Understanding hypercoagulability: determinants and impact on cardiometabolic disease

Han, J.

Part II.

Pathophysiological mechanisms of hypercoagulability and its link to cardiometabolic disease

Chapter 6

Sex-specific association between microvascular health and coagulation parameters: the Netherlands Epidemiology of Obesity (NEO) Study

Lushun Yuan, Jihee Han, Anouk I.M. van der Velden, Hans Vink, Renée de Mutsert, Frits R. Rosendaal, Astrid van Hylckama Vlieg, Ruifang Li-Gao, Ton.J Rabelink, Bernard M. van den Berg

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Abstract

Microvascular dysfunction is a growing determinant of sex difference in coronary heart disease (CHD). The dysregulation of the coagulation system is involved in CHD pathogenesis and can be induced by endothelial glycocalyx (EG) perturbation. However, little is known about the link between EG function and coagulation parameters in population-based studies on sex specificity. We sought to examine sex differences in the relationship between EG function and coagulation parameters in a middle-aged Dutch population. Using baseline measurements of 771 participants from the Netherlands Epidemiology of Obesity (NEO) study (age: 56 [IQR: 51-61] years, 53% women, BMI: 27.9 [IQR: 25.1-30.9] kg/m^2), associations between glycocalyx-related perfused boundary region (PBR) derived from sidestream dark-field imaging and coagulation parameters (factor VIII/IX/XI, thrombin generation parameters, and fibrinogen) were investigated using linear regression analyses, adjusting for possible confounders (including CRP, leptin, and GlycA), followed by sex-stratified analyses. There was a sex difference in the associations between PBR and coagulation parameters. Particularly in women, one SD PBR (both total and feed vessel, indicating poorer glycocalyx status) was associated with higher FIX activity (1.8%, 95% CI: 0.3-3.3, and 2.0%, 95% CI: 0.5-3.4) and plasma fibrinogen levels (5.1 mg/dL, 95% CI: 0.4-9.9, and 5.8 mg/dL, 95% CI: 1.1-10.6). Furthermore, one SD $PBR_{capillary}$ was associated with higher FVIII activity (3.5%, 95% CI: 0.4-6.5) and plasma fibrinogen levels (5.3 mg/dL, 95% CI: 0.6-10.0). We revealed a sex-specific association between microcirculatory health and procoagulant status, which suggests considering microvascular health in the early development of CHD in women.

***Keywords*:** coagulation factors, thrombin generation, fibrinogen, endothelial glycocalyx (EG), perfused boundary region (PBR)

Introduction

In previous decades, men were thought to be more susceptible to coronary heart disease (CHD) than women (1). However, the risk of CHD in women is frequently underestimated due to the under-recognition of CHD and distinct clinical presentations, which resulted in a poor prognosis (2). Nowadays, the systemic non-obstructive microvascular dysfunction, causing problems in small blood vessels feeding the muscular tissue, which can result in CHD, is believed to be more common in women than men. Therefore, this type of dysfunction is becoming a growing determinant of sex difference in CHD patients with normal or near normal coronary angiographic assessment (3, 4).

Previously, we examined endothelial surface perturbation using the non-invasive sidestream dark-field (SDF) imaging technique in a subpopulation of the Netherlands Epidemiology of Obesity (NEO) study (5-7). While SDF imaging provides high contrast images of the sublingual microvasculature allowing to determine lateral red blood cell (RBC) movement within the vessels of interest, with the newly developed software automatic analysis of RBC velocity allows to include flow changes between feed vessels (10-25µm) and capillaries (< 10µm) to be coupled to the perfused boundary region (PBR), vessel density, and capillary blood volume (8, 9). Already in various studies, it was previously shown that the detected changes in PBR, inversely related to the endothelial glycocalyx thickness, correlated with glycocalyx degradation products including circulating Syndecan-1, heparan sulfate (HS), hyaluronan, and soluble thrombomodulin and endothelial activation markers such as E-selection, soluble angiopoietin-2, and soluble Tie-2 (10-13). It was also found to be negatively correlated with coronary flow reserve (CFR), an assessment to evaluate coronary microvascular dysfunction (CMD). Besides, according to miCRovascular rarefaction in vascUlar Cognitive Impairment and heArt faiLure (CRUCIAL) study protocol (14), SDF imaging technique will be used to quantify microvascular health of cerebral and cardiac microvasculature, suggesting that this technique is a valid surrogate for cardiac microvasculature health examination (15).

The endothelial glycocalyx (EG) is a negatively charged gel-like surface matrix of proteoglycans and covalently bound glycosaminoglycans, glycoproteins, and glycolipids (16), and exerts an anti-inflammatory and antithrombotic role by covering various glycoprotein adhesion receptors for leukocytes (17), and platelets (18), or aiding in the sequestration of anti-adhesion factors (19). EG perturbation acts as a representative of impaired microvascular function (6, 9, 20, 21). In particular, several EG-bound proteins including specific sulfation patterns of HS proteoglycans or HS binding proteins such as anti-thrombin III (ATIII) and tissue factor pathway inhibitor (TFPI) could prevent the coagulation cascade *in vitro* and *in vivo* (22-24). In disease cases such as nephrotic syndrome, sepsis, brain injury, and severe COVID-19, derangement of EG induced by microvascular dysfunction could accelerate hypercoagulation (8, 25-28).

