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Sex difference in fibrin clot lysability: Association with coronary plaque composition

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ABSTRACT

Introduction: Fibrin clot lysability is associated with development of cardiovascular disease (CVD). We evaluated sex-differences in fibrin clot lysability and the association with coronary plaque composition determined by computed tomography angiography (CTA).

Methods: Middle-aged citizens without known CVD were randomly selected from a national registry. A coronary CTA assessed volumes of calcified-, non-calcified-, low-density non-calcified-, and total- plaque using a validated plaque quantification software. A non-enhanced cardiac CT scan assessed the Agatston score. Fibrin structure properties were determined using turbidimetric methods. Plasma concentrations of C-reactive protein and fibrinogen were assessed.

Results: 138 individuals (71 women) participated. Men more frequently had coronary plaques compared to women, $P < 0.05$. Coronary plaque features were comparable between men and women, $P > 0.05$. Women with total plaque volume $> 0\,mm^3$ had lower fibrin clot lysability compared to women with total plaque volume $= 0\,mm^3$, adjusted difference [95% confidence interval] 10.28 [1.42–19.15], $P = 0.02$, and a fibrinogen-dependent lower fibrin clot lysability compared to men with and without coronary plaques, 6.82 [−2.67–16.31], $P = 0.16$, and 8.73 [−0.43–17.89], $P = 0.06$, respectively. Fibrinogen correlated with all the coronary plaque features (correlation coefficient $r = 0.42$–0.57) only in women with total plaque volume $> 0\,mm^3$, all $P < 0.01$.

Conclusion: Asymptomatic women with coronary plaques assessed by coronary CTA have reduced fibrin clot lysability compared to both women without coronary plaques and men, suggesting a sex-dependent link between coronary atherosclerosis and fibrin clot lysability.

1. Introduction

Pathological [1] and epidemiological studies [2,3] have indicated sex-differences in morbidity and mortality [4] of cardiovascular diseases (CVD). Thus, there is an increasing interest in CVD among women [5].

Development of CVD is delayed in women compared to men [6]. However, a subgroup of women has a higher mortality after acute myocardial infarction (AMI) compared to age-matched men [2]. Several mechanisms for these findings have been proposed, i.e. influence of sex-hormones [7], sex-differences in the impact of CVD risk factors on coronary pathology [8], and in diagnosis and management of acute coronary syndromes [9,10]. However, sex-differences in the pathophysiological mechanisms in the subclinical phase of CVD are unclear.

Abbreviations and acronyms: Ag, Agatston score; AMI, acute myocardial infarction; BMI, body mass index; CAC, coronary artery calcification; CI, confidence interval; CRP, C-reactive protein; CT, computed tomography; CTA, computed tomography angiography; CVD, cardiovascular disease; HDL, high-density lipoprotein; IQR, interquartile range; LDL, low-density lipoprotein; LD-NCP, low-density non-calcified plaque; NCP, non-calcified plaque; RI, remodeling index; SD, standard deviation

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Studies have documented an association between altered fibrin clot lysability and development of CVD [11–16]. Recently, we have shown that middle-aged women with asymptomatic coronary artery calcification (CAC) have altered fibrinolytic properties both compared to men and compared to women without CAC [17,18]. In general, CAC is a marker of atherosclerotic burden and plaque growth [19], and is associated with increased risk of CVD [20]. Recently, increased calcium density in coronary plaques has been shown to contribute to plaque stability [21,22], while the low-attenuation non-calcified plaque component seemed associated with an unfavourable clinical outcome [23–25]. In this context, sex-differences in fibrin clot lysability and specific coronary plaque features and their associations are still unsettled.

Therefore, in the present cross-sectional cohort study we examined sex-differences in coronary plaque composition in middle-aged individuals free of known CVD. Furthermore, we addressed potential sex-differences in the association between fibrin clot lysability and coronary plaque composition.

2. Materials and methods

2.1. Design and study population

This cross-sectional cohort study recruited participants from the Danish Risk Score (DanRisk) study [26] initiated at four hospitals (Esbjerg, Vejle, Odense and Svendborg) in the Region of Southern Denmark. As previously described [26,27], participants born in either 1949 or in 1959 and free of known CVD, diabetes, and any regular medication were randomly chosen from the Danish national central person registry in 2009. The current study is based on results from the re-examination of the study participants performed in 2015, Fig. 1.

The participants filled in a questionnaire concerning their medical and family history of CVD. In addition, participants were personally interviewed about their medical and family history. Physical examination in the study included measuring height, weight, and BMI calculations. In addition, resting blood pressure after 5 min of rest was obtained.

