EuroIntervention 2020;16:387-394 published online August 2019

Special feature: Plaque characteristics

Association of coronary microvascular endothelial dysfunction with vulnerable plaque characteristics in early coronary atherosclerosis

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This paper also includes supplementary data published online at: https://eurointervention.pcronline.com/doi/10.4244/EIJ-D-19-00265

KEYWORDS

- clinical research
- intravascular ultrasound
- stable angina

Abstract

Aims: The aim of this study was to test the hypothesis that coronary microvascular endothelial dysfunction (CMED) is associated with epicardial coronary atherosclerosis.

Methods and results: We performed a cross-sectional analysis of a comprehensive invasive assessment of coronary physiology with a focus on endothelium-dependent coronary microvascular function and virtual-histology intravascular ultrasound (VH-IVUS) in a total of 148 consecutive patients with chest pain and angiographically normal coronary arteries or non-obstructive coronary artery disease (CAD). Endothelium-dependent coronary vascular reactivity was evaluated by graded doses of intracoronary acetylcholine (ACh). CMED was defined as a percent increase in coronary blood flow of ≤50% in response to ACh. Patients with CMED (n=87) showed more vulnerable plaque characteristics as compared to those without (n=61); they showed higher plaque burden in association with larger necrotic core volume and higher frequency of imaged arteries containing at least one VH-IVUS-derived thin-capped fibroatheroma (TCFA) (n=22 [25.3%] vs 5 [8.2%], p=0.008). Multivariate logistic regression analysis revealed that CMED was an independent predictor of VH-IVUS-derived TCFA (adjusted odds ratio 2.28 [95% confidence interval: 1.30-4.02], p=0.004).

Conclusions: Independently of conventional coronary risk factors, CMED was associated with vulnerable plaque characteristics in patients with non-obstructive CAD.

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SUBMITTED ON 12/03/2019 - REVISION RECEIVED ON 1st 29/05/2019 / 2nd 08/07/2019 - ACCEPTED ON 08/08/2019

DOI: 10.4244/EIJ-D-19-00265

Abbreviations

ACh	acetylcholine
CAD	coronary artery disease
CBF	coronary blood flow
CFR	coronary flow reserve
CMED	coronary microvascular endothelial dysfunction
IVUS	intravascular ultrasound
LAD	left anterior descending coronary artery
TCFA	thin-capped fibroatheroma
VH-IVUS	virtual-histology intravascular ultrasound

Introduction

Accumulating evidence has demonstrated that endothelial function plays essential roles in maintaining vascular homeostasis in health and disease and that endothelial dysfunction is the hallmark of atherosclerotic cardiovascular diseases[1]. We have previously demonstrated that epicardial coronary endothelial dysfunction is associated with the development and progression of the local coronary atherosclerotic lesion in patients with chest pain and non-obstructive coronary artery disease (CAD)[2,3]. Although functional and structural abnormalities of epicardial coronary arteries have been the focus of previous studies, those of coronary microvasculature have gained increasing attention in many clinical settings[4-6]. Previous studies have shown that patients with non-obstructive CAD with concomitant coronary microvascular endothelial dysfunction (CMED) are, as compared to those without CMED, associated with increased cardiac events including myocardial infarction, percutaneous or surgical revascularisation, and cardiac death[7,8]. Moreover, the prevalence of CMED in this clinical entity has been shown to be not negligible[6,9]. A comprehensive invasive assessment of coronary endothelial function is feasible and of diagnostic value to detect patients with CMED[4,6,9].

However, the role of CMED in the early stage of coronary atherosclerosis is largely unknown. The aim of the present study was to test the hypothesis that CMED is associated with epicardial atherosclerotic CAD. We performed a cross-sectional analysis over a decade of a comprehensive invasive assessment of coronary physiology with a focus on endothelium-dependent coronary microvascular function and virtual-histology intravascular ultrasound (VH-IVUS) in patients with chest pain and non-obstructive CAD.

Methods

More detailed methods are available in **Supplementary Appendix 1**.

STUDY DESIGN AND POPULATION

This retrospective, single-centre, cross-sectional study was performed at the Mayo Clinic in Rochester, MN, USA, using a prospective database of the Mayo Clinic Cardiac Catheterization Laboratory. Between July 2005, when VH-IVUS data became available, and February 2018, consecutive patients with angiographically normal coronary arteries or non-obstructive CAD who underwent both invasive coronary endothelial function testing and VH-IVUS examination in the left anterior descending coronary artery (LAD) for evaluation of chest pain were enrolled. Inclusion and exclusion criteria were as described previously[7,9,10]. The study population minimally overlapped with previous publications[2,9,10].

COMPREHENSIVE INVASIVE ASSESSMENT OF CORONARY PHYSIOLOGY

Invasive assessment of endothelium-dependent and endothelium-independent coronary vascular responses in the LAD was performed in a comprehensive manner using a 0.014-inch Doppler guidewire (FloWire®; Philips Volcano, Rancho Cordova, CA, USA), as described previously[2,7,11]. Endothelium-independent coronary flow reserve (CFR) was obtained at maximum hyperaemia induced by intracoronary bolus injections of adenosine at incremental doses (18 to 72 μg). A CFR value of <2.0 was considered abnormal[12,13]. Endothelium-dependent coronary vascular reactivity was assessed by selective infusion of incremental doses of acetylcholine (ACh) into the LAD at 10^{-6}, 10^{-5}, and 10^{-4} mol/L[2,7,11]. Coronary blood flow (CBF) was calculated by the following equation: CBF=π(time-averaged peak velocity)(coronary artery diameter/2)[2,14]. CMED was defined as a percent increase in CBF of ≤50%, and epicardial coronary endothelial dysfunction as a percent decrease in coronary artery diameter of ≥20%, from baseline in response to a maximum dose of ACh[2,7,11]. To examine the dose-effect relationship, CMED was further subclassified into mild (percent change in CBF in response to a maximum dose of ACh of ≤50% but ≥0%) or severe (that of <0%)[7].

GREYSCALE AND VIRTUAL-HISTOLOGY INTRAVASCULAR ULTRASOUND

Greyscale and VH-IVUS images were obtained and analysed as described previously[2,3,15]. VH-IVUS-derived thin-capped fibroatheroma (TCFA) was defined as plaque burden of >40% with confluent necrotic core of >10% in contact with the vessel lumen on at least three consecutive VH-IVUS frames[13,16,17]. The high accuracy of VH-IVUS-derived tissue characterisation has been histologically validated in humans[18,19].

STATISTICAL ANALYSIS

Bonferroni's correction was used for multiple comparisons. Multivariate logistic regression analysis was performed to identify independent risk factors associated with the presence of TCFA. Potential variables based on the literature[13] with $p<0.20$ in the univariate analysis were included in multivariate logistic regression models. Statistical analyses were performed using SPSS Statistics, Version 25 (IBM Corp., Armonk, NY, USA) and GraphPad Prism version 6.00 (GraphPad Software, La Jolla, CA, USA). A two-tailed p-value of <0.05 was considered statistically significant.

