gene transcription. *Submitted for publication*

Safdar H, Cleuren AC, Cheung KL, Gonzalez FJ, Vos HL, Inoue Y, Reitsma PH, van Vlijmen BJ. Regulation of the *F11, Klkb1, Cyp4V3* gene cluster in livers of metabolically challenged mice. *Submitted for publication*

Cleuren AC, Postmus I, Vos HL, Reitsma PH, van Vlijmen BJ. Progestins possess poor anti-estrogenic activity on murine coagulation gene transcription despite evident anti-estrogenic activity on uterine tissue. *Thromb Res. 2011;128:200-201*

Cleuren AC, van der Linden IK, de Visser YP, Wagenaar GT, Reitsma PH, van Vlijmen BJ. 17α-Ethinylestradiol rapidly alters transcript levels of murine coagulation genes via estrogen receptor α. *J Thromb Haemost. 2010;8:1838-1846*

Cleuren AC, van Hoef B, Hoylaerts MF, van Vlijmen BJ, Lijnen HR. Short-term ethinyl estradiol treatment suppresses inferior caval vein thrombosis in obese mice. *Thromb Haemost. 2009;102:993-1000*

Tchaikovski SN, van Vlijmen BJ, Cleuren AC, Thomassen MC, Tchaikovski V, Tans G, Rosing J. Pregnancy-associated changes in the hemostatic system in wild-type and factor V Leiden mice. *J Thromb Haemost. 2009;7:312-318*

Nagai N, Hoylaerts MF, Cleuren AC, van Vlijmen BJ, Lijnen HR. Obesity promotes injury induced femoral artery thrombosis in mice. *Thromb Res. 2008;122:549-555*

Nagai N, Lijnen HR, Cleuren AC, Rosendaal FR, van Hoef B, Hoylaerts MF, van Viljmen BJ. Factor V Leiden mutation is associated with enhanced arterial thrombotic tendency in lean but not in obese mice. *Thromb Haemost. 2007;98:858-863*

Cleuren AC, van Vlijmen BJ, Reitsma PH. Transgenic mouse models of venous thrombosis: fulfilling the expectations? *Semin Thromb Hemost. 2007;33:610-616*