2.2. Ethical considerations

The study was conducted in accordance with the ethical standards of the Regional Scientific Ethics Committee for Southern Denmark and with the ethical guidelines of the 1975 Declaration of Helsinki and its later amendments. The protocol was approved by the Regional Scientific Ethics Committee for Southern Denmark, reference number: S20130169 (45023). All participants provided written informed consent for study inclusion and publication of obtained data.

2.3. Coronary CTA

2.3.1. Acquisition

A standard non-contrast sequential cardiac CT using a Philips 64-slice scanner (Brilliance 64, Philips Healthcare, Koninklijke, Netherlands), with a mAs 50 for standard participants with adjustments made for participant size to assess CAC by the Agatston score (Ag) [28] was undertaken prior to the coronary CTA [18]. The participants underwent coronary CTA with Optiray™ 350 mgI/ml (Mallinckrodt, Surrey, United Kingdom) using the Siemens Dual Source CT-scanner (Definition FLASH Dual Source, Siemens Healthcare, Forchheim, Germany). Targeting a heart rate ≤ 60 beats/min, pre-scan oral beta-receptor blockers were administered. Sublingual nitroglycerin was administered immediately before the scanning in all patients. The following settings applied: gantry rotation time 280 ms, collimation 2 × 64 × 0.6 mm, reconstructed slice thickness was 0.6 mm with an increment of 0.3 mm in a matrix of 512 × 512 pixels and a field of view < 250 mm, in 65, 70, and 75% of R-R interval, 120 kV tube voltage with a quality reference mAs of 320 (using CAREDose4D Automatic Exposure Correction) was employed. A prospectively ECG-triggered (step and shoot) acquisition protocol with ECG-based radiation pulsing was applied in all participants. ECG-pulsing was set to 100% dose for 65–75% of the R-R-interval. A "test bolus" with 8 ml of contrast media was used to determine the optimal time of coronary artery enhancement. The individual Dose-Length-Product was registered in all participants and effective dose in milliSieverts (mSv) calculated (k-factor = 0.017) [29].

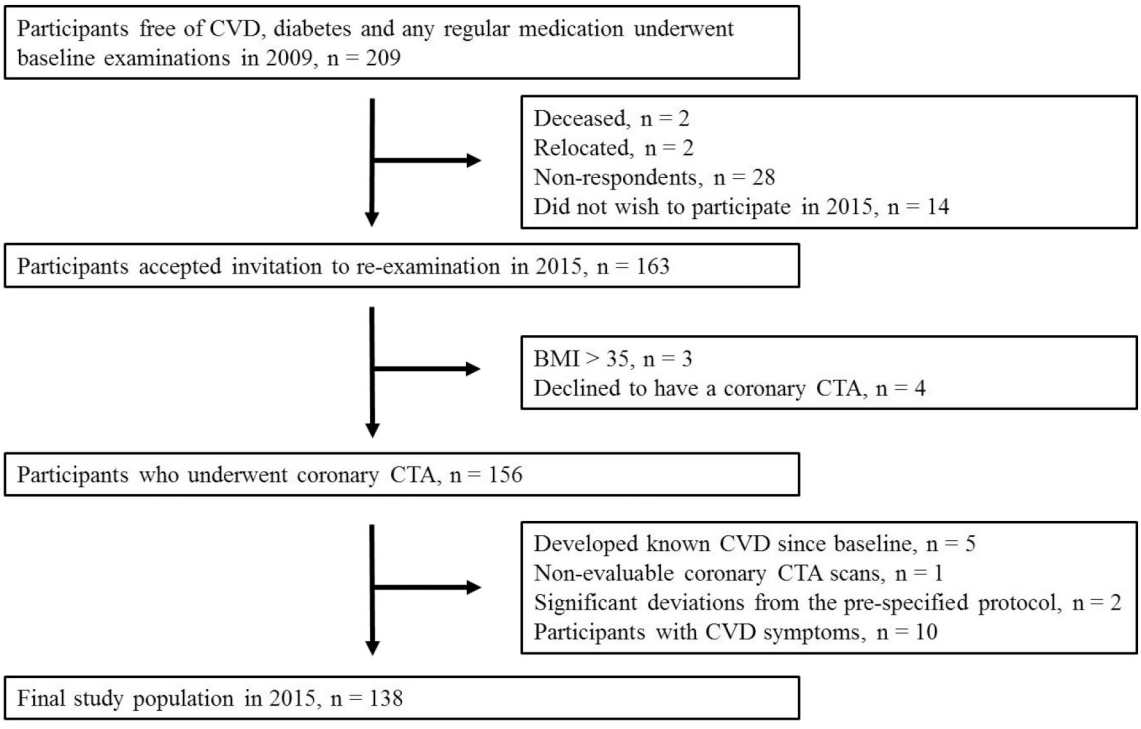

Fig. 1. Flowchart of study enrollment.