Results

PATIENT CHARACTERISTICS

Between July 2005 and February 2018, a total of 190 patients, who were referred for evaluation of chest pain and found to have angiographically normal coronary arteries or non-obstructive

CAD (<40% stenosis), underwent comprehensive invasive coronary endothelial function testing and VH-IVUS examination of the LAD. Forty-two patients were excluded because of the predefined reasons listed in **Supplementary Figure 1**. Of the remaining 148 patients, 87 (59%) met the criteria for CMED. As shown in **Table 1**, patients with CMED were slightly older and had a higher proportion of men as compared to those without. Other clinical characteristics were comparable between the two groups, including coronary risk factors, type of chest pain, medications, laboratory data, and left ventricular ejection fraction. In both groups, more than 60% were women, less than 10% had diabetes mellitus, approximately 60% had hyperlipidaemia, approximately 40% were obese, and a little less than 50% were on statin therapy. The plasma levels of fasting blood glucose, lipid profile, and a systemic inflammatory marker, high-sensitivity C-reactive protein, were unremarkable and comparable between the two groups.

Table 1. Clinical characteristics of the study patients.

	CMED (–) (n=61)	CMED (+) (n=87)	*p*-value
Age, years	50.8±11.0	54.5±10.0	0.033
Male, n (%)	8 (13.1)	29 (33.3)	0.005
Body mass index, kg/m^2	29.7±6.0	29.2±5.9	0.662
Coronary risk factors, n (%)			
Diabetes mellitus	3 (4.9)	8 (9.2)	0.329
Hypertension	29 (47.5)	50 (57.5)	0.233
Hyperlipidaemia	34 (55.7)	57 (65.5)	0.229
Family history	42 (68.9)	50 (57.5)	0.160
Smoking history	23 (37.7)	37 (42.5)	0.556
Obesity	28 (45.9)	32 (36.8)	0.266
Type of chest pain, n (%)			
Exertional	19 (31.1)	18 (20.7)	0.148
Resting	17 (27.9)	31 (35.6)	0.321
Both	25 (41.0)	38 (43.7)	0.744
Medications, n (%)			
ACEi/ARB	10 (16.4)	12 (13.8)	0.662
β-blocker	20 (32.8)	34 (39.1)	0.434
Calcium channel blocker	20 (32.8)	30 (34.5)	0.830
Nitrates	28 (45.9)	32 (36.8)	0.266
Statin	25 (41.0)	40 (46.0)	0.547
Aspirin	36 (59.0)	46 (52.9)	0.459
Oestrogen replacement therapy	8 (18.2)	5 (7.9)	0.138
Laboratory data			
Fasting blood glucose, mg/dL	100±14	101±22	0.780
Total cholesterol, mg/dL	188±45	181±41	0.318
LDL cholesterol, mg/dL	105±39	96±36	0.136
HDL cholesterol, mg/dL	61±18	59±19	0.489
Triglyceride, mg/dL	115±68	122±66	0.578
Creatinine, mg/dL	0.86±0.15	0.90±0.16	0.138
eGFR, mL/min/1.73 m^2	84±15	82±15	0.321
hs-CRP, mg/dL	0.14 (0.06-0.46)	0.18 (0.07-0.41)	0.839
Left ventricular EF, %	63±5	62±7	0.397

Values are mean±SD, median (IQR), or n (%). ACEi: angiotensin-converting enzyme inhibitor; ARB: angiotensin II receptor blocker; CMED: coronary microvascular endothelial dysfunction; EF: ejection fraction; eGFR: estimated glomerular filtration rate; HDL: high-density lipoprotein; hs-CRP: high-sensitivity C-reactive protein; LDL: low-density lipoprotein

COMPREHENSIVE INVASIVE CORONARY PHYSIOLOGY STUDY

As summarised in **Supplementary Table 1** and **Supplementary Figure 2**, patients with CMED showed abnormal endothelial function at both microvascular and epicardial levels. Endothelium-dependent CBF increases in response to ACh were evident in a dose-dependent manner in patients with normal coronary microvascular endothelial function, while those were completely abolished in patients with CMED **(Supplementary Figure 2A, Supplementary Figure 2B)**.

In contrast to these endothelium-dependent coronary vascular responses to ACh, endothelium-independent responses to nitroglycerine at both epicardial and microvascular levels were comparable between the two groups **(Supplementary Figure 2A-Supplementary Figure 2D)**. The percentages of patients with abnormal endothelium-independent CFR (<2.0) were also comparable between the two groups, although the mean CFR was slightly but significantly lower in patients with CMED than in those without **(Supplementary Table 1)**. Notably, most patients with abnormal CFR had CMED (n=7/10 [70.0%]: 3 had mild and 4 had severe CMED), whereas most patients with normal coronary microvascular endothelial function had normal CFR (n=58/61 [95.1%]). In addition, the above-mentioned differences in endothelium-dependent coronary vascular responses were not attributable to differences in basal coronary flow rate or haemodynamic parameters because baseline CBF, mean arterial pressure, and heart rate at each point were all comparable between the two groups **(Supplementary Table 1, Supplementary Figure 2E, Supplementary Figure 2F)**.

GREYSCALE INTRAVASCULAR ULTRASOUND FINDINGS

A total of 10,271 greyscale IVUS frames with corresponding VH-IVUS frames from 148 patients (arteries) were analysed. The mean number of analysed frames per artery and the mean length of analysed arteries were similar between the two groups **(Supplementary Table 1)**. Patients with CMED exhibited larger plaque volume index, plaque burden, and plaque thickness, as compared to those without **(Supplementary Table 1)**. When patients with CMED were further divided into mild or severe groups according to the severity of CMED, a dose-effect relationship was found between coronary microvascular endothelial function and IVUS-derived plaque characteristics; plaque burden and plaque volume index increased in accordance with the severity of CMED **(Figure 1A, Figure 1B)**. Change in CBF in response to a maximum dose of ACh showed a significant negative correlation with plaque burden ($r=-0.301$, $p<0.001$) and plaque volume index ($r=-0.287$, $p<0.001$) **(Figure 1C, Figure 1D)**.

