Understanding hypercoagulability: determinants and impact on cardiometabolic disease

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Chapter 9

Summary and general discussion

This thesis is aimed to better understand the mechanisms that lead to hypercoagulability and its role in cardiometabolic disease. **Part I** explores the genetic architecture of individual coagulation factor levels (FVIII, FIX, FXI, and fibrinogen), thrombin generation potential, and venous thromboembolism (VTE). **Part II** examines the complex interplay between individual coagulation factor levels, thrombin generation potential, endothelial function, and high-density lipoprotein (HDL) characteristics, as well as the impact of these coagulation parameters on the risk of type 2 diabetes. This discussion chapter summarizes the main findings and addresses implications and future perspectives.

Summary of main findings

Part I of this thesis starts with **Chapter 2,** which provides an overview of genetic variants that increase VTE risk. Since the 1960s, when the first genetic risk factor for VTE was identified (1), a large number of genetic risk factors has been found through various approaches. These genetic findings have contributed to an improved understanding of the genetic susceptibility to VTE. Also, adding genetic findings to clinical prediction models has improved the ability to identify individuals at a high risk of VTE. However, questions remain unanswered, e.g., whether the added value of genetic findings in the prediction model is clinically relevant, whether this added value outweighs the additional burden and cost from genetic testing, and which target population could benefit from genetic prediction models. Furthermore, the underlying mechanism by which these genetic factors increase the risk of VTE often remains unclear. To provide insights into the mechanism, the study presented in **Chapter 3** investigated the association between 61 recently identified VTE-associated genetic variants, individual coagulation factor levels, and thrombin generation potential. We observed that 31 genetic variants are associated with at least one of these coagulation parameters, of which four variants showed robust associations after multiple testing corrections. As individual coagulation factors and thrombin generation potential we focused on are well-established contributors to VTE risk, our findings provide insight into how these genetic factors influence VTE risk by disrupting the coagulation cascade. **Chapters 4** and **5** were aimed to evaluate the genetic architecture of individual coagulation factor levels and thrombin generation potential through a meta-analysis of genome-wide association studies (GWAS). In **Chapter 4**, we performed the first GWAS for FIX activity and identified 10 genetic loci associated with FIX activity. Notably, a subset of these loci has been associated with glucose levels and liver enzymes, implying a relationship between FIX activity and metabolism. Therefore, we further examined the functional role of specific genetic variants in metabolic and hemostatic phenotypes. We demonstrated genetic associations between FIX activity levels and hemostatic phenotypes, including activated partial thromboplastin time, FVIII, FXI, and FXII activity. Additionally, we identified associations with metabolic phenotypes, such as triglycerides, gamma-glutamyl transferase, and low-density lipoprotein cholesterol levels. Lastly, we provided insight into the causal role of FIX activity in the risk of VTE, stroke, and peripheral artery disease using Mendelian randomization analysis. In **Chapter 5**, we explored genetic determinants of the thrombin generation potential. We performed a meta-analysis of GWASs for thrombin generation potential measured at low or high tissue factor concentrations. Discovery analyses identified several hundred genetic variants and several dozens of these were replicated in independent cohorts. We further found functional impacts of identified genetic variants by integrating transcriptomics, proteomics, and coagulation factor activity data. We also highlighted that thrombin generation potential is associated with atherosclerosis, lipid metabolism via phenome-wide association study, and clinical outcomes (i.e., atherosclerosis and type 2 diabetes) via Mendelian randomization

analyses. Our study contributes to a better understanding of the genetic landscape of hypercoagulability and its relationships with downstream phenotypes and clinical outcomes.