2.4. Data analysis

Analyses of data were performed on a dedicated workstation, Syngo.via (Siemens Healthcare, Ballerup, Denmark). Scan data were evaluated visually by a trained CT-cardiologist, blinded to all the clinical data. Coronary artery calcification was assessed by summing up the scores from all foci in the coronary arteries, expressed in Agatston units (U), using Syngo Calcium scoring (Siemens Healthcare). Coronary CTA datasets were categorized as being normal or as having non-obstructive - or obstructive stenosis (> 50% of lumen). Location of lesions was reported using a 17-segment model [30].

Images were deemed non-evaluable if artifacts affected ≥3 coronary segments. All coronary CTA datasets with coronary lesions were analyzed quantitatively by a dedicated plaque software program. The phases with most optimal image quality were transferred to a research workstation for plaque assessment.

2.5. Semi-automated plaque quantification on coronary CTA

Coronary segments > 2 mm with plaques (tissue structures > 1 mm^2) were analyzed using a validated semi-automated plaque software (Autoplaque© research software, version 2.0 (Cedars-Sinai Medical Center, Los Angeles, California, USA)) [31,32]. Software assessment allowed detailed investigation of plaques by color-coding of Hounsfield Units ranges. Coronary CTA images were examined in multiplanar reformats, and a circular region of interest (normal reference blood pool) was placed in the aorta. Atherosclerotic plaques were characterized according to their Hounsfield Units. Based on the relative amount of calcified and non-calcified components, plaques were classified as calcified plaque (yellow overlay) characterized by predominate calcified tissue (any structure with a density of 130 Hounsfield Units or more that could be visualized separately from the contrast-enhanced coronary lumen) and non-calcified plaque (NCP) characterized by any structure with a density of 130 Hounsfield Units or less (red overlay), that could be visualized separately from the contrast-enhanced coronary lumen, see Fig. 2. Scan-specific thresholds for calcified plaque and NCP volumes were automatically generated. Adjustments were made if necessary. Low-density NCP (LD-NCP) was defined with attenuation < 30 Hounsfield Units. The part of the NCP that was not classified as LD-NCP was defined as NonLD-NCP. Coronary plaque burden was defined as (plaque volume / vessel volume) × 100%. Remodeling index was calculated as maximum lesion vessel area/area of a proximal normal reference point. Spotty calcification was defined as calcifications < 3 mm and occupying < 90° of the vessel circumference. Low observer variability for coronary plaque assessment using Autoplaque has been demonstrated earlier [33]. All analyses were performed by an experienced reader who was blinded to all other patient data.

The population was separated into two groups based on coronary total plaque volume: total plaque volume = 0 mm^3 or total plaque volume > 0 mm^3.

2.6. Blood collection, handling and biochemical analysis

Blood samples were drawn from an antecubital vein into sterile vacuum plastic tubes containing either 0.109 mol/l citrate or no anticoagulants, and the plasma or serum isolated after 20 min centrifugation at 2000 *g* at 20 °C were frozen and stored in aliquots at −80 °C until analysis.

Lipids were analyzed using Cholesterol, Direct LDL, Ultra HDL and Triglycerides kits employing the Architect C16000 analyzer using kits and analyzer from Abbott (Wiesbaden, Germany). Concentrations of CRP and fibrinogen were determined on a BN-II nephelometer using antibodies and reagents from Siemens Healthcare Diagnostics GmbH (Marburg, Germany).

Fibrin clot lysis was recorded using turbidity measurements as previously described [16]. In brief, citrate-stabilized plasma was mixed with thrombin, $CaCl_2$, and Tween 80, and incubated overnight at room temperature in a microtiter plate sealed with adhesive tape. All clots were made in duplicate. To initiate fibrinolysis t-PA and flufenamic acid were added onto the clots. The 405 nm optical density (OD) was then followed on a Tecan Sunrise plate reader (Grödig/Salzburg, Austria) every 5 min for 4 h at 25 °C. The rate of fibrinolysis per hour was determined from the slope of the curve at the time when the slope became constant, and was normalized with respect to the maximum absorbency value before lysis initiation.