Specifically, women were found to have already higher fibrinogen plasma levels than men of the same age and ethnic group (29). Increased fibrinogen levels could push the balance from fibrinolysis to clotting (30) and have been reported as a potential risk factor for CHD (31-34). Furthermore, other coagulation factors were also associated with increased risk of CHD (33, 35). However, little is known about the link between EG function and coagulation parameters in population-based studies as well as whether a sex-specific determinant is present. Based on *in vitro* and *in vivo* evidence, we hypothesized that poorer endothelial glycocalyx function could lead to a procoagulant state in a population-based study, which might demonstrate sex specificity and be involved in the risk of CHD. The aim of our study was therefore to investigate the association between microvascular health, assessed by the new SDF imaging parameters, and levels of procoagulant factor activities (Factor VIII [FVIII], FIX, and FXI), fibrinogen concentration, and thrombin generation parameters in the general Dutch population and to further perform analyses stratified by sex, revealing sex differences in vascular vulnerability and its potential roles in the risk of CHD.

Methods

Study population and study design

This study was performed in a population-based prospective cohort, the Netherlands Epidemiology of Obesity (NEO) study (7). All 6,671 participants gave written informed consent and the Medical Ethical Committee of the Leiden University Medical Center (LUMC) approved the study design. Initiated in 2008, the NEO study was designed to study pathways that lead to obesity-related diseases. Detailed information about the study design and data collection have been described elsewhere (7). Briefly, men and women aged between 45 and 65 years with a self-reported body mass index (BMI) of 27 kg/m^2 or higher living in the greater area of Leiden (in the west of the Netherlands) were eligible to participate in the NEO study. In addition, all inhabitants aged between 45 and 65 years from one municipality (Leiderdorp) were invited irrespective of their BMI, representing the BMI distribution of the Dutch general population. Prior to their visits, participants completed questionnaires at home with demographic, lifestyle, and clinical information. At the baseline visit, fasting blood samples were drawn from the antecubital vein after the NEO participants had rested for 5 minutes. The present study is a cross-sectional analysis using a subpopulation of 918 NEO participants in whom SDF imaging was performed between January and October 2012, as part of the baseline visit at the Leiden University Medical Center NEO study center. Individuals were excluded from the analyses (Figure 1), when: 1) with missing SDF imaging parameters (n = 110); 2) with missing values of confounding factors (n = 8); 3) with current use of anticoagulant therapy (using vitamin K antagonists or heparin) (n = 7); 4) with missing values of coagulation parameters (n = 18); 5) with outliers values (z score > 5) in outcomes (i.e., coagulation factors and thrombin generation parameters) (n = 4), and 771 participants were included for all analyses using coagulation parameters as outcomes.

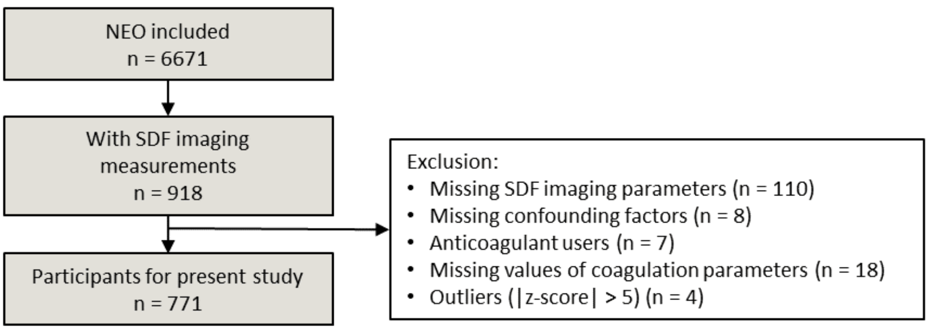

Figure 1. Study flow chart. Abbreviations: NEO, Netherlands Epidemiology of Obesity; SDF, sidestream dark field.

SDF microcirculation imaging

Intravital microscopy was performed earlier on individuals in a supine position using an SDF camera (MicroVision Medical Inc., Wallingford, Pennsylvania) and acquired using Glycocheck software (Microvascular Health Solutions Inc., Salt Lake City, Utah) as described elsewhere (6, 9). The software automatically identifies all available measurable microvessels distributed at a 1μm interval between 4-25μm and RBC velocity was included as a new parameter using the new software. After reanalyzing, the following validated parameters (8) were included in this study, divided into two categories: glycocalyx-related parameters, i.e., total PBR (PBR_{Total}, 4-25μm), PBR feed vessel ($PBR_{feed\ vessel}$, 10-19μm), and PBR capillary ($PBR_{capillary}$, 4-9μm); microcirculatory-perfusion-related parameters, i.e., feed vessel RBC velocity ($RBCV_{feed\ vessel}$), capillary RBC velocity ($RBCV_{capillary}$), total valid vessel density (with measurable RBC velocity; D_{Total}), perfused feed vessel density ($D_{feed\ vessel}$), perfused capillary density ($D_{capillary}$), and capillary blood volume (CBV_{static}). Detailed information about the new software used in the NEO study is described previously (8). PBR_{Total}, $PBR_{feed\ vessel}$, and $PBR_{capillary}$ were expressed in μm. Levels of $RBCV_{feed\ vessel}$ and $RBCV_{capillary}$ were expressed in μm/s. Levels of D_{Total}, $D_{feed\ vessel,}$ and $D_{capillary}$ were expressed in μm /mm^2. CBV_{static} was expressed in pL/mm^2.