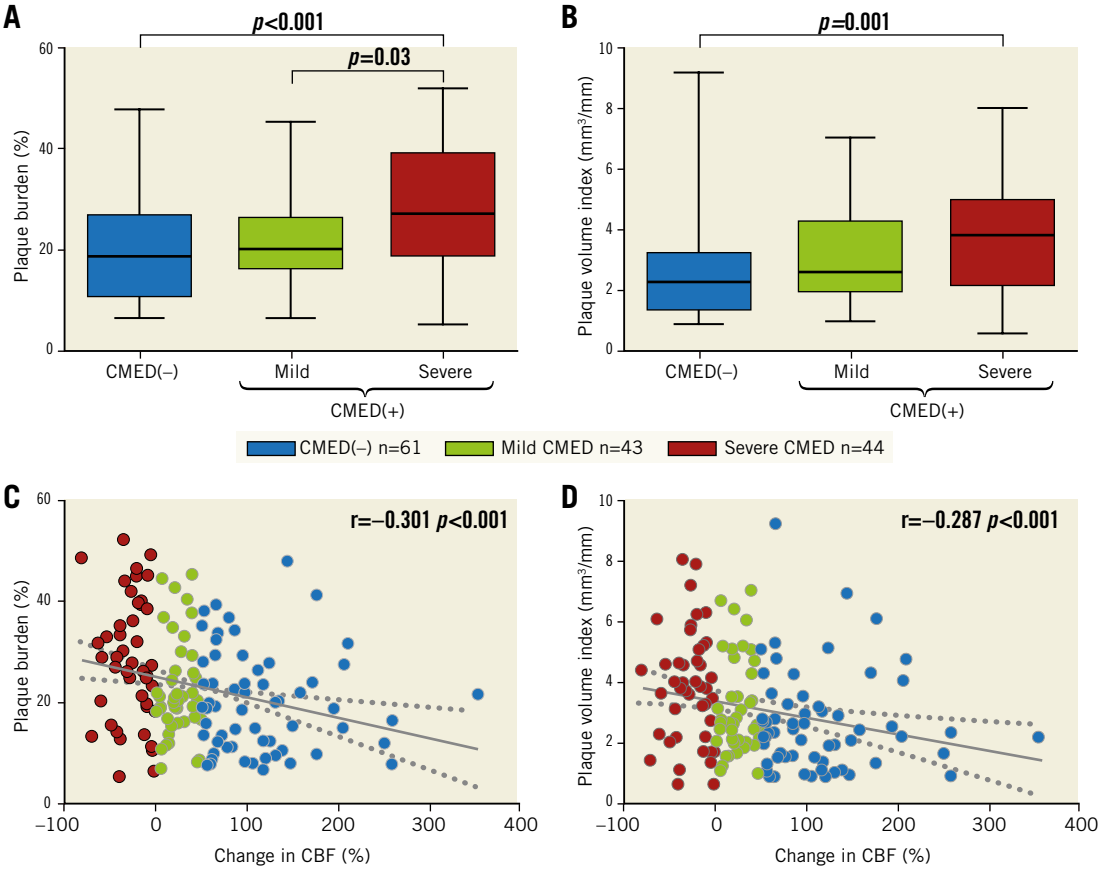

Figure 1. *Association of coronary microvascular endothelial function with plaque burden and plaque volume. A) & B) Greyscale IVUS-derived plaque burden and plaque volume index in box and whisker plots. C) & D) Correlations between change in CBF in response to a maximal dose of acetylcholine and plaque burden and plaque volume index. Dotted lines indicate 95% confidence intervals. CBF: coronary blood flow; CMED: coronary microvascular endothelial dysfunction*

VIRTUAL-HISTOLOGY INTRAVASCULAR ULTRASOUND FINDINGS

Representative images of VH-IVUS in the absence or presence of CMED are presented in **Figure 2A** and **Figure 2B**. Although both patients had angiographically silent atherosclerotic lesions with similar plaque burden and lumen area, they showed distinct differences in the plaque composition, as illustrated. Patients with CMED, as compared to those without, exhibited higher fibrous and necrotic core volume indices in accordance with the severity of CMED **(Supplementary Table 1, Figure 3A, Figure 3B)**. Moreover, patients with CMED had a higher frequency of imaged arteries containing at least one VH-IVUS-derived TCFA in a dose-effect dependent manner **(Supplementary Table 1, Figure 3C)**. In contrast, no association was found between endothelium-independent CFR and coronary plaque characteristics **(Supplementary Figure 3)**.

We performed additional analyses restricted to the coronary segments without epicardial coronary endothelial dysfunction in order to examine whether CMED was associated with more advanced plaque characteristics, independently of the presence of epicardial coronary endothelial dysfunction. A total of 409 segments from 107 patients (arteries) without epicardial coronary endothelial dysfunction were analysed **(Supplementary Figure 4A)**. As shown in **Supplementary Figure 4B**-**Supplementary Figure 4D**, analysed epicardial segments of LAD coronaries with CMED showed greater plaque burden with more vulnerable characteristics even in the absence of epicardial coronary endothelial dysfunction.

MULTIVARIATE LOGISTIC REGRESSION ANALYSIS

Finally, we performed a multivariate logistic regression analysis to identify predictors of the presence of VH-IVUS-derived TCFA. Among potential variables that might affect plaque vulnerability, CMED was an independent predictor of the presence of VH-IVUS-derived TCFA **(Table 2)**.

Discussion

The novel findings of the present study were twofold. First, CMED was associated with more advanced plaque characteristics in patients with chest pain and non-obstructive CAD even in the absence of epicardial coronary endothelial dysfunction. Second, CMED was an independent predictor of the presence of VH-IVUS-derived TCFA, which is characteristic of rupture-prone vulnerable plaques. These results indicate a potential role of

