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Thromboinflammation in high-risk human populations

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CHAPTER

1

General introduction and outline of the thesis

1. Endothelium: a key regulator in inflammation and Coagulation

The biological systems of coagulation and inflammation are intricately intertwined and delicately balanced, with a great deal of crosstalk between each other. Whereby inflammation not only leads to coagulation activation, but coagulation also considerably affects inflammatory activity. When any one component is out of balance, the entire balance is thrown off, resulting in a wide range of disorders with varying degrees of excess inflammation and thrombosis.

The endothelium is the fundamental regulator in both systems. Quiescent endothelial cells reveal an anti-coagulant surface phenotype by repressing tissue factor (TF) expression and releasing anti-thrombogenic factors (e.g, TF pathway inhibitor). Endothelial cells become activated and rapidly shift in response to inflammation in either acute illnesses such as sepsis and COVID-19 or chronic inflammatory states such as diabetes and obesity, disrupting the hemostatic balance which could lead to a procoagulant state [1]. TF plays a critical role in procoagulant phenotype switching and endothelial dysfunction. In experimental *in vitro* situations, a wide range of inflammatory cytokines, including tumor necrosis factor (TNF)- α , IL-1, and IFN-I beta, together with C-reactive protein (CRP) and lipopolysaccharides (LPS) have been shown to increase TF expression in endothelial cells [2-5]. Such an increase in TF initiates the extrinsic coagulation pathway which allows complex forming with circulating factor VIIa, enhancing the catalytic activity of the latter and triggering coagulation by activating coagulation factor X [6, 7] and participating in the prothrombinase complex (FVa: FXa). The prothrombinase complex (FVa: FXa) activates prothrombin (PT) to thrombin. Once the generated thrombin concentration exceeds a threshold beyond physiological conditions, it leads to a pro-inflammatory state and triggers a wide spectrum of endothelial responses [8]. This includes the induction of adhesion molecules that facilitate leukocyte binding and transmigration, such as E-selectin, P-selectin, intracellular cell adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1), as well as the disruption of the endothelial barrier function, the release of proinflammatory cytokines and complement activation [9-13].

Additionally, previous studies showed that the TF-FVIIa complex could stimulate proteinase-activated receptor (PAR) signaling, which induces the release of inflammatory cytokines and chemokines [14].

Endothelial cells of all blood vessels are covered with an endothelial glycocalyx, a tight matrix of glycosaminoglycans, such as heparan sulfate (HS) and chondroitin sulfate (CS) anchored in the cell membrane by a protein backbone, together as proteoglycans, and intertwined with hyaluronan (HA), a glycosaminoglycan not covalently linked [15, 16]. The endothelial glycocalyx together with associated proteins, such as lipase, growth factors, and chemokines, forming a bioactive surface layer [17], and plays a critical role in maintaining vascular integrity and homeostasis, regulating endothelial mechanotransduction, vascular permeability, coagulation, and inflammation [18, 19].

Under physiological conditions, the endothelial glycocalyx composition inhibits blood coagulation. For example, 3-O sulfate modification of HS inhibits Factor Xa activity, and further blocks thrombin generation [20]; besides, other components such as syndecan-1 and hyaluronic acid also interfere with clot formation (inhibits both intrinsic and extrinsic pathway) and affect fibrin polymerization [21]. Tissue factor pathway inhibitor (TFPI) in turn, binds to endothelial cells via HS and as such prevents initiation of coagulation by blocking the actions of the FVIIa-TF complex. HS binding and sequestering protein anti-thrombin III (ATIII) to the cell surface is capable of inhibiting thrombin activity produced by the coagulation cascade, whereas thrombomodulin (TM), also bound to HS binds thrombin, inhibiting fibrin generation. Subsequently, thrombin-TM complexes activate protein C, and activated protein C (APC) inactivates coagulation factors Va and VIIIa, thereby inhibiting further thrombin generation [22]. Intracellular Weibel-Palade bodies (WPB) in endothelial cells store von Willebrand factor (vWF), a protein enhancing the interaction with platelets. HS chains are reported to act as a relevant binding factor for vWF fibers at the endothelial cell surface [23]. In addition to its anti-coagulation function, the endothelial glycocalyx is involved in inflammation. As HS compositional changes can induce binding and signaling in inflammatory conditions, numerous studies have shown

that HS, via its protein-binding properties, regulates inflammatory responses [24-27]. For example, CCL2 is a well-known proinflammatory chemokine, playing a critical role in macrophage recruitment and polarization during inflammation [28]. HS, particularly 3-O-sulfated HS domains, can interact with C-C Motif Chemokine Ligand 2 (CCL2), which further regulates vascular integrity and homeostasis through the CCL2/CCR2 signaling pathway [29, 30]. Also, N- and 6-O-sulfated HS domains were remarkably increased on glomerular endothelium under inflammatory conditions, enhancing leukocyte adhesion *in vitro* [31]. Finally, loss of HA could lead to impaired microvascular perfusion and endothelial stability via disturbed angiopoietin1- TEK receptor tyrosine kinase (TIE2) signaling, stabilizing VE-cadherin cell-cell interactions [32].