Coagulation factor activity

Blood samples for measurements of coagulation factors activity were collected and processed within 4 hours and drawn into tubes containing 0.106M trisodium citrate (Sarstedt, Etten Leur, the Netherlands) without the measurement of residual platelets, as discussed earlier (7). Fasting fibrinogen levels were measured according to the method of Clauss, as described earlier (36). Fasting FVIII, FIX, and FXI activity were measured with a coagulometric clot detection method on an ACL TOP 700 analyzer (Werfen, Barcelona, Spain), and the Werfen plasma was used as reference plasma to calibrate factor measurements (Werfen). Levels of FVIII, FIX, and FXI activity were expressed in percentages (%). Levels of fibrinogen were expressed in mg/dL.

Thrombin generation

All blood samples were collected and processed within 4hrs. Tubes were centrifuged for 10 minutes at 2500g at 18°C and aliquoted plasma was stored at -80°C until further use, only after first time thawing for 3 min 37°C (waterbath), as discussed earlier (7). Thrombin generation was measured according to the protocols earlier described by Hemker *et al.* (37): calibrated automated thrombogram (CAT; Thrombinoscope BV, Maastricht, the Netherlands). Briefly, 20 μL of PPP-Reagent LOW (86194,TS31.00, STAGO, France) and thrombin calibrator (86192, TS30.00, STAGO, France) were dispensed into the wells of a round-bottom 96-well plate (#3655, Thermo Scientific, Uden, the Netherlands). Thermostable inhibitor of contact activation (TICA, PS-0177-oxoxox, Maastricht, the Netherlands) was added to plasma samples from participants in the NEO study and normal pooled plasma (NPP, as an internal control for each plate). Then 80 μL of mixed plasma was added to the plate and the plate was placed in the Fluorometer for a 10-minute, 37°C incubation. Thrombin formation was initiated by adding 20 μL of the fluorogenic substrate with calcium (FluCa-kit, 86197, TS 50.00, STAGO, France). The final reaction volume was 120 μL. Thrombin formation was determined every 10 seconds for 50 minutes and corrected for the calibrator using Thrombinoscope software. Thrombin generation parameters included lag time, time to Peak (ttPeak), peak thrombin generation (Peak), endogenous thrombin potential (ETP), and thrombin generation velocity (VelIndex). Levels of lag time and ttPeak were expressed in minutes (min); levels of Peak height were expressed in nM; levels of ETP were expressed in nM × min; levels of VelIndex were expressed in nM/min.

Serum inflammatory markers

Serum concentrations of C-reactive protein (CRP) were determined using a high sensitivity CRP assay (hs-CRP, TINA-Quant CRP HS system, Roche, Germany and Modular P800, Roche, Germany) (36). Serum leptin concentration was measured using a human leptin competitive RadioImmunoAssay (RIA) (HL-81HK, Merck Millipore, Darmstadt, Germany). The leptin concentration was counted using a gamma counter, as described elsewhere (38). Glycoprotein acetyls (GlycA) concentrations were measured in plasma that had undergone one previous freeze-thaw cycle, using a high-throughput proton nuclear magnetic resonance (NMR) spectroscopy (Nightingale Health Ltd., Helsinki, Finland) (39).

Statistical analyses

Descriptive baseline characteristics of the study population were expressed as median with interquartile range (IQR) for non-normally distributed variables, mean with standard deviation (SD) for normally distributed variables, or percentages (%) for dichotomous variables.

First, the distributions of confounding factors and SDF imaging parameters were evaluated, and z-transformation (i.e., with mean=0 and SD=1) was performed on age, BMI, and SDF imaging parameters. After checking the distributions of outcome variables, thrombin generation velocity (VelIndex) showed a right-skewed distribution and, $\log_2$ transformation was performed on VelIndex. Afterwards, linear regression analysis was used to investigate the associations between SDF imaging parameters (exposures) and pro-coagulation factors (i.e., FVIII, FIX, and FXI), fibrinogen, and thrombin generation parameters (outcomes). The analyses were adjusted for several potential confounding factors. First, crude analyses were performed (model 1). Second, we adjusted the models for the confounding factors age (unit: years, continuous variable) and sex (categories: women and men, dichotomous variable) (model 2). Third, models were adjusted for other confounding factors: BMI (unit: kg/m^2, continuous variable), current smoking status (categories: current users and non-current users, dichotomous variable), menopausal status (categories: premenopausal and postmenopausal, dichotomous variable), current use of the oral contraceptive pill (categories: current users and non-current users, dichotomous variable), and current use of hormone replacement therapy (categories: current users and non-current users, dichotomous variable) (model 3). Fourth, we adjusted for serum C-reactive protein (CRP) (unit: mg/L, continuous variable), serum leptin concentration (unit: µg/L, continuous variable) and glycoprotein acetyls (GlycA, unit: mmol/L, continuous variable) in a separate model as these systemic inflammation markers might be potential confounders of the association between microvascular health and coagulation parameters (model 4). We calculated differences in the mean levels of fibrinogen, FVIII, FIX, FXI, and thrombin generation parameters, with 95% confidence intervals (CI), associated with SDF imaging parameters. Furthermore, the association between SDF imaging parameters and coagulation factor levels was examined through sex-stratified analysis. Mean coagulation factor levels were calculated for sex-stratified SDF imaging parameters. As a sensitivity analysis, all analyses were performed separately in premenopausal and postmenopausal women.