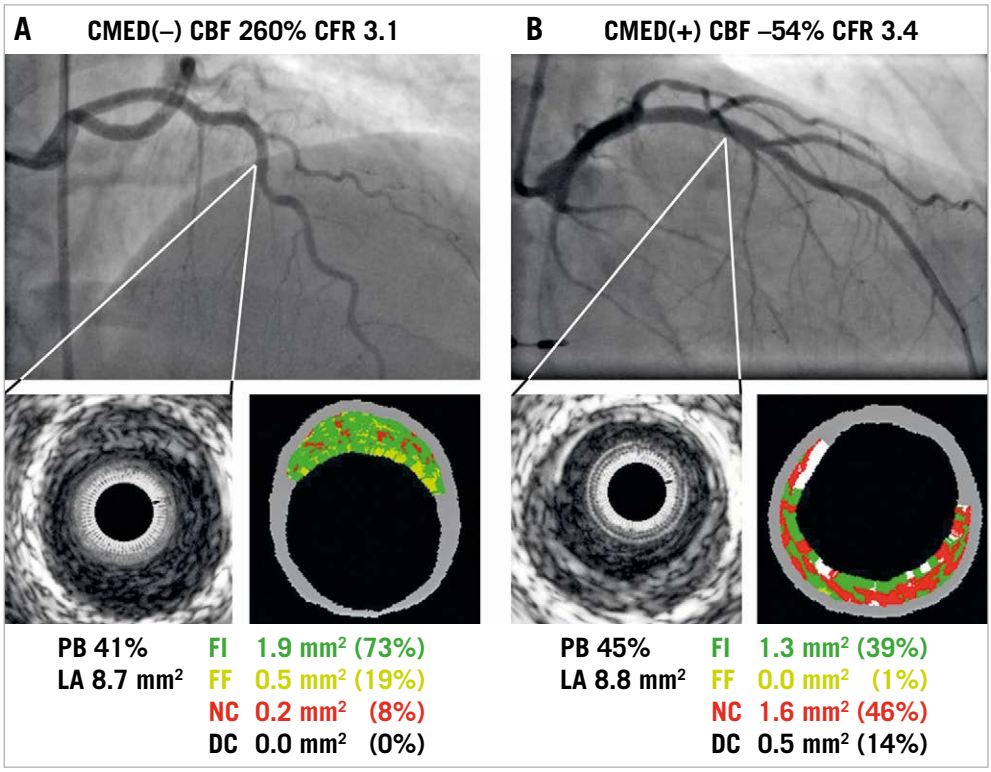

Figure 2. *Representative images of coronary angiography, greyscale and VH-IVUS in patients with or without CMED. A) A 53-year-old woman with chest pain showed no significant epicardial coronary artery stenosis and normal coronary microvascular endothelial function. VH-IVUS revealed an angiographically silent fibrous plaque. B) A 59-year-old woman with chest pain showed severe CMED in the absence of significant epicardial coronary artery stenosis and in association with VH-IVUS-derived thin-capped fibroatheroma. CBF: coronary blood flow; CFR: coronary flow reserve; CMED: coronary microvascular endothelial dysfunction; DC: dense calcium; FF: fibro-fatty; FI: fibrous; LA: lumen area; NC: necrotic core; PB: plaque burden*

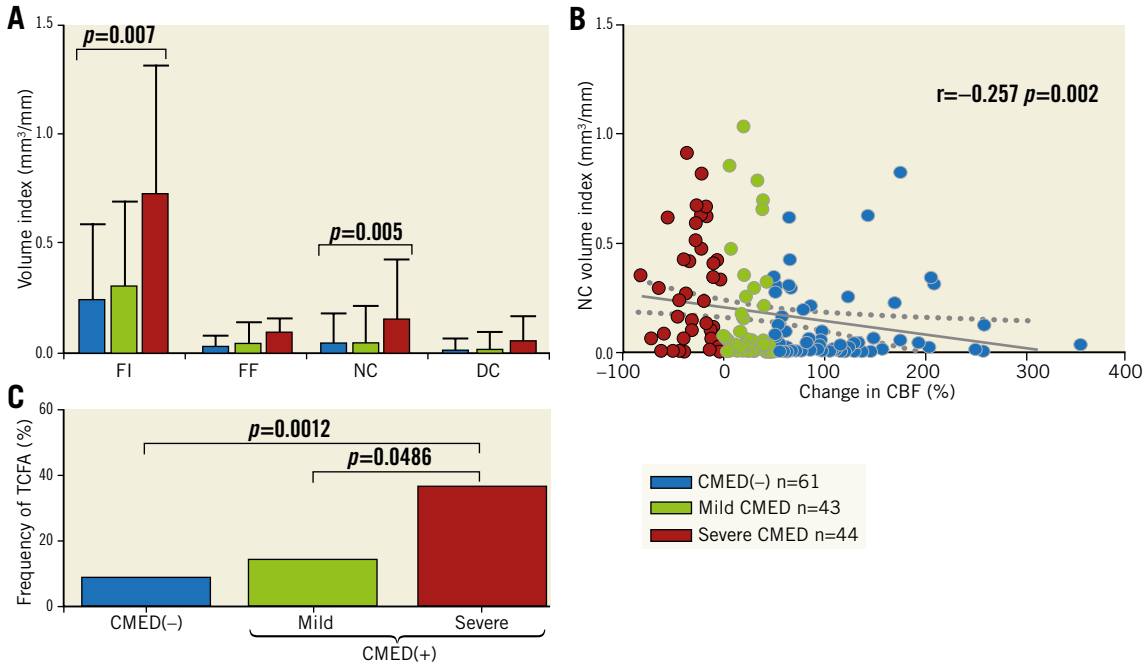

Figure 3. *Association of coronary microvascular endothelial function with plaque composition and vulnerability. A) VH-IVUS-derived tissue composition. Values are expressed as median with IQR. B) A correlation between change in CBF in response to a maximum dose of acetylcholine and VH-IVUS-derived NC volume index. C) Frequency of imaged arteries containing at least one VH-IVUS-derived TCFA. CBF: coronary blood flow; CMED: coronary microvascular endothelial dysfunction; DC: dense calcium; FF: fibro-fatty; FI: fibrous; NC: necrotic core*

Table 2. Predictors of the presence of TCFA.

Variables	Univariate		Multivariate 1		Multivariate 2	
	OR (95% CI)	*p*-value	OR (95% CI)	*p*-value	OR (95% CI)	*p*-value
Age	1.06 (1.01-1.11)	0.010	1.06 (1.01-1.11)	0.017	–	–
Male	2.51 (1.04-6.07)	0.041	2.86 (1.07-7.65)	0.037	–	–
Diabetes mellitus	1.77 (0.44-7.15)	0.425	–	–	–	–
Hypertension	1.97 (0.82-4.72)	0.130	–	–	1.45 (0.57-3.69)	0.442
Hyperlipidaemia	4.48 (1.46-13.8)	0.009	–	–	3.38 (1.06-10.8)	0.039
Statins	1.48 (0.64-3.41)	0.360	–	–	–	–
CFR <2.0	1.13 (0.23-5.65)	0.882	–	–	–	–
CMED	2.64 (1.51-4.61)	0.001	2.28 (1.27-4.11)	0.006	2.28 (1.30-4.01)	0.004

Variables with a *p*-value of <0.20 in the univariate analysis were included in each multivariate logistic regression model. CFR: coronary flow reserve; CI: confidence interval; CMED: coronary microvascular endothelial dysfunction; OR: odds ratio; TCFA: thin-capped fibroatheroma

CMED in the progression of coronary atherosclerosis in the early stage of the disease.

Patients with CMED, whether accompanied by epicardial coronary endothelial dysfunction or not, showed more vulnerable plaque characteristics as compared to those without. We have previously demonstrated associations of segmental epicardial coronary endothelial dysfunction with early structural changes in the local vascular wall in patients with non-obstructive CAD using multimodality imaging. Local coronary segments with epicardial endothelial dysfunction showed more advanced characteristics and accelerated progression of the coronary plaques as compared to those without[2,3]. Such anatomical links at the epicardial level can account for the development and progression of the site-specific coronary plaques in the early stage of the disease. However, the role of CMED in this context was not fully investigated. One possible mechanism for the association of CMED with advanced plaque characteristics can be derived from our recent observation that CMED is associated with low endothelial shear stress in the epicardial coronary artery[11]. Steady laminar or pulsatile shear stress provides atheroprotective effects on the vascular wall and, conversely, altered oscillatory or low shear stress with disturbed flow promotes atherogenesis through endothelial and vascular smooth muscle cell proliferation, inflammation, lipoprotein uptake, and leukocyte adhesion. Indeed, altered shear stress on the coronary artery wall, whether high or low, has been implicated in the local progression of atherosclerotic coronary plaque[20]. In the present study, a mechanistic role was, at least in part, attributable to the microcirculation because the same coronary segments with downstream CMED showed more advanced plaque characteristics even in the absence of epicardial coronary endothelial dysfunction **(Supplementary Figure 4)**. Taken together, these observations may provide insight into the underlying mechanisms by which CMED, probably in association with altered patterns of endothelial shear stress, contributes to the development of epicardial coronary atherosclerosis, even though these focal lesions are located upstream to the microcirculation.