2.7. Statistics

Continuous variables are presented as means and 95% confidence interval (CI), median and interquartile ranges (IQR), or as numbers (%) as appropriate. The distribution of data was examined by the Shapiro-Wilks test as well as by visually assessing histograms and QQ-plots. If possible, non-normally distributed variables were log-transformed to obtain normality. Dichotomous variables are shown as numbers and percentages. Student's *t*-test was used for comparison of normally distributed continuous variables, and the Mann-Whitney test for comparison of non-normally distributed continuous variables. The χ^2-test was performed for comparison of dichotomous variables. Kendall's rank correlation test was used to evaluate association between coronary plaque features and total plaque tertiles in men and women. *P*-values < 0.05 were considered statistically significant. Covariates were identified as variables which correlated with fibrin clot lysability using the Spearman correlation test. A linear regression model was conducted using fibrin clot lysability as the dependent variable and combination of sex and total plaque volume (women with total plaque volume = 0 mm^3, women with total plaque volume > 0 mm^3, men with total plaque volume = 0 mm^3, and men with total plaque volume > 0 mm^3) as exposures, and women with total plaque volume > 0 mm^3 as reference group. The model was adjusted for the identified covariates triglycerides and fibrinogen. To ensure model validation, QQ-plots for residuals were inspected for normality. Stata 15.0, StataCorp, Texas, USA was used for statistical analyses.

3. Results

3.1. Population characteristics

The population characteristics are shown in Table 1.The study included 138 participants (women n = 71), Fig. 1. Diastolic blood pressure and triglycerides were significantly higher in men compared to women, whereas HDL cholesterol was higher in women. All other variables were comparable between the sexes. There was no sex-difference in the usage of medication, Table 1.

The median radiation dose (IQR, range) was 3.52 mSv (2.67–5.65, 1.72–12.29) mSv for the coronary CTA. Women had lower median (IQR) CT radiation dose and median (IQR) contrast usage compared to men, 2.72 (2.24–3.32) vs. 5.47 (3.69–6.65) and 70 (65–80) vs. 90 (75–95), respectively, P < 0.05. Otherwise CT acquisition parameters were comparable between the sexes.

3.2. Sex-difference in coronary plaque characteristics

Men had more frequently coronary plaques compared to women, n = 38 (57%) vs. n = 26 (37%), P = 0.02. In participants with total plaque volume > 0 mm^3, no difference in coronary plaque characteristics were found between the sexes, Table 2.

The volume fractions of LD-NCP, calcified plaque and NonLD-NCP according to total plaque volume tertiles are given in Fig. 3. In men, the calcified plaque fraction was positively associated with total plaque volume tertiles, P < 0.05.

