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.

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