The second significant observation of this study was that CMED was an independent predictor of VH-IVUS-derived TCFA in patients with chest pain and non-obstructive CAD. The patients in both groups showed an angiographically similar degree of CAD; however, they showed distinct differences in the angiographically silent plaque characteristics. Patients with CMED exhibited a greater volume of necrotic core and a higher incidence of VH-IVUS-derived TCFA in a dose-effect dependent manner. VH-IVUS-derived TCFA emerged as a surrogate for plaque vulnerability; its clinical importance as a precursor of future coronary events has been repeatedly reproduced[16,21]. Moreover, this study builds on a recent report showing that coronary microvascular dysfunction is independently associated with a higher prevalence of optical coherence tomography-defined TCFA in patients with advanced coronary lesions, albeit a different method (index of microcirculatory resistance) was used to evaluate coronary microvascular function[22]. Unlike our study, an endothelium-independent CFR value of <2.0, as evaluated by the same invasive method as our study, was shown to be an independent predictor of the presence of VH-IVUS-derived TCFA in a prior study[13]. This study did not assess endothelium-dependent coronary microvascular function and included patients with more advanced stages of CAD including acute coronary syndrome with resultant differences in the predictive value of CFR for VH-IVUS-derived TCFA. Considering the possibility that CMED might precede epicardial coronary endothelial dysfunction as well as endothelium-independent coronary microvascular dysfunction in the early phase of CAD[11], CMED may enable us to capture patients at risk for future coronary events at an earlier stage of the disease than does endothelium-independent coronary microvascular dysfunction.

CLINICAL IMPLICATIONS: PRIMARY CORONARY MICROCIRCULATORY DYSFUNCTION

This study supports the novel concepts of "primary coronary microcirculatory dysfunction"[23] and the "vulnerable patient"[24] and provides important implications for practice and research. Patients with chest pain but without angiographical abnormalities are often underdiagnosed and offered no specific treatment or follow-up under the umbrella of "normal" coronary arteries. Contrary to this

otherwise common practice, patients with CMED may be at opposite poles; they may be predisposed to the development of more vulnerable coronary atherosclerosis and thus likely destined for future coronary events[7,8]. Identifying patients at risk for future coronary events could provide significant benefit if they have CMED and may provide physicians with useful information for decision making and risk stratification beyond conventional coronary risk factors. It may be speculated that the development of novel therapies to improve CMED will attenuate the progression of coronary atherosclerosis and that patients with CMED may benefit from early aggressive medical management aimed at improving endothelial function and atherosclerosis upon detection of CMED. To this end, a comprehensive invasive assessment of coronary physiology has been shown to be feasible and of diagnostic value[4,6,9]. Further research is needed to address how to modulate CMED to improve clinical outcomes of patients with this condition and whether decision making driven by CMED benefits them.

Study limitations

Several limitations should be mentioned in this study. First, although the sample size was relatively large and derived from the prospective database, the results obtained from this retrospective, single-centre, cross-sectional study should be regarded as hypothesis-generating rather than definite evidence. Further research is warranted, especially to establish detailed pathophysiological mechanisms and the cause-effect relationship between CMED and vulnerable plaque characteristics. Second, some of the patient characteristics in the present study were not typical for patients with CAD in general; more than 60% were women and fewer than 10% had diabetes mellitus. Although these characteristics were compatible with those of prior studies that enrolled similar patients with chest pain and non-obstructive CAD[4,25,26], a generalisation of the present findings to a wider range of patients with CAD awaits further investigation. Third, ischaemia-inducing testing was not routinely performed in the study patients. However, we have previously demonstrated that contemporary non-invasive stress tests have limited diagnostic accuracy for detecting coronary microvascular dysfunction in patients with chest pain and non-obstructive CAD[9,27]. Fourth, a previous study suggested the usefulness of the combined use of VH-IVUS and optical coherence tomography to detect TCFA accurately *in vivo*[28]. The gold standard for assessment of plaque composition is true histology, which obviously cannot be performed *in vivo*. However, multiple studies have demonstrated the high predictive accuracy of VH-IVUS in classifying coronary plaque components and phenotypes, including TCFA[18,29]. Moreover, a large clinical trial has linked VH-IVUS-defined plaque characteristics to adverse clinical outcomes[16].

Conclusions

This study highlighted that, independently of conventional coronary risk factors and epicardial coronary endothelial dysfunction, CMED was associated with vulnerable plaque characteristics in patients with early coronary atherosclerosis.

Impact on daily practice

This study shows that CMED is not uncommon and associated with more advanced plaque characteristics in patients with non-obstructive CAD. CMED is an independent predictor of the presence of VH-IVUS-derived TCFA, which is characteristic of rupture-prone vulnerable plaques. A comprehensive invasive assessment of coronary endothelial function is feasible and of diagnostic value to detect patients with CMED.

Acknowledgements

We appreciate the effort of the members of the Mayo Clinic Cardiac Catheterization Laboratory and especially the registered cardiovascular invasive specialist Jonella M. Tilford for her excellent technical assistance. Appreciation is also extended to the MSD Life Science Foundation, Public Interest Incorporated Foundation scholarship, Japan.

Funding

This work was supported by the Mayo Foundation.

Conflict of interest statement

The authors have no conflicts of interest to declare.

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Supplementary data

Supplementary Appendix 1. Detailed methods.

Supplementary Figure 1. Flow chart showing the enrolment of the study patients.

Supplementary Figure 2. Endothelium-dependent and endothelium-independent coronary vascular responses.

Supplementary Figure 3. No association of endothelium-independent coronary microvascular function with plaque composition and vulnerability.

Supplementary Figure 4. Association of coronary microvascular endothelial function with plaque characteristics in coronary segments without epicardial endothelial dysfunction.

Supplementary Table 1. Coronary artery characteristics of the study patients.