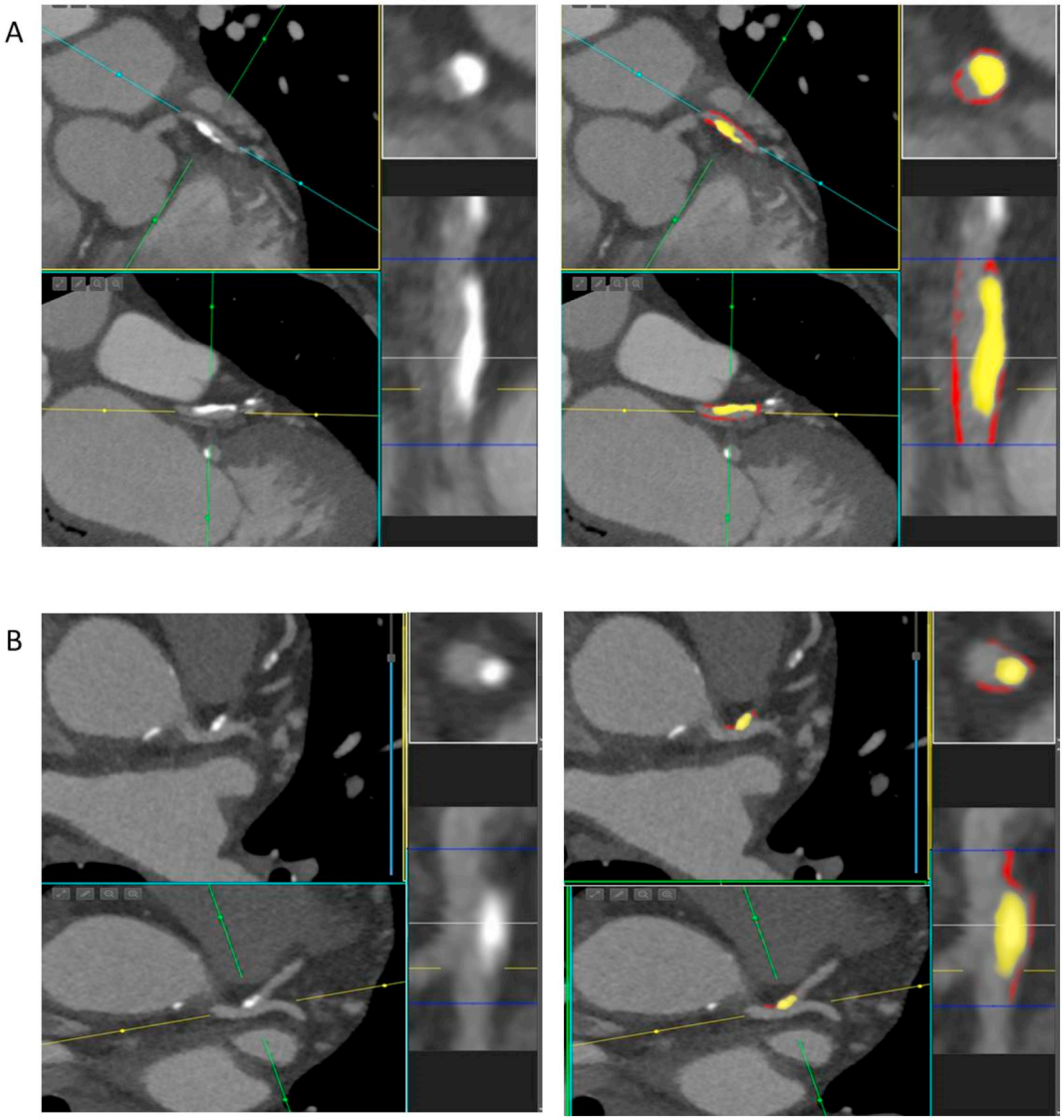

Fig. 2. Illustration of quantitative coronary plaque analysis with and without overlay. Red overlay indicates non-calcified plaque (NCP), and yellow overlay indicates calcified plaque. (A) Stretched and horizontal views of non-obstructive mixed plaques in the left anterior descending artery segment 1. (B) Stretched and horizontal views of non-obstructive mixed plaque in the distal left main artery. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.3. Sex-difference in fibrin clot lysability and fibrinogen in relation to coronary plaque

Fibrin clot lysability in relation to the occurrence of coronary plaques in men and women is depicted in Fig. 4.Women with a total plaque volume $> 0\,mm^3$ had reduced mean [95% CI] fibrin clot lysability, 39.3% [32.2–46.4], both compared to men and women without coronary plaques, 53.8% [46.4–61.3] and 53.7% [46.5–61.0], respectively, and compared to men with a total plaque volume $> 0\,mm^3$, 50.9% [44.0–57.9], all $P < 0.05$. No significant difference in fibrin clot lysability between women and men without coronary plaque was found.

Fibrin clot lysability correlated with fibrinogen ($r = -0.460$, $P < 0.001$) and triglycerides ($r = -0.176$, $P = 0.039$).

Table 3 shows the differences in fibrin clot lysability in women with total plaque volume $= 0\,mm^3$, men with total plaque volume $= 0\,mm^3$ and men with total plaque volume $> 0\,mm^3$ compared to women with total plaque volume $> 0\,mm^3$. Three different models are used: a) unadjusted analysis, b) adjusted for triglycerides only, and c) adjusted for triglycerides and fibrinogen. When adjusting for the triglycerides only, fibrin clot lysability was significantly lower in women with total plaque volume $> 0\,mm^3$ compared with women with total plaque volume $= 0\,mm^3$ and men, $P < 0.05$. When adjusting for both triglycerides and fibrinogen, fibrin clot lysability remained significantly low in women with total plaque volume $> 0\,mm^3$ compared to women with total plaque volume $= mm^3$.

Fibrinogen correlated with all the coronary plaque features (correlation coefficient $r = 0.42$–0.57) in women with total plaque volume $> 0\,mm^3$, all $P < 0.01$. There were no correlation between fibrinogen and coronary plaque features in men ($P > 0.05$). Fibrinogen was higher in women with total plaque volume $> 0\,mm^3$, 10.1 [9.5–10.8], compared to both men with total plaque volume $= 0\,mm^3$,

Table 1
Population characteristics.