To our knowledge, the present study is the first to investigate the association between the microvascular health index derived from SDF imaging and the levels of coagulation factors as well as the parameters of thrombin generation on a population level; we have no prior information on effect sizes to perform a power calculation. Noteworthy, most of previous studies with relatively small sample sizes have identified associations between the microvascular health index and disease outcomes, e.g., COVID-19 in 38 participants (13), coronary artery disease in 115 participants (40), and sepsis in 51 participants (8). To address the power that we could achieve using 771 individuals, we used the following setting: significant level 0.05, power 80%, with the linear regression PBR_{Total}~ fibrinogen as an example; we may need 394, 54, and 24 samples for a small, medium and large effect size, respectively (41). Therefore, a sample size of 771 individuals may have the statistical power to capture even small effect sizes.

Results

Participant characteristics

Table 1 shows the characteristics of individuals included in the present study. Of 771 included participants, 53% were women, with a median age of 56 years (interquartile range, 51–61 years), and 12.5% were current smokers. Participants had a median BMI of 27.9 kg/m^2 (interquartile range, 25.1-30.9). Among women, 84% had a postmenopausal status, 7.6% used oral contraceptives, and 3.4 % hormone replacement therapy. Apart from this, we observed a higher level of inflammatory markers and procoagulant status. While capillary glycocalyx is very important for proper endothelial function, the PBR dimensions are measured in vessels with diameters ranging from 4 – 25 microns Therefore the contribution of capillary glycocalyx to PBR_{total} is very limited. PBR_{total} is comparable to $PBR_{feed\ vessel}$ and both are the main parameters presenting possible disturbances of the endothelial surface layer, which in women are both significantly increased (median, women vs. men: PBR_{total}, 2.35 vs. 2.3; $PBR_{feed\ vessel}$, 2.31 vs. 2.24). The density of perfused capillaries is higher in women vs. men, as measured by both a higher capillary density ($D_{capillary}$) and higher capillary blood volume (CBV_{static}), which is associated with a lower capillary RBC velocity ($RBCV_{capillary}$), suggests a more advantages capillary presence in women.

Association between microvascular health and levels of coagulation parameters in the total population

Linear regression analyses were used to investigate the association between one standard deviation (SD) difference in microvascular parameters and the coagulation factors. In model 3 of the total population, both glycocalyx-related parameters, PBR_{Total} and $PBR_{feed\ vessel}$ were associated with higher fibrinogen levels (Table S1). Nevertheless, with an additional adjustment for systemic inflammatory markers (model 4), only the association of the $PBR_{feed\ vessel}$ remained (Table S1 and Figure S1) and we observed that one SD (i.e., 0.3µm) in the $PBR_{feed\ vessel}$ was associated with an higher fibrinogen concentration of 3.2 mg/dL (95% CI: 0.02-6.4). Interestingly, the microcirculatory perfusion parameters, in particular all three vessel density parameters, were positively associated with the endogenous thrombin potential (ETP) in all models with very similar effect size estimations between model 3 and model 4 (Table S2 and Figure S2). In model 4 of the total population, one SD in vessel density (D_{Total}, $D_{feed\ vessel}$, $D_{capillary;}$ 65.84, 42.17, 41.39 µm/mm^2, respectively), corresponded to higher ETP levels (31.9 nM × min, 95% CI: 9.6-54.2; 23.7 nM × min, 95% CI: 1.2-46.2; 25.2 nM × min, 95% CI: 2.9-47.5, respectively).