In **Part II**, we shift our focus towards pathophysiological mechanism leading to hypercoagulability, particularly through microvascular health and HDL particles, and the effect of hypercoagulability on the risk of type 2 diabetes. In **Chapter 6**, we aimed to elucidate the effect of microvascular health on individual coagulation factor levels and thrombin generation potential. Endothelial glycocalyx, a thin layer covering the inner surface of blood vessels, plays an important role in maintaining vascular homeostasis. Its perturbation implies microvascular dysfunction, which is seen in procoagulant and proinflammatory conditions (2-8). Sidestream dark-filed imaging is used to assess the condition of endothelial glycocalyx with several markers including perfused boundary region markers that measure the extent to which red blood cells penetrate the glycocalyx. A larger perfused boundary region marker indicates a thinner or more degraded glycocalyx. We examined the association between these markers and individual coagulation factor levels as well as thrombin generation potential. We observed that perfused boundary region markers were positively associated with fibrinogen levels in the total population. We further demonstrated a sex-specific positive association between perfused boundary region and FIX, FVIII, and fibrinogen in women but not men. In **Chapter 7**, we examined the anti-thrombotic effect of various HDL particles. Although HDL particles vary in composition and size, previous research mainly focused on HDL-cholesterol levels showing inconsistent effects on VTE risk (9-13), which suggests that mere HDL-cholesterol levels might not be a proper marker for studying the anti-thrombotic effect of HDL particles. Investigating whether the composition and size of HDL particles were associated with individual coagulation factor levels and thrombin generation potential, we observed particle size-dependent associations; large HDL particles were consistently and negatively associated with these coagulation parameters, while small HDL particles were consistently and positively associated. We further investigated whether the particle size-dependent associations were mediated by perfused boundary region based on findings in **Chapter 6.** However, this was not supported by the data. In **Chapter 8**, we examined the association between individual coagulation factor levels, thrombin generation potential, and the incidence of type 2 diabetes. Although hypercoagulability has been identified in patients with type 2 diabetes (14-16), only a few studies have investigated the role of coagulation factors in the incidence of type 2 diabetes (17-22). We showed that elevated FIX activity and thrombin generation potential were associated with an increased risk of type 2 diabetes. We further sought to explain the underlying mechanism of these associations via glycoprotein acetylation (GlycA), which is suggested to be a biomarker of inflammation and originates from glycoproteins including many coagulation factors. However, performing mediation analysis, we observed the proportion mediated was marginal, which suggests that the role of GlycA is negligible in the association between coagulation factors and the incidence of type 2 diabetes.

Implications and future perspectives

Is it still worthwhile doing GWAS?

GWAS have contributed to uncovering genetic loci associated with cardiometabolic disease including VTE, as discussed in **Chapter 2**, and biomarkers for hypercoagulability including the thrombin generation potential presented in **Chapter 5**. These findings helped explore a genetic landscape of cardiometabolic disease and hypercoagulability, yet unstudied phenotypes and missing heritability for studied phenotypes need further research. The first

GWAS on FIX activity in **Chapter 4** discovered genetic loci associated with FIX activity that contributed to a better understanding of the genetic architecture of FIX activity.

The Identification of additional genetic variants becomes feasible with increased sample sizes, advanced sequencing technology, and a better reference genome for imputation. For example, while the previous GWAS for thrombin generation potential included 1,967 participants and identified one locus associated with thrombin generation potential (23), the meta-analysis of GWASs presented in **Chapter 5** included 19,159 participants and identified multiple novel loci. Furthermore, using whole-genome sequencing data and Trans-Omics for Precision Medicine (TOPMED) as a reference panel for imputation with an increased coverage of genome compared with previous GWASs, recent GWAS for FVIII activity identified additional novel genetic loci associated with FVIII activity (24). This progress enhances our understanding of the genetic architecture underlying hypercoagulability and cardiometabolic disease.

It has been argued that understanding the downstream function of genetic variants, which are replicated in independent populations but mechanistically underexplored, should come first to add value to clinical practice rather than identifying additional novel genetic signals. Many genetic variants identified so far have not been mechanistically determined for several reasons, which hinders the further application of genetic findings in clinics. First, it is often uncertain whether the identified genetic variants are causal or only associated with the causal variants due to linkage disequilibrium. Also, over 90% of identified genetic variants are located in non-coding regions (25), which makes it difficult to identify target genes and understand the function of genetic variants. Furthermore, the pleiotropic effects of genetic variants hinder the clear characterization of genetic function with target phenotypes.