Variable	Men (n = 67)	Women (n = 71)	P-value
Age,			
55-Years	43 (64)	35 (49)	
65-Years	24 (36)	36 (51)	0.08
Tobacco use			
Never	30 (45)	35 (49)	
Prior/current	37 (55)	36 (51)	0.60
Systolic blood pressure, mm Hg	138 [134–143]	135 [131–138]	0.09
Diastolic blood pressure, mm Hg	81 [79–83]	73 [70–75]	< 0.001
Body mass index, kg/m^2	27.6 [27.9–28.3]	26.9 [25.9–30.0]	0.14
Total cholesterol, mmol/l	5.36 [5.15–5.57]	5.54 [5.32–5.75]	0.88
LDL cholesterol, mmol/l	3.37 [3.18–3.57]	3.27 [3.05–3.50]	0.26
HDL cholesterol, mmol/l	1.30 (1.10–1.40)	1.60 (1.40–1.80)	< 0.001
Triglycerides, mmol/l	1.56 (1.14–2.57)	1.21 (0.90–1.94)	< 0.01
C-reactive protein, mg/l	0.74 (0.46–1.50)	0.74 (0.26–2.01)	0.37
Fibrinogen, μmol/l	9.0 (7.7–10.1)	9.5 (8.4–10.7)	0.09
Diabetes	0 (0)	1 (1)	0.33
Antihypertensive treatment	8 (12)	8 (11)	0.90
Lipid-lowering treatment	5 (7)	9 (13)	0.31
Anti-thrombotic treatment	0 (0)	1 (1)	0.62

Values are presented as mean [95% CI], median (IQR) or n (%). BMI was calculated based on height and weight. LDL: low-density lipoprotein; HDL: high-density lipoprotein.

Table 2
Plaque characteristics in study participants with total plaque volume > 0 mm^3 determined by coronary computed tomography angiography.

Variable	Men (n = 38)	Women (n = 26)	P-value
Number of affected segments, n			
1–2	15 (22)	10 (14)	
3–4	11 (16)	10 (14)	
≥5	12 (18)	6 (8)	0.10
Ag, U	99 (14–139)	60 (18–164)	0.88
Coronary plaque components, mm3			
NCP volume	201 (110–366)	179 (76–341)	0.24
Calcified plaque volume	25 (6–42)	19 (4–52)	0.52
LD-NCP volume	19 (9–39)	19 (8–47)	0.16
Total plaque volume	241 (134–397)	194 (81–352)	0.23
Remodeling index	1.6 (1.3–2.0)	1.7 (1.5–2.0)	0.53
Spotty calcification			
Yes	30 (79)	18 (69)	
No	8 (21)	8 (31)	0.28

Values are presented as median (IQR) or n (%). Ag: Agatston score; NCP: non-calcified plaque; LD-NCP: low-density non-calcified plaque.

9.0 [CI 8.3–9.8], and men with total plaque volume > 0 mm^3, 9.2 [8.6–9.8], P < 0.05, while fibrinogen levels were comparable between women with and without coronary plaque.

4. Discussion

The main finding of this cross-sectional study of middle-aged asymptomatic individuals without known CVD is that women with coronary plaques by coronary CTA have reduced fibrin clot lysability compared to women without coronary plaques independent of the potential confounders fibrinogen and triglycerides, and a fibrinogen-dependent lower fibrin clot lysability compared to men.

Clinical studies have identified vulnerable plaque features by coronary CTA as plaques with low-attenuation, spotty calcification, and vascular remodeling [24,34,35]. Therefore, coronary CTA is capable of predicting adverse CVD events by quantifying these vulnerable atherosclerotic plaque features [25]. In accordance with previous reports in symptomatic subjects [36,37], the current study, performed in a randomly selected asymptomatic population, showed that men more frequently had coronary plaques compared to women. However, we did not observe any difference in the volumes of plaque sub-types between the sexes. Nasir et al. reported that symptomatic women with coronary plaques by CTA have relatively less calcified plaques and a larger fraction of non-calcified plaque compared to men [36]. We report of a positive association between the fraction of calcified coronary plaque component and total coronary plaque volumes in men and not in women, which might be in accordance with the sex-differences in coronary plaque composition reported by Nasir et al.