Table 1. Characteristics of the study population

	Total (100%)	Women (53%)	Men (47%)
Demographic factors			
Age, y	56 (51-61)	56 (50.75-61)	57 (51-61)
Body mass index, kg/m^2	27.9 (25.1-30.9)	27.8 (24.5-31.3)	28.0 (25.7-30.6)
Smoking (% current user)	12.5	8.1	17.4
Menopause status (% of post or perimenopausal)	NA	83.8	NA
Medication (current use)			
Oral contraceptive pill (%)	NA	7.6	NA
HRT (%)	NA	3.4	NA
Systemic inflammatory markers			
C-reactive protein, mg/L	1.37 (0.74-3.06)	1.61 (0.8-3.5)	1.25 (0.64-2.26)
Leptin, µg/L	14.7 (8-27.15)	25.2 (16-37)	8.4 (5.55-12.9)
Glycoprotein acetyls, mol/L	1.23 (1.13-1.34)	1.21 (1.11-1.32)	1.24 (1.14-1.35)
Coagulation parameters			
Fibrinogen, mg/dL	289 (258-331)	301 (266-346)	279 (247-313)
Factor VIII (%)	123 (101-147)	123 (102-148)	123 (101-145)
Factor IX (%)	119 (108-134)	119 (107-136)	119 (110-131)
Factor XI (%)	117 (103-129)	120 (107-134)	112 (100-123)
Lag time, min	6.72 (6-7.75)	6.5 (5.75-7.58)	7.08 (6.17-7.92)
Time to peak, min	14.7 (13.4-16.0)	14.3 (13.1-15.7)	15.0 (13.8-16.3)
Peak, nM	83 (62-106)	85 (61-111)	82 (63-102)
ETP, nM·min	1149 (9156-1397)	1175 (917-1440)	1125 (914-1329)
Velocity, nM/min	10.4 (7.5-14.6)	10.8 (7.4-15.3)	10.2 (7.8-13.8)
SDF imaging parameters			
PBR of total vessel, µm	2.33 (2.19-2.5)	2.35 (2.2-2.54)	2.3 (2.17-2.44)
PBR of feed vessel, µm	2.28 (0.3)	2.31 (0.3)	2.25 (0.29)
PBR of capillary, µm	1.2 (1.13-1.27)	1.2 (1.14-1.27)	1.2 (1.13-1.26)
RBC velocity in feed vessel, µm/s	55.8 (43.9-68.9)	51.2 (39.7-64.4)	60.2 (49.5-73.1)
RBC velocity in capillary, µm/s	55.7 (41.9-70.5)	50.7 (37.7-66.8)	60.4 (47.7-72.6)
Total vessel density, µm/mm^2	239 (195-287)	241 (194-293)	239 (195-282)
Feed vessel density, µm/mm^2	132 (107-161)	130 (104-158)	135 (111-165)
Capillary density, µm/mm^2	101 (80-131)	104 (82-137)	97 (75-122)
Capillary blood volume, pL/mm^2	2.52 (1.4-4.35)	2.81 (1.41-4.76)	2.29 (1.38-3.92)

Data are shown as mean (± standard deviation), median (25th percentile–75th percentile) or percentage. Abbreviations: ETP, endogenous thrombin potential; HRT, hormonal replacement therapy; NA, not applicable; PBR, perfused boundary region.

Effect modification by sex of the association between microvascular health and coagulation factors

After sex stratification, we observed differences between men and women between the glycocalyx-related parameters and coagulation factor levels (Figure 2 and Figure 3). Consistent associations were found between PBR_{Total} and $PBR_{feed\ vessel}$ and FIX activity as well as plasma fibrinogen concentration specifically in women, which remained after adjusting for inflammatory markers: one SD in PBR_{Total} or $PBR_{feed\ vessel}$ (0.25 µm and 0.3 µm, respectively) was associated with higher fibrinogen levels (5.1 mg/dL, 95% CI: 0.4-9.9 and 5.8 mg/dL, 95% CI: 1.1-10.6; respectively) and higher FIX activity (1.8%, 95% CI: 0.3-3.3 and 2.0%, 95% CI: 0.5-3.4; respectively) (Figure 2 and Table S3); while no associations were observed in men (Figure 3 and Table S4). For $PBR_{capillary}$, in addition to the association with fibrinogen concentration, $PBR_{capillary}$ was also associated with FVIII activity, which remained after further adjustment for systemic inflammatory markers (model 4): one SD in $PBR_{capillary}$ (0.11 µm) was associated with higher fibrinogen levels (5.3 mg/dL, 95% CI: 0.6-10.0) and FVIII activity (3.5%, 95% CI: 0.4-6.5) (Figure 2 and Table S3).

There were differences in the associations between microcirculatory-perfusion-related parameters and coagulation factor levels stratified by sex. For women, the associations between vessel density and ETP were observed in the crude and age- and sex-adjusted model (Figure S3 and Table S5); however, only $D_{capillary}$ was positively associated with ETP after further adjusting for demographic and lifestyle factors (model 3) and systemic inflammatory markers (model 4), in which one SD of $D_{capillary}$ (42.26 µm/mm^2) was associated with higher levels of ETP (32.0 nM × min, 95% CI: 0.1-63.8). In men, only one consistent negative association was observed between the $RBCV_{feed\ vessel}$ and thrombin generation parameter time to peak (ttPeak) (Figure S4 and Table S6), in which one SD of the $RBCV_{feed\ vessel}$ (61.52 µm/s) was associated with lower levels of ttPeak (-0.2, 95% CI: -0.4- -0.02). In addition, one SD of D_{Total} (68.08 µm/mm^2) was associated with higher ETP levels (31.9 nM × min, 95% CI: 1.3-62.6).

Sensitivity analyses were performed to test the robustness. Using premenopausal women yielded even more notable results than the main analysis for total women that PBR difference was associated with higher fibrinogen concentration, higher FXI and FVIII activity, and higher thrombin generation parameters (ETP, peak, and VelIndex) (Figure S5, Table S7, Figure S7, and Table S9). However, the results in postmenopausal women were less significant but comparable to the main analysis for all women (Figure S6, Table S8, Figure S8, and Table S10).