Multiple approaches have been suggested to characterize the functionality of identified genetic loci, including using public repositories and performing experiments, as reviewed in previous literature (26, 27). A Bayesian approach can be employed to prioritize causal variants among identified genetic signals by calculating the probability of each being causal. Furthermore, candidate genes can be prioritized by assessing whether the variant influences gene expression (expression quantitative loci, eQTL), protein levels (protein QTL), or protein function by disturbing protein-coding sequences. Overlap with transcription factor binding sites or histone markers can also indicate that a variant may act as a regulatory element, such as a promotor, enhancer, or splicing site. Public functional data repositories developed by large-scale projects (e.g., GTEx, ENCODE, and Roadmap Epigenomics), facilitate the annotation of genetic functions (28-30). However, as these datasets are primarily derived from cells or tissues in a stable state, further molecular assays are needed to investigate genetic function in specific cell types and under cellular conditions where genetic variants function (31, 32). Lastly, evaluating whether the changes caused by genetic variants have clinically relevant impacts is crucial. *In vivo* or *in vitro* experiments using knock-out or knock-down models can be useful to estimate the biological impact of genetic variants.

GWAS remains worthwhile for advancing our understanding of the genetic architecture of diseases and phenotypes, especially with the integration of advanced technologies such as whole-genome sequencing. The combination of these technologies and large-scale international collaboration can help identify novel genetic loci that could lead to better insights into disease mechanisms. However, it is also important to understand the functional roles of these identified genetic variants to ensure their clinical applicability. By conducting functional validation and molecular experiments, we can bridge the gap between genetic discovery and its clinical implementation. Therefore, while GWAS provides novel insights

into the genetics of cardiometabolic disease, an effort to understand the causal mechanisms of genetic variants is crucial to make these findings more worthwhile in clinical practice.

How can we use genetic findings in clinical practice?

Genetic findings have been integrated into clinical practice in various ways. One application is in risk prediction, where genetic findings are used to identify high-risk individuals, even without definitive evidence of causality for the genetic effects. Furthermore, genetic variants with established causal effects have contributed to the development of therapeutic strategies for cardiometabolic disease. These findings also enhance our understanding of disease pathogenesis, enabling the identification of novel drug targets involved in the process of genetic variants influencing disease or physiological conditions. However, translating genetic findings into clinical practice remains challenging. Below, we discuss approaches for translating genetic findings into clinical practice, their potential benefits and limitations.

First, genetic findings are used to estimate individual genetic risk for disorders or pathological conditions, which can then enhance preventive management strategies in clinical practice. This approach may be particularly beneficial for individuals at high risk of disease as prophylaxis after early screening may help prevent the disease and its subsequent complications, as discussed in **Chapter 2**. However, the clinical relevance of the genetic prediction model remains uncertain due to the lack of impact studies demonstrating their effectiveness in diagnoses and prediction of cardiometabolic disease. Also, the feasibility of its implementation is questionable as sequencing needs additional time, labor, and expenses. Furthermore, the generalizability of genetic prediction models to diverse populations is also limited. For instance, a genetic risk score based on five well-known genetic variants for VTE effectively identifies high-risk individuals with European ancestry (33), but is less accurate for those with African ancestry (34). These discrepancies arise from differences in genetic architecture between populations, underscoring the importance of research on population-specific genetics, especially underrepresented populations. Further research that involves underrepresented populations could lead to the development of clinically relevant risk prediction models tailored to specific populations, eventually contributing to the mitigation of clinical biases (35). As discussed in **Chapter 2** and recent literature (36), further research is needed to determine the clinical relevance of genetic risk predictions, including when, for whom, for which condition, and for what purpose the genetic prediction provides added value in clinical practice.

Second, genetic findings can help drug development by identifying drug targets, evaluating drug responses, repurposing existing drugs for other disorders, and providing evidence for clinical trials. Several drugs for cardiometabolic disease have already been developed using this approach. For example, the finding that *PCSK9* variants are associated with low-density lipoprotein cholesterol levels and the risk of cardiovascular disease (37, 38) has led to the development of lipid-lowering drugs, which function as PCSK9 inhibitors (39-42). Also, *VKORC1* and *CYP2C9* variants, which are associated with drug responses and metabolism, have been studied to guide the determination of anticoagulant dosage to prevent side effects (43-46). Furthermore, genetic findings have been used to identify opportunities for drug repurposing, as demonstrated by Finan et al., who showed a substantial number of drugs could potentially target cardiometabolic diseases for which they were not originally targeted (47). Additionally, the possibility of successful drug development increases when supported by genetic evidence (48) while clinical trials for drug targets lacking genetic evidence have a higher chance of early termination than those with genetic evidence (49). However, as previously discussed, many genetic variants remain uncharacterized regarding causality,