Impairment of the endothelial glycocalyx during various pathological conditions, therefore, can result in the initiation of both the inflammation and coagulation system which results in thromboinflammation. Thromboinflammation, the activating interplay of thrombosis and inflammation not only drives cardiovascular disease but also induces acute severe illness, such as sepsis, acute respiratory distress syndrome (ARDS), and disseminated intravascular coagulation (DIC). In the current thesis, I specifically will discuss the role of thromboinflammation in the participants of the Netherlands Epidemiology of Obesity (NEO) study and high-risk patient populations during the COVID-19 pandemic and South-Asian Surinamese participants with type 2 diabetes mellitus (T2DM).

2. Thromboinflammation and gender

Accumulating evidence shows that sex-specific pathophysiological mechanisms play a growing role in the development of cardiovascular disease (CVD). Thromboinflammation, as one of the risk factors for CVD, also exhibits certain sex differences.

C-reactive protein (CRP), the most studied inflammatory marker, is primarily released from the liver following cytokine stimulation [33]. CRP has been shown to predict cardiovascular events in both men and women independently [34, 35]. However, various groups have reported remarkable sex differences in CRP, in which the CRP concentration in women is much higher than in men [36-42]. The well-documented sex differences in body fat distribution and sex hormone concentrations might be key determinants in sex dimorphism [43, 44]. For instance, CRP concentration was found to be higher in premenopausal women than in men, owing to the increasing effect of estrogens [36]. Meanwhile, the use of oral contraceptive drugs and postmenopausal hormone replacement therapy was also able to raise CRP levels [45, 46]. CRP is tightly correlated with both subcutaneous and visceral adipose tissue. Postmenopausal and perimenopausal women have more ectopic fat accumulation (i.e., visceral adipose tissue) than premenopausal women, and women have more subcutaneous fat than men, which contributes to higher CRP concentrations in women [47].

Women not only appear to have higher plasma levels of inflammatory factors, but they also have higher fibrinogen plasma levels than men of the same age and ethnic group [48], pushing the balance from fibrinolysis to clotting [49]. Plasma fibrinogen was reported to be associated with morbidity and mortality in coronary heart disease (CHD) patients [50-52] and to the extent of coronary atherosclerosis [53]. Besides, the levels of coagulation factors II, VII, X, IX, XI, and XII are higher in women than in men [54]. These elevated coagulation factors have been associated with an increased risk of CHD in multiple studies [55, 56].

Sex differences in presence of inflammatory- and coagulation factors therefore might lead to differences in microvascular health and contribute to a different clinical presentation and outcome of CVD. In previous decades, men were thought to be more susceptible to coronary heart disease (CHD) than women [57]. However, the risk of CHD in women is frequently underestimated due to the under-recognition of CHD and distinct clinical presentations, which eventually when discovered resulted in a poor prognosis [58].

Therefore, it is important to study the significance of microvascular dysfunction in the pathophysiology of CHD and how this may differ by sex.

3. Thromboinflammation and diabetes

T2DM is characterized by insulin resistance and insufficient compensatory insulin secretion, the mechanism of which varies by ethnicity.

Thromboinflammation is commonly observed in patients with diabetes [59]. A previous epidemiological study in the general population demonstrated that increased levels of fasting glucose, HbA1c, and postprandial glucose response were associated with higher activity of FVIII, FIX, and FXI, and to some extent also with increased concentration of fibrinogen, which provided some evidence between hyperglycemia and coagulation [60]. Another study revealed that platelets of patients with diabetes had an increased capacity of mediating microvascular thrombosis and inflammation during ischemia-reperfusion injury [61].