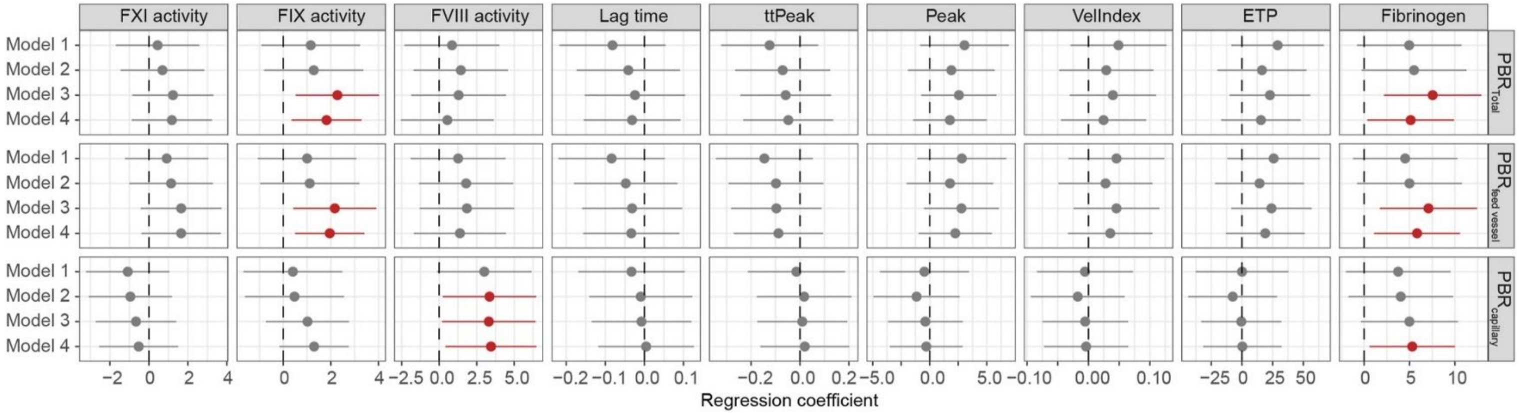

Figure 2. Association between glycocalyx-related SDF imaging parameters and levels of coagulation parameters in women. After sex stratification, differences between the glycocalyx-related parameters and coagulation factor levels could be observed in women. Model 1: crude model. Model 2: model 1 + age. Model 3: model 2 + body mass index, current smoking status, menopausal status, current use of an oral contraceptive pill, and current use of hormone replacement therapy. Model 4 = model 3 + serum C-reactive protein, serum leptin concentration, and serum glycoprotein acetyl concentration. The effect size and 95% CI are depicted by a horizontal line with a dot. A nonsignificant association is represented in gray, and a substantial positive association is represented in red. Perfused boundary region $(PBR)_{Total}$: PBR of total vessels from 4 to 25 µm. $PBR_{feed\ vessel}$: PBR of feed vessels from 10 to 19 µm. $PBR_{capillary}$: PBR of capillaries from 4 to 9 µm. ETP, endogenous thrombin potential; FIX, factor IX; FVIII, factor VIII; FXI, factor XI; ttPeak, time to peak.

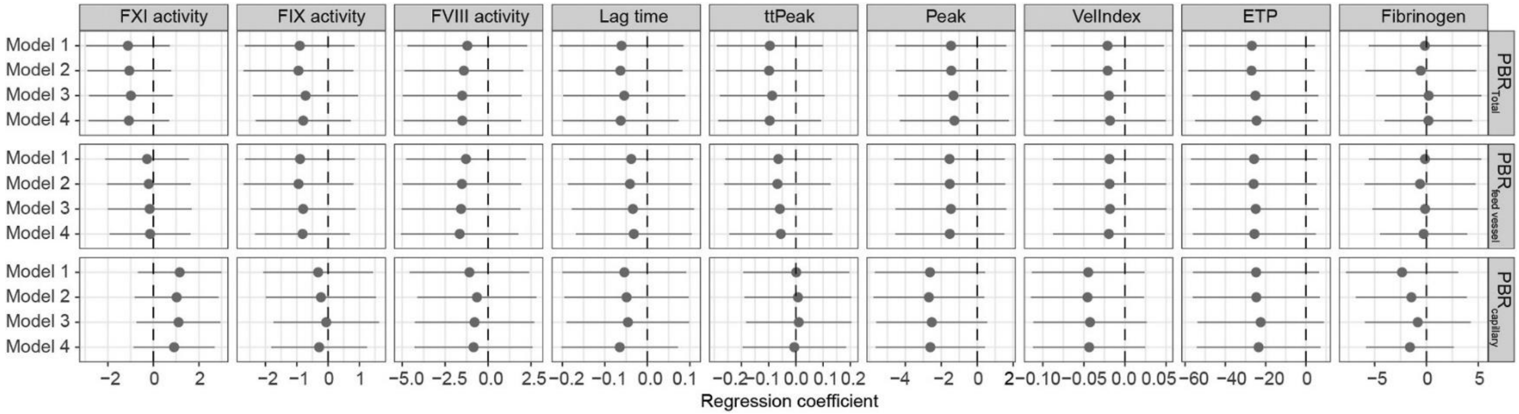

Figure 3. Association between glycocalyx-related SDF imaging parameters and levels of coagulation parameters in men. After sex stratification, differences between the glycocalyx-related parameters and coagulation factor levels could be observed in men. Model 1: crude model. Model 2: model 1 + age. Model 3: model 2 + body mass index + current smoking status. Model 4: model 3 + serum C-reactive protein, serum leptin concentration, and serum glycoprotein acetyl concentration. The effect size and 95% CI are depicted by a horizontal line with a dot. A nonsignificant association is represented in gray, and a substantial positive association is represented in red. Perfused boundary region $(PBR)_{Total}$: PBR of total vessels from 4 to 25 µm. $PBR_{feed\ vessel}$: PBR of feed vessels from 10 to 19 µm. $PBR_{capillary}$: PBR of capillaries from 4 to 9 µm. ETP, endogenous thrombin potential; FIX, factor IX; FVIII, factor VIII; FXI, factor XI; ttPeak, time to peak.