target genes, and function, which limits the application of genetic findings in drug development. Moreover, even when a target gene is identified, it encodes a protein that may not always be suitable for drug development. Out of 20,300 protein-coding genes, 4,479 (22%) either serve as a drug target or have the potential for drug development (47), which suggests the pool of druggable targets is limited. Despite promising opportunities in drug development with genetic findings, further research is required to improve the process of drug development based on the genetic evidence.

Lastly, we can use genetic findings to study genetic correlations or causal relationships between traits, which contributes to the identification of similarities in genetic architecture between complex traits and a better understanding of biological mechanisms (50). For example, in **Chapter 4,** we investigated genetic correlations between FIX activity and multiple hemostatic and metabolic phenotypes, showing FIX activity was genetically associated with FXI, FVIII, and activated partial thromboplastin time of hemostatic factors, and gamma-glutamyl transferase, low-density lipoprotein cholesterol, triglyceride levels of metabolic factors. However, as the genetic correlation does not imply causality, additional investigations are needed to establish causal relationships. Mendelian randomization, which uses genetic variants as instrumental variables, provides insights into the causal relationships between exposures and outcomes. This method minimizes the possibility of reverse causation and residual confounding in observational studies. In **Chapter 4**, we also performed Mendelian randomization analyses to infer the causal relationships between genetically correlated traits. Our findings demonstrated that genetically influenced FIX activity was not associated with any of the investigated metabolic phenotypes, whereas genetically influenced BMI, GGT, and triglyceride levels were associated with FIX activity, which suggests the importance of further investigation into complex mechanisms between metabolism and coagulation system. However, Mendelian randomization is valid only under stringent assumptions, including the requirement that variants used as instrumental variables have strong associations with the phenotype. Also, it estimates the impacts of genetically predicted exposures on outcomes, reflecting cumulative effects over a lifetime. Because it does not consider environmental changes in a lifetime, the genetically predicted effects might be inconsistent with the actual effects, or less meaningful when disease or pathophysiological conditions occur only in specific situations. Therefore, conducting genetic correlation and Mendelian randomization analyses requires appropriate implementation and careful interpretation to derive clinically relevant insights from genetic findings.

How can we further understand the mechanisms leading to hypercoagulability and its role in cardiometabolic health?

Hypercoagulability results from complex mechanisms. From the basic perspective, multiple coagulation factors are involved in the coagulation system. These factors work intricately together to regulate each other and maintain homeostasis. In addition, these coagulation factors interact with various other factors, including anticoagulants that inhibit coagulation factors (51, 52), endothelial cells that initiate the coagulation process (53), and platelets that enhance the coagulation process (54). For example, endothelial cells play a crucial role in producing and activating coagulation factors, and the degradation of endothelial glycocalyx contributes to endothelial dysfunction, which is an early marker of microvascular disorders (2-8). Studying the association between glycocalyx perturbation markers and coagulation parameters in **Chapter 6**, we observed that glycocalyx perturbation markers were associated with procoagulants and the associations were more prominent in women than men. These

findings suggest that monitoring the markers of early microvascular disturbance may eventually help prevent hypercoagulability-related cardiometabolic disorders, particularly in women. Furthermore, the complexity increases when we consider the process of production of these factors, transport to the sites where they are active, and clearance when they malfunction or are no longer needed. Thus, while this thesis focused on coagulation factors to understand hypercoagulability, further research is required to investigate all related factors and to gain a deeper understanding of the mechanisms leading to hypercoagulability.