The supplementary data are published online at: https://eurointervention.pcronline.com/doi/10.4244/EIJ-D-19-00265

Supplementary data

Supplementary Appendix 1. Detailed methods

Study design and population

This retrospective, single-centre, cross-sectional study was performed at the Mayo Clinic in Rochester, MN, USA, using a prospective database of the Mayo Clinic Cardiac Catheterization Laboratory. This database had been enrolling all patients who underwent diagnostic coronary angiography and invasive coronary endothelial function testing since December 1992. Inclusion and exclusion criteria were as described previously. Between July 2005, when virtual-histology intravascular ultrasound (VH-IVUS) data became available, and February 2018, consecutive patients with angiographically normal coronary arteries or non-obstructive coronary artery disease (CAD) who underwent both invasive coronary endothelial function testing and VH-IVUS examination in the left anterior descending coronary artery (LAD) for evaluation of chest pain were enrolled. Patients with the following were excluded: acute coronary syndrome, ≥40% angiographic stenosis of any coronary artery, prior coronary artery bypass graft, prior percutaneous coronary intervention to the LAD, prior Q-wave myocardial infarction, vasospastic angina, valvular heart disease, uncontrolled hypertension, peripheral vascular disease, and estimated glomerular filtration rate of <30 mL/min/1.73 m^2. Vasospastic angina was defined as transient total or subtotal coronary artery occlusion (>90% constriction) with angina and ischaemic electrocardiogram changes in response to acetylcholine (ACh). Some of the study patients were included in our previous studies. This study complies with the Declaration of Helsinki and was approved by the Mayo Clinic Institutional Review Board. All patients provided written informed consent.

Medical records and the prospective database, including the results of routine laboratory tests and

echocardiography, were reviewed to obtain clinical information. Diabetes mellitus was defined as a fasting blood glucose of ≥126 mg/dL or the current use of insulin or hypoglycaemic agents; hypertension, blood pressure of ≥140/90 mmHg or the current use of antihypertensive agents; hyperlipidaemia, a total cholesterol of ≥200 mg/dL or the current use of lipid-lowering agents; a positive family history, the presence of premature CAD in at least one first-degree relative; a positive smoking history, former or current tobacco smoking; obesity, body mass index of ≥30 kg/m^2.

Comprehensive invasive assessment of coronary physiology

Invasive assessment of endothelium-dependent and endothelium-independent coronary vascular responses in the LAD was performed in a comprehensive manner, as described previously. The LAD was selected because of accessibility and supplying the largest territory of the myocardium. All patients withheld calcium channel blockers and long-acting nitrates for at least 36−48 hours prior to catheterisation for the assessment of baseline coronary physiology. Using a 0.014-inch Doppler guidewire (FloWire®; Philips Volcano, Rancho Cordova, CA, USA) within a 2.2 Fr coronary infusion catheter (Ultrafuse; SciMed Life Systems, Minneapolis, MN, USA) placed in the middle LAD, coronary flow reserve (CFR) was obtained at maximum hyperaemia induced by intracoronary bolus injections of adenosine at incremental doses (18 to 72 μg). This CFR reflects predominantly endothelium-independent vasodilatation in the microcirculation by adenosine. A CFR value of <2.0 was considered to be abnormal. Then, endothelium-dependent coronary vascular reactivity was assessed by selective infusion of incremental doses of ACh into the LAD at 10^{-6}, 10^{-5}, and 10^{-4} mol/L. Doppler measurements and coronary angiography were obtained at baseline and at each dose with close monitoring of patient symptoms, arterial blood pressure,

heart rate, and electrocardiogram. The infusion of ACh was stopped before reaching the final dose for safety at the discretion of the attending physician when patients experienced significant chest pain in association with ischaemic electrocardiogram changes and severe vasoconstriction on angiography. Finally, an intracoronary bolus of nitroglycerine (100−200 μg) was injected into the LAD to examine endothelium-independent responses.

Doppler flow velocity spectra were analysed to determine the time-averaged peak velocity. Coronary blood flow (CBF) was calculated by the following equation: CBF= π(time-averaged peak velocity)(coronary artery diameter/2) [2]. All measurements were performed by an independent investigator in the segments 5 mm distal to the tip of the Doppler guidewire offline using a quantitative coronary angiography program (Medis, Leiden, the Netherlands) in order to ensure consistency. CMED was defined as the percent increase in CBF of ≤50%, and epicardial coronary endothelial dysfunction as the percent decrease in coronary artery diameter of ≥20%, from baseline in response to a maximum dose of ACh. To examine the dose-effect relationship, CMED was further subclassified into mild (percent change in CBF in response to a maximum dose of ACh of ≤50% but ≥0%) or severe (that of <0%).

Greyscale and virtual-histology intravascular ultrasound

Greyscale and VH-IVUS images were obtained and analysed as previously described. After intracoronary administration of nitroglycerine, a 20-MHz, 2.9 Fr phased-array IVUS catheter (Eagle Eye® Gold; Philips Volcano) was advanced into the middle LAD and pulled back to the coronary ostium at a speed of 0.5 or 1.0 mm/s during the electrocardiogram-gated image acquisition at the peak of the R wave. Quantitative measurements were performed for each frame

in accordance with the American College of Cardiology Clinical Expert Consensus Document on IVUS to obtain vascular volume, lumen volume, plaque volume, plaque burden, plaque thickness, minimum lumen area, and remodelling index. Vascular volume was defined as external elastic membrane (EEM) cross-sectional area (CSA) multiplied by length; plaque volume, vascular volume minus lumen volume; plaque burden, plaque area divided by EEM CSA; remodelling index, lesion EEM CSA divided by reference EEM CSA.

VH-IVUS-derived tissue composition was characterised as fibrous (FI), fibro-fatty (FF), necrotic core (NC), and dense calcium (DC), and was expressed as absolute values and percentages of all four composition types. VH-IVUS-derived thin-capped fibroatheroma (TCFA) was defined as plaque burden of >40% with confluent NC of >10% in contact with the vessel lumen on at least three consecutive VH-IVUS frames. The high accuracy of VH-IVUS-derived tissue characterisation is histologically validated in humans. Despite not having a high enough resolution to visualise a thin fibrous cap (<65 μm), VH-IVUS can identify TCFA with as high diagnostic accuracy as optical coherence tomography.

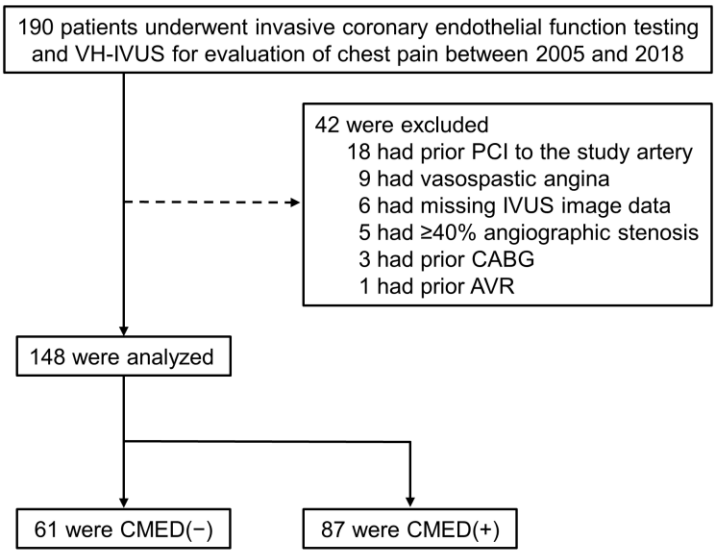
Frames with poor image quality, a minimum lumen diameter of ≤2.0 mm, or a major side branch were discarded. All measurements were averaged per artery and per segment as defined by the section of vessel bordered by two side branches. All volumetric data were divided by the length in order to compensate for differences in length of each examined artery and expressed as a volume index (mm^3/mm). Two experienced investigators (S. Godo and M.T. Corban) performed offline analysis of IVUS images in a blinded and independent fashion with respect to the clinical information, using pcVH version 2.2, Volcano Image Analysis Software version 3.1, and Volcano s5i Imaging System (Volcano Corporation, Rancho Cordova, CA, USA). The inter- and intra-

observer agreement were well validated as described elsewhere.