Previous reports have shown that increased fibrinogen levels are associated with alterations in fibrin structure leading to reduced fibrin clot lysability [15,38], and eventually increased risk of thrombosis [39,40]. It is commonly accepted that women have higher fibrinogen levels compared to age-matched men [41], and increased plasma fibrinogen levels have been associated with the risk of cardiovascular events [42]. In this relation, fibrin clot lysability is influenced by several factors including fibrinogen, known CVD risk factors [43], and treatment with aspirin [44]. In addition, fibrinogen glycation in patients with diabetes mellitus has been shown to influence fibrin clot lysability [45], and women with diabetes and CVD have longer lysis time compared to men [46]. An increased presence of fibrin and fibrin degradation products in vulnerable plaques has been reported [47], and the presence of fibrin in coronary plaques causing sudden cardiac death has been described [48,49].Otherwise, the causal association between reduced fibrin clot lysability and increased risk of thrombosis is still not fully understood [50]. However, studies on fibrin structure and clot lysability have primarily been restricted to patients with CVD and/or diabetes [11–14,16,45,46,51], and the interplay between fibrin clot lysability and coronary plaque composition has not been evaluated before. We have previously shown that asymptomatic women with progression of CAC over a five-year period have reduced fibrin clot lysability compared with women without CAC progression and compared with men [18], findings that were independent of fibrinogen. The current study expands our knowledge, as it was demonstrated that women with coronary plaques had a fibrinogen independent lower fibrin clot lysability compared to women without coronary plaques and a fibrinogen dependent lower fibrin clot lysability compared to men. Furthermore, fibrinogen correlated with adverse coronary plaque features only in women with coronary plaques. This suggests different pathophysiological mechanisms in atherosclerotic plaque composition between the sexes.

The onset of menopause causing hormonal changes is known to influence development of CVD [52] and the hemostatic system [53,54]. Women with coronary plaques in our study were significantly older than women without coronary plaques. However, presumably most of these women had entered menopause and no significant difference was observed in fibrin clot lysability between women born in 1949 and 1959 neither with nor without coronary plaque. This indicates that the results on fibrin clot lysability in this study were not affected by the hormonal status of the women included in the study.

4.1. Limitations

Due to the small sample size, subgroup analyses were not feasible. We have previously shown that women receiving oral contraceptives have increased fibrin clot lysability [54], and five women received hormone replacement therapy in the current study. Repeat analysis after exclusion of these five individuals did not change our findings. Furthermore, there was no difference in medication use between

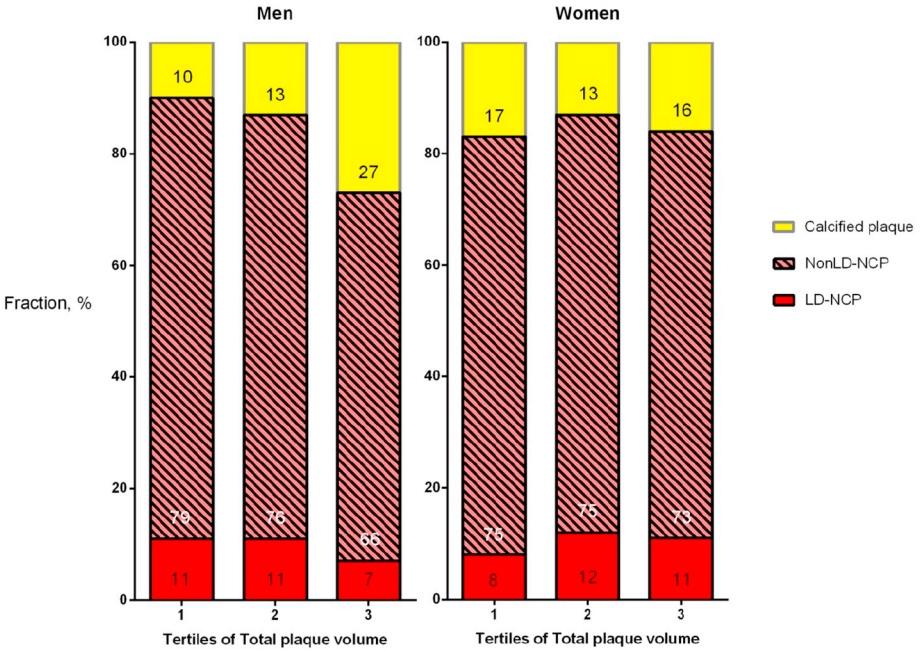

Fig. 3. Coronary plaque fraction as percentage of total plaque volume tertiles in men and women.

women with and without coronary plaques, and only two women received anti-thrombotic treatment. Repeat analysis after exclusion of these two individuals did not change our findings. As this is an observational study, any causality between coronary plaque volumes and fibrin clot lysability should be considered with caution. However, our results might be in line with studies on vessel pathology, that reported on a higher frequency of coronary plaque erosion with a lesser content of calcium, thereby increasing the risk for plaque-erosion, -rupture, and intracoronary thrombosis in women compared to men [1,55], resulting in a higher CVD mortality in women with coronary plaques compared to men [2,56].