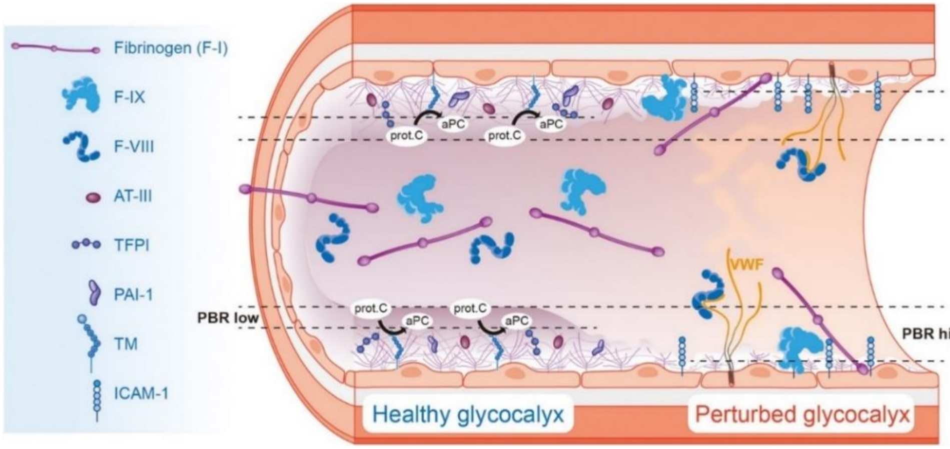

Figure 4. Proposed pathophysiologic concept of the association between microvascular health and endothelial response to coagulation. Here, a healthy glycocalyx (ie, perfused boundary region low, marked by the arbitrary dotted lines) provides a robust anticoagulant barrier through binding of antithrombin III [22], presence of thrombomodulin [26], PAI-1 [50], and tissue factor pathway inhibitor [23] to heparan sulfate proteoglycans [19]. The thrombin-TM complex activates protein C to produce APC, which inactivates factor (F)VIIIa and FVa in the presence of protein S, thereby inhibiting further thrombin (F-II) formation [51]. The procoagulant endothelial surface is a result of multiple mechanisms involving effects such as gene transcription as well as protein expression and release. We hypothesized that in the event of a perturbed glycocalyx (ie, perfused boundary region high, marked by arbitrary dotted lines further apart), a procoagulant surface appears, with increasing binding possibilities of fibrinogen (interacting with ICAM-1) [52,53], FXI [54,55], and FVIII (to surface-expressed von Willebrand factor) [47] and reduced presence of anticoagulant factors. Although FIX can bind directly to the cell surface or abluminal collagen, binding of fibrinogen and FVIII to the surface depends more on the activation state of endothelial cells. This increased interaction of coagulation FIX and FVIII activity and fibrinogen concentration, as observed in the present study, together with possible diminished surface anticoagulation pathways, would play a critical role in increased systemic microvascular dysfunction and development of coronary heart disease especially in women. aPC, activated protein C; AT-III, antithrombin III; ICAM-1, intercellular adhesion molecule-1; PAI-1, plasminogen activator inhibitor-1; PBR, perfused boundary region; TFPI, tissue factor pathway inhibitor; TM, thrombomodulin; VWF, von Willebrand factor.

Discussion

In this population-based cross-sectional study the PBR in feed vessels was positively associated with plasma fibrinogen levels in the total population. Remarkably, we discovered a sex difference in the associations between PBR and coagulation parameters, in which higher PBR (both total and feed vessel, indicating perturbed glycocalyx) was associated with higher FIX activity and plasma fibrinogen levels in women. Furthermore, in women, higher $PBR_{capillary}$ (i.e., poorer glycocalyx status in capillaries) was associated with higher FVIII activity and fibrinogen levels and higher $D_{capillary}$ was associated with higher levels of ETP, while none of these associations were present in men.

SDF imaging technique could provide two types of parameters concerning endothelial glycocalyx and microcirculatory perfusion function. We proposed that the change in the endothelial surface properties, i.e. the endothelial glycocalyx layer (PBR), is a likely functional unit that could present as a marker for microvascular dysfunction. In the present study, we observed an association between early (pre-clinical) microvascular health changes and coagulation factor activation and discovered a striking sex difference in microvascular health, in which women showed a perturbed EG concomitant with a more pro-coagulable endothelial surface. This association was not observed in men. These findings highlight the importance of sex differences in microcirculatory perturbation in CHD.