Several mechanisms might be involved in the diabetes-associated thromboinflammation process. One mechanism is associated with platelet aggregation/activation, such as platelet-derived chemokine C-X-C motif ligand 14 (CXCL14). CXCL14 is one of the potential players in the development of thromboinflammation in diabetes expressing proinflammatory properties through its involvement in thrombus formation, platelet migration, and monocyte migration [62, 63]. Besides, platelets can interact with inflammatory cells by regulating lipids in a paracrine manner [64]. A recent study showed that the platelet atypical chemokine receptor 3 (ACKR3)/ CXC-chemokine receptor 7 (CXCR7) interaction is capable of favoring antiplatelet lipids over an atherothrombotic lipidome and regulating thromboinflammation [65].

Another mechanism at play in diabetes involves the endothelial glycocalyx in platelet adhesion to the endothelium of damaged vessels [66]. Our previous study showed that loss of endothelial hyaluronan, a key component of the extracellular matrix, could lead to disturbed glomerular endothelial stabilization [18]. Besides, the endothelial glycocalyx is perturbed upon treatment with human diabetic serum [67]. The impaired endothelial glycocalyx could lead to increased ICAM1 and decreased eNOS expression [68], which might contribute to platelet activation [69, 70], thus forming a procoagulant cell surface and further promoting thromboinflammation.

Increasing studies investigate the biological functions of high-density lipoproteins (HDL) in pathophysiological conditions including cholesterol efflux mediation, capacity of anti-oxidation, anti-inflammation, and anti-thrombosis [71, 72]. Given the causal relationship between HDL function and diabetes [73-76], it may yet be another novel mechanism causing thromboinflammation in subjects with diabetes. Numerous epidemiological studies have shown that low levels of plasma HDL cholesterol (HDL-C) were associated with an elevated risk of T2DM and cardiovascular disease [77-79]. Also, the function of HDL was associated with incidence of cardiovascular disease and prognosis of heart failure [80-82]. Interestingly, accumulation of symmetric dimethylarginine, a marker associated with diabetes, could lead to HDL dysfunction, switching to an endothelial-damaging phenotype, and then mediating glycocalyx breakdown [83].

4. Thromboinflammation and COVID-19

Severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) infection caused the coronavirus disease 2019 (COVID-19), and its worldwide spreading led to a global pandemic since late 2019 [84]. Although the majority of COVID-19 patients are asymptomatic or only showed mild symptoms [85], it is worth noting that part of the patients suffered from respiratory illness in the early disease stage, and rapidly progressed

into respiratory failure [86, 87]. Additionally, there was an increased incidence of thromboembolism events during hospitalization in the intensive care unit (ICU) [88]. ARDS and coagulopathy are major contributors to the mortality of COVID-19 [89], while endothelial dysfunction is reported to participate in both ARDS and coagulopathy [90, 91]. Severe pulmonary endothelial damage was observed in autopsy of COVID-19 non-survivors [92], therefore, COVID-19 could be regarded as an endothelial disease. Endothelial dysfunction with subsequent COVID-19-related thromboinflammation might play a vital role in the pathogenesis of ARDS and coagulopathy.

Hyperinflammation is involved in the severe illness of COVID-19 resulting in a cytokine storm regarding to increased levels of pro-inflammatory cytokines [85, 93-97]. Although the observed cytokine storm in COVID-19 patients was lower than those with non-COVID-19 severe cases with ARDS, sepsis, and influenza virus infection [98, 99], COVID-19 patients exhibited decreased innate antiviral defenses accompanied by exploded inflammatory cytokine production [100]. NLR family pyrin domain containing 3 (NLRP3) inflammasome was activated by SARS-CoV-2 in the lungs of patients who died from COVID-19-associated ARDS. *In vitro* studies on primary human monocytes revealed that SARS-CoV-2 infection activated the NLRP3 inflammasome [101, 102]. Furthermore, IL-1 and IL-18, products of NLRP3 inflammasome, are elevated in the COVID-19 patients with critical illness and are associated with poor clinical outcomes [102].

In addition to the hyperinflammation, SARS-CoV-2 infection could also lead to a prothrombotic state. Patients with severe COVID-19 were associated with endogenous activation of coagulation and fibrinolysis; but different from sepsis-induced hypercoagulant status, patients with COVID-19 showed a loss of coagulation-initiating mechanisms [103]. Moreover, emerging evidence demonstrated that the complement system had a role in the maladaptive immune response that promotes hyperinflammation and thrombotic microangiopathy, increasing COVID-19 mortality [104]. SARS-CoV-2 was found to trigger complement-mediated endothelial damage, cause deregulations of the coagulation cascade, and further result in adverse clinical presentations [105-107]. Despite

the indirect effect of the complement activation, COVID-19-associated coagulopathy is also suggested to be caused by endothelial dysfunction. Endothelial activation markers such as von Willebrand factor (vWF) and Angiopoietin 2 (ANG2) were elevated in patients with SARS-CoV-2 infection and associated with disease severity [108, 109]. In COVID-19 patients, elevated circulating ANG2 levels have been linked to decreased respiratory function, hypercoagulable status, acute kidney injury, and increased mortality [109-112]. ANG2 also was shown to mediate the endothelial glycocalyx breakdown [113], and the MYSTIC study revealed that glycocalyx health, as measured by perfused boundary region (PBR), an inversed reflection of endothelial glycocalyx layer, was a prognostic predictor for COVID-19 and disease severity [109].