Statistical analysis

Values are expressed as mean±SD, median (IQR), or n (%). Differences between two groups were compared using the Student's t-test, Mann-Whitney U test, or Pearson's chi-square test as appropriate. Bonferroni's correction was used for multiple comparisons. Correlations between two variables were assessed using Pearson's or Spearman's r correlation coefficient as appropriate. Dose-response curves between groups were compared by two-way analysis of variance (ANOVA), followed by Bonferroni's multiple comparisons test. Multivariate logistic regression analysis was performed to identify independent risk factors associated with the presence of TCFA. Potential variables based on the literature with $p<0.20$ in the univariate analysis were included in multivariate logistic regression models. Statistical analyses were performed using SPSS Statistics, Version 25 (IBM Corp., Armonk, NY, USA) and GraphPad Prism version 6.00 (GraphPad Software, La Jolla, CA, USA). A two-tailed p-value of <0.05 was considered statistically significant.

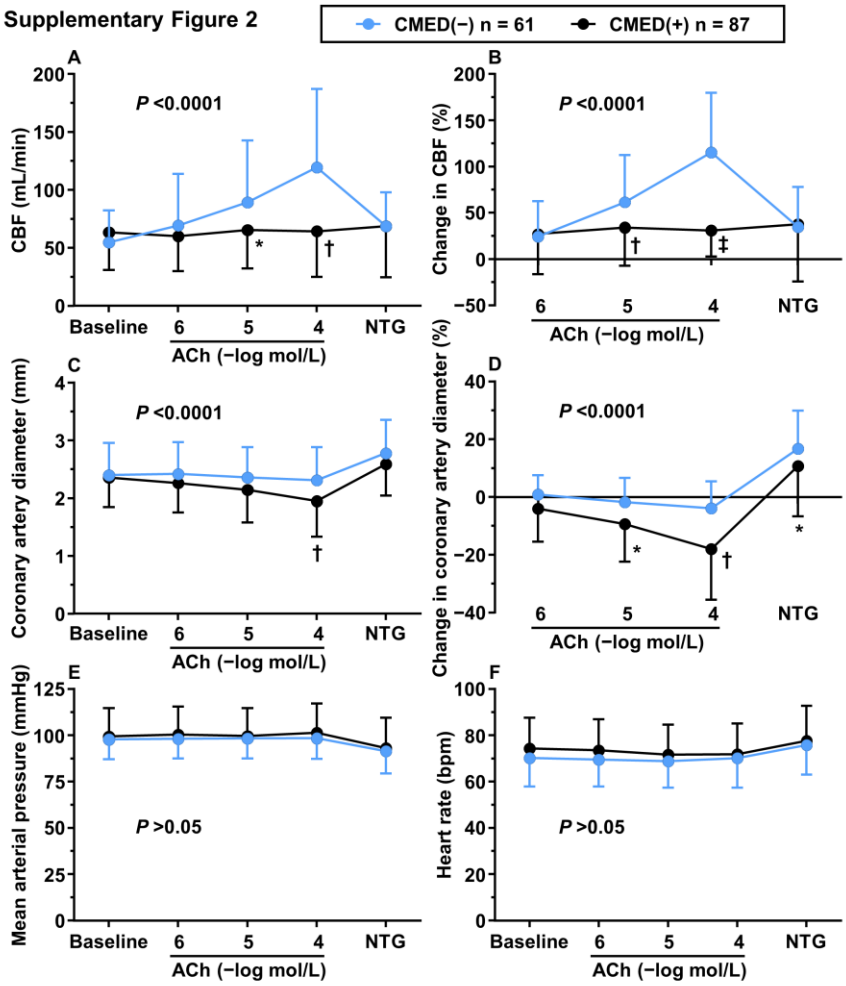
Supplementary Figure 1



Supplementary Figure 1. Flow chart showing the enrolment of the study patients.

The flow diagram summarises the selection process of the study population.

AVR: aortic valve replacement; CABG: coronary artery bypass graft; CMED: coronary microvascular endothelial dysfunction; IVUS: intravascular ultrasound; PCI: percutaneous coronary intervention; VH-IVUS: virtual-histology IVUS



Supplementary Figure 2. Endothelium-dependent and endothelium-independent coronary vascular responses.

A) & B) Endothelium-dependent and endothelium-independent CBF responses to ACh and NTG, respectively.

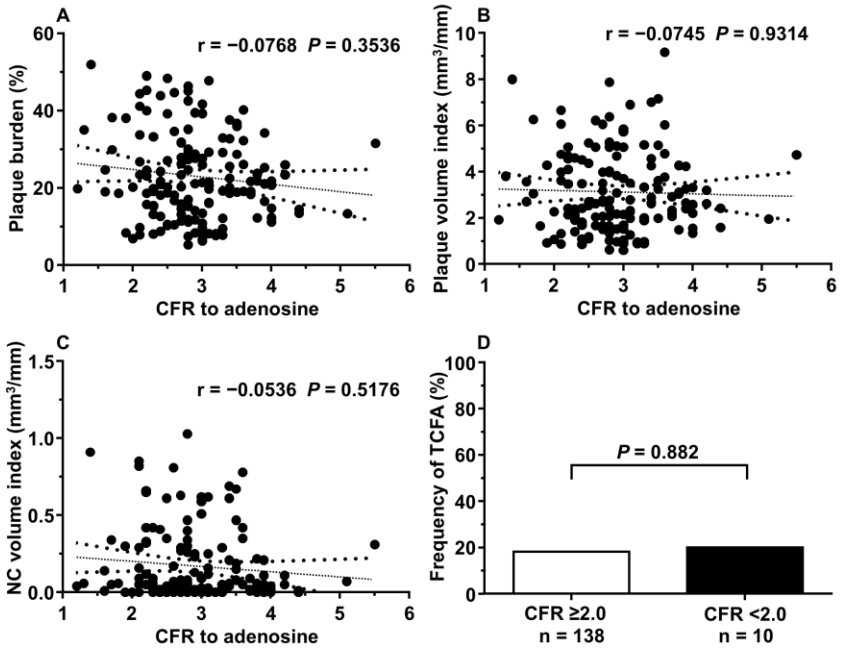
C) & D) Endothelium-dependent and endothelium-independent epicardial coronary artery diameter changes to ACh and NTG, respectively.