In conclusion, in asymptomatic middle-aged individuals, women with coronary plaques have a fibrinogen dependent reduced fibrin clot lysability compared to men and a fibrinogen independent reduced fibrin clot lysability compared to women without coronary plaques. These results suggest sex-difference in the pathophysiological mechanism of CVD development, linking atherosclerotic vessel wall changes to fibrin clot lysability.

Conflict of interest

Damini Dey is a patent-holder of Autoplaque © research software, and receives software royalties from Cedars-Sinai Medical Center, Los Angeles, California, USA. All other authors have reported that they have no relationships relevant to the contents of this paper to disclose.

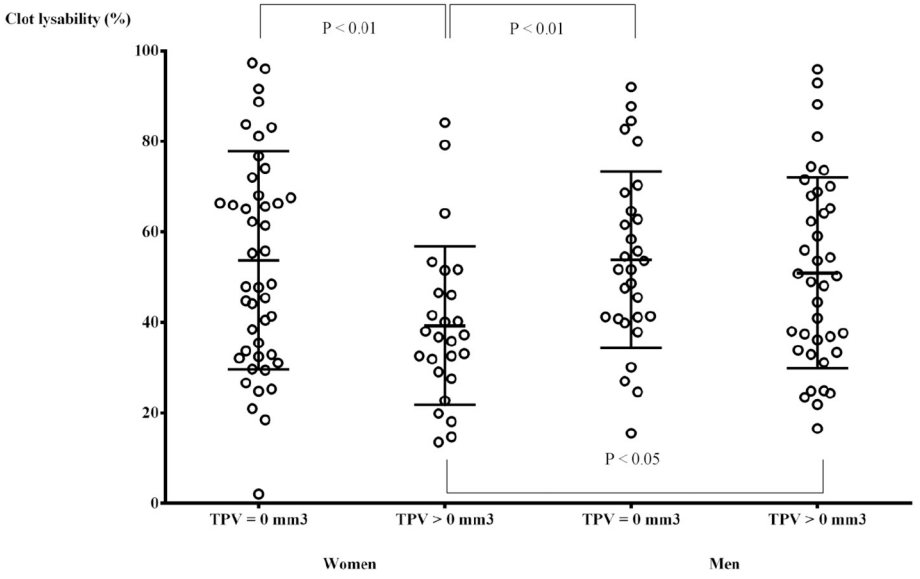

Fig. 4. Clot lysability in women and men in relation to coronary plaque. Mean and SD are indicated in bars.

Table 3
Differences in fibrin clot lysability in women with total plaque volume = 0 mm^3, men with total plaque volume = 0 mm^3 and men with total plaque volume > 0 mm^3 compared to women with total plaque volume > 0 mm^3 as the reference group.

Variable	Unadjusted difference[a]	Adjusted difference[b]	Adjusted difference[c]	P-value[c]	95% CI[c]
Women, TPV = 0 mm^3	14.43	14.41	10.28	0.02	1.42–19.15
Men, TPV = 0 mm^3	14.54	14.87	8.73	0.06	−0.43–17.89
Men, TPV > 0 mm^3	11.64	11.88	6.82	0.16	−2.67–16.31

TPV: Total plaque volume. CI: Confidence interval.
All models included fibrin clot lysability as the dependent variable and combinations of sex and TPV (TPV = 0 mm^3 or TPV > 0 mm^3).
[a] Coefficient from unadjusted linear regression model.
[b] Adjusted coefficient from adjusted linear regression model adjusting for triglycerides.
[c] Adjusted coefficient from adjusted linear regression model adjusting for triglycerides and fibrinogen.

CRediT authorship contribution statement

Ramshanker Ramanathan: Writing - original draft. **Jørgen B. Gram:** Writing - review & editing. **Johannes J. Sidelmann:** Writing - review & editing. **Damini Dey:** Writing - review & editing. **Bjarne L. Nørgaard:** Writing - review & editing. **Niels Peter R. Sand:** Writing - review & editing.

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