Investigation of the complex relationships between all hypercoagulability-related factors will further contribute to understanding and eventually preventing hypercoagulability and cardiometabolic disease. Components involved in production, transport, activation, and clearance of coagulation factors are often related to cardiometabolic health. For instance, HDL particles play a role in anti-inflammation, cholesterol efflux, anti-oxidation, and anti-thrombotic processes, with their functionality in each process varying based on the composition and size of HDL. The study presented in **Chapter 7** demonstrated size-dependent associations between HDL particles and coagulation parameters. Therefore, characterization of these distinct associations may provide opportunities to control hypercoagulability and develop preventive strategies for cardiometabolic disease. To effectively apply this knowledge in preventing cardiometabolic disease, it is also crucial to identify specific hypercoagulability-related disorders and the role of hypercoagulability in the disorders. Although hypercoagulability is observed in patients with cardiometabolic disease (20, 55-61), it remains uncertain which disorders are especially associated with hypercoagulability and whether hypercoagulability is a cause or a consequence of the disease. For instance, **Chapter 8** showed positive associations between coagulation parameters and the incidence of type 2 diabetes. In **Chapter 4** and **5**, we provided evidence for the causal effects of hypercoagulability on VTE, ischemic stroke, peripheral artery disease, and type 2 diabetes using Mendelian randomization analyses. Although observational studies and Mendelian randomization analyses have certain limitations in establishing definitive causality, as discussed earlier, our findings provide evidence for the causal effects of hypercoagulability on specific cardiometabolic diseases. Further investigation is required to validate these findings and clarify the underlying mechanisms of these diseases, ultimately helping identify potential therapeutic targets.

What are the remaining methodological challenges and opportunities in understanding the complex mechanism of hypercoagulability?

Challenges in understanding the complex mechanism of hypercoagulability include the fact that the associated factors are related reciprocally, rather than in one direction. This highlights the importance of studying the process over time, rather than relying on snapshots to understand the mechanism leading to hypercoagulability comprehensively. The associations observed in this thesis are based on cross-sectional data, which limits the ability to address causality. This limitation warrants further longitudinal data with biological factors measured at multiple time points. Nevertheless, collecting these biological factors during follow-up is challenging (62). Unlike medical history, which can be extracted from the healthcare system without direct involvement from participants, these factors are measured from blood or tissue samples requiring participants to visit the research center physically. Therefore, the chance of participants dropout and missing samples increases with the duration of the data collection period. Additionally, maintaining accuracy and consistency in measurements over time can be difficult, and the process of sample collection, storage,

and data generation increases cost and labor burden. These challenges in regularly collecting biological markers hinder understanding the complex relationships between the factors, the mechanism leading to hypercoagulability, and its role in cardiometabolic health. Further research is needed to develop new methods to collect and manage samples, which are convenient and cost-effective while still maintaining comparability with the accuracy and representativeness of measurements obtained through current methods.

In the Understanding Society Innovation Panel, the feasibility of using dried blood spot cards was studied (63). Blood samples were collected in various settings, including collection by trained nurses using venepuncture and dried blood spot cards, and self-collection by participants using the cards. Although the response rate of self-collection was lower than that of nurse collection, the measurements obtained through the card showed good quality and similar ability to stratify patients as with the measurements collected via venepuncture. However, as only a few markers have been evaluated, further research is needed to implement this method in hypercoagulability and cardiometabolic health research. Another potential approach to study the mechanism leading to hypercoagulability in a less pervasive but more convenient way is using gut microbiome data. Unlike blood samples, participants can collect samples themselves at home and send them to researchers, alleviating the burden of in-person visits. Only limited studies on the role of gut microbiome in hypercoagulability have been studied (64, 65), so additional research is necessary to identify microbiome related to hypercoagulability and cardiometabolic health. Despite the potential of these methods to reduce the burden of in-person visits and the need for trained researchers to collect the samples, several challenges abovementioned remained. Further research is needed to address these challenges and to optimize methods to study the complex relationships between factors and thus understand the mechanism underlying hypercoagulability and cardiometabolic disease.

Conclusion

The elucidation of the underlying mechanisms that lead to hypercoagulability ultimately aims to contribute to developing better preventive and treatment strategies for related disorders, such as cardiometabolic disease. The current thesis identified genetic and pathophysiological markers, including endothelial function and HDL particles, associated with coagulation parameters and the role of coagulation parameters in type 2 diabetes. Further investigation is required to understand the biological mechanism underlying these associations and to explore additional markers and their roles in hypercoagulability and cardiometabolic disease.

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