E) & F) Haemodynamic data. Values are expressed as mean±SD.

P-values are from two-way ANOVA of CMED(−) vs CMED(+). **P*<0.05, †*P*<0.001, ‡*P*<0.0001 vs CMED(−).

ACh: acetylcholine; bpm: beats per minute; CBF: coronary blood flow; CMED: coronary microvascular endothelial dysfunction; NTG: nitroglycerine

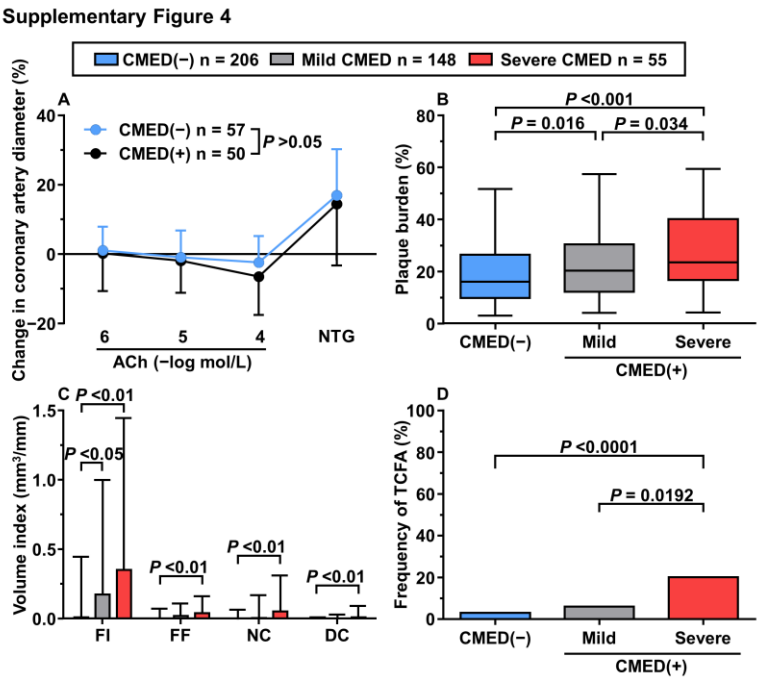
Supplementary Figure 3



Supplementary Figure 3. No association of endothelium-independent coronary microvascular function with plaque composition and vulnerability.

Correlations between CFR to adenosine and plaque burden (A), plaque volume index (B), and NC volume index (C). Dotted lines indicate 95% confidence intervals. D) Frequency of imaged segments containing VH-IVUS-derived TCFA.

CFR: coronary flow reserve; NC: necrotic core



Supplementary Figure 4. Association of coronary microvascular endothelial function with plaque characteristics in coronary segments without epicardial endothelial dysfunction.

A) – D) Patients with epicardial coronary endothelial dysfunction (n=41) were excluded; the per-segment analyses were performed in the remaining 107 patients with 409 coronary segments.

A) Endothelium-dependent and endothelium-independent epicardial coronary artery diameter changes to ACh and NTG, respectively. Values are expressed as mean±SD. The p-value is from two-way ANOVA of CMED(−) vs CMED(+).

B) Greyscale IVUS-derived plaque burden in box and whisker plots.

C) VH-IVUS-derived tissue composition. Values are expressed as median with IQR.

D) Frequency of imaged segments containing VH-IVUS-derived TCFA.

ACh: acetylcholine; bpm: beats per minute; CMED: coronary microvascular endothelial dysfunction; DC: dense calcium; FF: fibro-fatty; FI: fibrous; LA: lumen area; NC: necrotic core; NTG: nitroglycerine

Supplementary Table 1. Coronary artery characteristics of the study patients.

	CMED (−) (n=61)	CMED (+) (n=87)	*p*-value
Epicardial coronary endothelial function			
Change in coronary artery diameter in response to ACh, %	−4.2±9.4	−18.2±16.8	<0.001
Endothelial dysfunction, n (%)	4 (6.6)	37 (42.5)	<0.001
Diameter stenosis by QCA, %	14.7±9.5	15.3±10.9	0.692
Coronary physiology measurements			
Baseline CBF, mL/min	54.9±27.3	63.4±32.6	0.099
Change in CBF in response to ACh, %	114.5±63.7	−1.7±31.5	<0.001
CBF in response to ACh, mL/min	117.4±68.0	62.6±39.7	<0.001
CBF in response to NTG, mL/min	68.7±29.1	69.0±44.3	0.969
CFR	3.1±0.8	2.8±0.7	0.006
CFR <2.0, n (%)	3 (4.9)	7 (8.0)	0.456
Greyscale IVUS findings			
Number of frames/arteries	4,118/61	6,153/87	
Length, mm	34.1±15.1	38.0±18.0	0.160
Vascular volume index, mm^3/mm	12.6±3.0	13.1±3.2	0.333
Lumen volume index, mm^3/mm	10.0±2.4	9.6±2.8	0.442
Plaque volume index, mm^3/mm	2.6±1.6	3.5±1.8	0.003
Plaque burden, %	19.7±10.1	25.2±11.5	0.002
Plaque thickness, mm	0.37±0.20	0.48±0.23	0.003

Remodelling index	1.08±0.10	1.11±0.15	0.139
Maximum plaque burden, %	37.2±14.4	43.9±14.3	0.004
Maximum plaque thickness, mm	0.71±0.40	0.90±0.37	0.006
Minimum lumen area, mm^2	6.4±2.3	6.1±2.4	0.559
Virtual-histology IVUS findings			
Fibrous, mm^3/mm	0.24 (0.02–0.58)	0.42 (0.13–0.93)	0.004
Fibro-fatty, mm^3/mm	0.03 (0.00–0.08)	0.07 (0.02–0.15)	0.003
Necrotic core, mm^3/mm	0.04 (0.00–0.17)	0.07 (0.01–0.34)	0.017
Dense calcium, mm^3/mm	0.01 (0.00–0.06)	0.02 (0.00–0.14)	0.053
Fibrous, %	19.0 (5.7–40.1)	33.9 (14.4–52.0)	0.014
Fibro-fatty, %	2.6 (0.3–5.6)	4.0 (1.9–7.1)	0.008
Necrotic core, %	2.9 (0.7–8.5)	4.4 (1.8–14.6)	0.031
Dense calcium, %	0.8 (0.1–3.1)	1.1 (0.2–6.3)	0.149
TCFA, n (%)	5 (8.2)	22 (25.3)	0.008

Values are mean±SD, median (IQR), or n (%).

ACh: acetylcholine (at a maximum dose); CBF: coronary blood flow; CFR: coronary flow reserve; CMED: coronary microvascular endothelial dysfunction; IVUS: intravascular ultrasound; NTG: nitroglycerine; QCA: quantitative coronary angiography; TCFA: thin-capped fibroatheroma