Previously, studies reported the association between CHD and the underlying systemic presence of a hypercoagulable state (42-44). Our results showed that in women, one SD in PBR_{Total} and $PBR_{feed\ vessel}$ was associated with a 5.1 and 5.8 mg/dL increase in fibrinogen concentration. Based on a large individual participant meta-analysis study that assessed the association between fibrinogen concentration and CHD risk (45), such increase in PBR would suggest a 12-14% increase in CHD risk in women, which could be clinically relevant. In line with our observations, Brands *et al.* reported that reduced endothelial glycocalyx barrier properties were only found in women with CHD, while not present in men (40). In addition, in the REasons for Geographic and Racial Differences in Stroke Study (REGARDS), a large population-based observational study, higher levels of FVIII and FIX were associated with increased risk of CHD (35, 46). According to these associations, per SD difference in PBR could correspond to a 2-5% increase in the risk of CHD in women. FVIII and vWF are two distinct but related glycoproteins that circulate in plasma as a tightly bound complex (FVIII/VWF) (47). As one of the endothelial activation markers, vWF can bind to HS at the endothelial cell surface (26, 48). Based on the observed tight correlation between FVIII and vWF in the Multiple Environmental and Genetic Assessment of risk factors for venous thrombosis (MEGA) study (49), we deduced an association between EG health and vWF in our present study, to further indicate the link between EG dysfunction and endothelial activation. Our current findings imply a role of microvascular health in CHD risk in women through the association between higher microvascular PBR (perturbed glycocalyx) and differences in both FIX and FVIII activity, together with the already high plasma fibrinogen levels (Figure 4).

The present study also included various thrombin generation parameters which represent the global coagulation cascade in addition to coagulation factor activity. Interestingly, no associations were observed between the microvascular health parameters measured and the dynamic parameters of thrombin generation (such as lag time and time to peak); except for an association between vessel density and ETP. Thrombin was found to be involved in both pro-thrombotic and inflammatory endothelial processes (50). While previous studies reported an association between *in vivo* thrombin generation potential and severity of coronary vessel disease (51), increased thrombin generation potential was a characteristic in patients with clinically stable CHD (52). Although we found sex differences in the association between vessel density and ETP, when we compared the findings in the total population, men, and women, we found that the associations in men and women were close to significance; therefore, this observation might reflect a general feature of microvascular health and thrombin formation rather than a sex specific feature. Furthermore, comparing with previous studies using thrombin generation assay, the participants in the present study were preclinical and did not have CHD yet, which might limit the effect of thrombin and findings related to thrombin generation. In the context with the associations between endothelial glycocalyx (PBR) and coagulation factors, the associations between vessel densities and ETP were very marginal.

To the best of our knowledge, this is the first study to show the association between microvascular health derived from SDF imaging and the levels of coagulation factors and thrombin generation parameters in a large population-based study. In addition, adjustment for extensive potential confounding factors including systemic inflammatory markers strongly suggested a sex-specific role in monitoring microvascular health change in women. Additionally, the present study used the new software with red blood cell (RBC) velocity being included as a new parameter to better quantify the microcirculatory difference.

Limitations of the present study should also be addressed. First, it is an observational, cross-sectional study and residual confounding may still be present. Second, the present study population is mainly comprised of European white participants in middle age (i.e., between 45 and 65 years). Therefore, it is not clear whether the results of the present study can be generalizable to other ethnicities or age groups. Third, the majority of women are postmenopausal; although sensitivity analysis revealed similar findings in premenopausal women, future studies including more premenopausal women need to be done. Fourth, a recent study reported the variability of microcirculatory measurements in healthy volunteers (53) showing that when three consecutive measurements are averaged, SDF imaging with GlycoCheck software can be used with acceptable reliability and reproducibility for microcirculation measurements on a population level. In our current study, repeated measurement of SDF imaging was not performed for each participant and limited information was extracted from the smallest capillaries (diameter 4 μm). However, the measurement by itself is already an average from at least ten recordings on one individual at the same time point rather than averaging three consecutive measurements on the same individual, which could only lead to random error and subsequent underestimation of the effect size. Fifth, due to the technical limitation of the *ex vivo* thrombin generation assay, we could not avoid batch effects and this assay could not represent the real circumstances of the coagulation cascade in humans. Sixth, as a result of sample availability to measure the circulating EG disruption and EC activation markers, our study lacks possible mechanistic correlations between EG dysfunction, increased procoagulant state and increased CHD risk in females. Seventhly, although we focussed on the role of EG perturbation in coagulation in the present study, it is important to note that the formation of a pro-coagulant surface is a result of multiple mechanisms, among others, such as gene transcription changes and protein expression and release. Finally, in model 4 we adjusted for systemic inflammatory markers (such as CRP, leptin, and GlycA) in the association between microvascular health and coagulation factor levels. It should be noted that these systemic inflammatory markers could act as mediators instead of confounders in the estimated associations. If so, adjustment for systemic inflammatory markers could lead to an underestimation of the association between microvascular health and coagulation factor levels.

In conclusion, our data reveal a hitherto unreported sex-specific association between microcirculatory health and procoagulant status. Microcirculatory differences between men and women identified in our study implied that microvascular health changes might be the earliest detectable clue prior to the general higher procoagulant status in women ultimately developing CHD, which is also independent of increased systemic inflammatory state (Figure 4). Our study suggested the potential clinical utility of monitoring microcirculatory change specifically in women to prevent the development of CHD.

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