Early studies of lipid metabolism in patients revealed a role for high-density lipoproteins (HDL) protective factors in a variety of endothelial functions such as antioxidant, anti-inflammatory, anti-thrombotic, and even anti-infectious properties [114, 115]. Thromboinflammation in COVID-19 might be also induced by HDL dysregulation. Recently, it was observed that the metabolic lipid profile in COVID-19 patients in the ICU was different when compared to healthy controls or patients with cardiogenic shock in ICU [116]. It is worth noting that increasing evidence suggested that low serum HDL-cholesterol (HDL-C) levels at hospital admission are associated with disease severity and mortality in COVID-19 [117, 118]. However, other studies revealed that the HDL lipidome and proteome rather than quantitative HDL-C concentration play a more representative role in HDL function during disease [80, 119]. In addition to HDL-C concentrations in COVID-19, several studies showed significant inflammatory remodeling of the HDL proteome, associated with COVID-19 disease severity in both adult and pediatric COVID-19 patients [119-121].

Outline of this thesis

In this thesis, we address the role of thromboinflammation in different high-risk populations, such as women versus men in the general Dutch population, type 2 diabetes mellitus, and COVID-19 patients.

Recent research reveals that microvascular dysfunction more commonly in women is a growing determinant of sex difference in coronary heart disease. In **Chapter 2**, we examined the sex differences in the relationship between microvascular health and coagulation parameters in a middle-aged Dutch population and revealed a hitherto unreported sex-specific association between microcirculatory health and procoagulant status, which suggests considering microvascular health in the early development of coronary heart disease in women.

HDL particles exhibit large heterogeneity in size, density, and composition. The composition of HDL can partly affect various functions including mediating cholesterol efflux, anti-oxidation, anti-inflammation, and anti-thrombotic processes, which is becoming an important determinant in the development of microvascular complications in T2DM. In **Chapter 3**, we used ^1H nuclear magnetic resonance (NMR) spectroscopy and Bruker IVDr Lipoprotein Subclass Analysis (B.I.LISATM) software to determine the changes in plasma HDL (both particle size and lipid composition) in healthy individuals (Dutch white Caucasian [DwC], Dutch South Asian [DSA]) and individuals with T2DM. We also investigated the role of HDL in anti-thrombotic capacity, determined as the ability to suppress TNF- α induced thrombin generation in endothelial cells *in vitro*.

Lipids play an essential role in both the intrinsic and extrinsic pathways of prothrombin activation and dyslipidemia is one of the hallmarks of T2DM. Oxidized lipids could contribute to thrombus initiation and growth in oxidative stress-induced cardiovascular diseases. Following the previous study, in **Chapter 4**, we used the LC/MS-based LipidalyzerTM platform to measure the same participants mentioned above to study the biological

mechanisms underlying the link between dyslipidemia and T2DM in different ethnic groups.

Evidence of pulmonary microvascular thrombosis and inflammation were found on autopsy of COVID-19 non-survivors, leading to the increasing concern on thromboinflammation in the disease pathogenesis. In **Chapter 5**, we explored how serum factors affect vascular integrity in patients with severe COVID-19 (glycocalyx function, barrier function, and anti-coagulation capacity). Serum from COVID-19 patients in the ICU could induce endothelial dysfunction, characterized by endothelial glycocalyx degradation, endothelial barrier failure, and hypercoagulable status, which could be targeted earlier in the disease by supplementation of heparin sulfate mimetics. As HDL has both anti-inflammatory and anti-thrombotic capacity regarding thromboinflammation, therefore, in **Chapter 6**, we measured blood HDL subclasses and lipid content concentrations in longitudinally collected serum samples from ICU and non-ICU, together with age-matched healthy controls using ^1H NMR spectroscopy and the validated B.I.LISATM software to identify HDL composition concerning disease progression, and endothelial function, and investigate whether specific HDL compositional changes could lead to different outcomes in the course of the disease.

In **Chapter 7**, we summarize and discuss the observations in this thesis, as well as future perspectives.

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