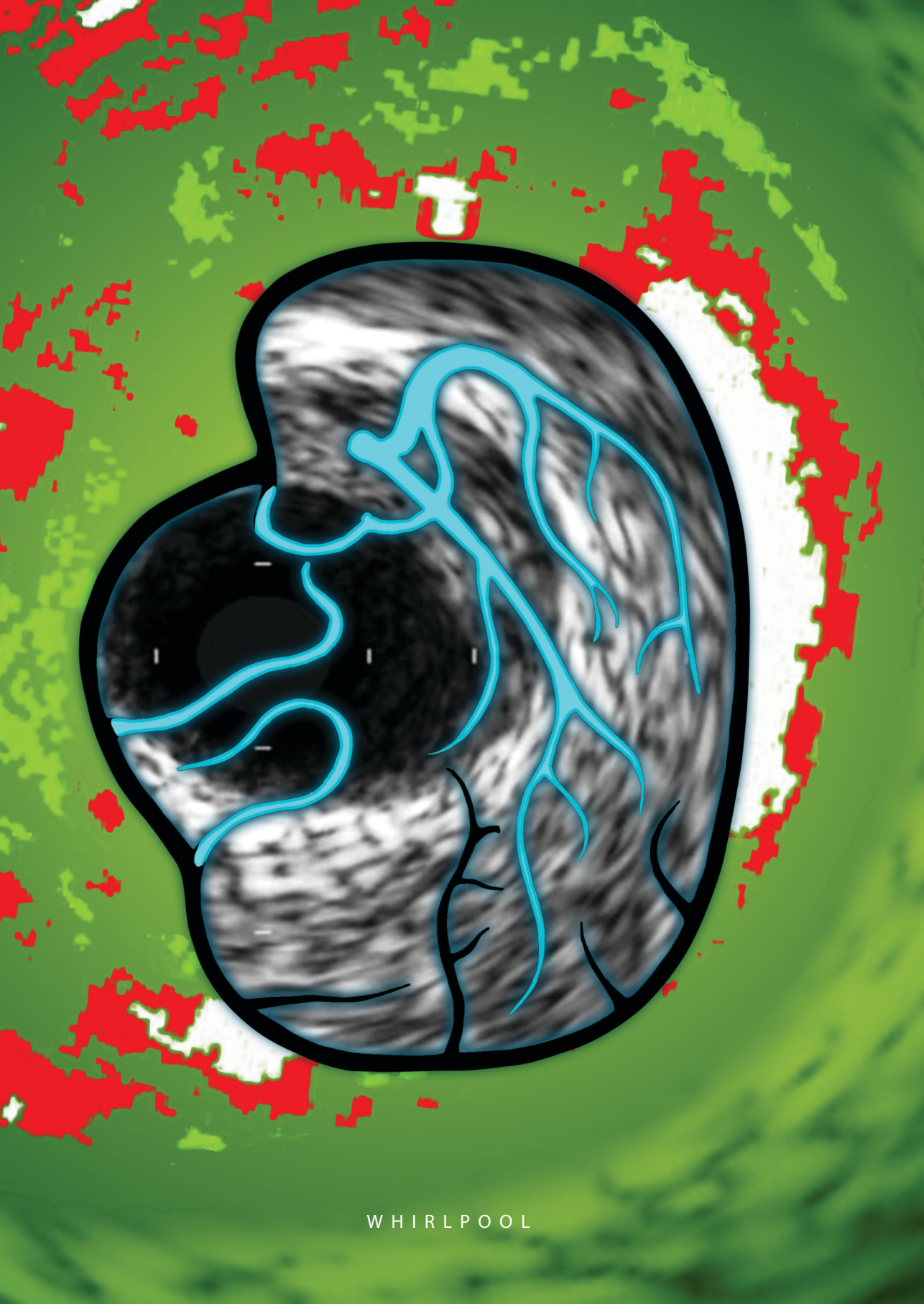
WHIRLPOOL

Part III

IMPLICATIONS OF RENAL FUNCTION FOR ISCHEMIC HEART DISEASE

CHAPTER 10

Plasma Cystatin C and Neutrophil Gelatinase-Associated Lipocalin in Relation to Coronary Atherosclerosis on Intravascular Ultrasound and Cardiovascular Outcome Impact of Kidney Function The AtheroRemo-IVUS Study

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ABSTRACT

Background

We investigated whether plasma cystatin C (CysC) and neutrophil gelatinase-associated lipocalin (NGAL) are associated with intravascular ultrasound (IVUS)-derived characteristics of coronary atherosclerosis and 1-year adverse coronary events in patients with normal and mildly-to-moderately impaired kidney function.

Methods

Between 2008-2011, virtual histology (VH)-IVUS of a non-culprit coronary artery was performed in 581 patients undergoing coronary angiography. Creatinine, CysC and NGAL were measured in pre-procedural blood samples. Presence of VH-IVUS-derived thin-cap fibroatheroma (TCFA) lesions, lesions with plaque burden (PB)≥70% and lesions with minimal luminal area (MLA)≤4 mm^2 was assessed. Major adverse coronary events (MACE) comprised the composite of all-cause mortality, acute coronary syndrome, or unplanned coronary revascularization. Analyses were stratified using $eGFR_{Cr}$ of 90 ml/min/1.73m^2 as the cut-off.

Results

In patients with normal kidney function, those with higher CysC levels had fewer lesions with PB≥70% and fewer VH-TCFA lesions (adjusted odds ratios(ORs) and 95% confidence intervals(CIs): 0.46 [0.30-0.69] and 0.59 [0.44-0.83], respectively, per standard deviation(SD) ln[ng/mL] CysC). Those with higher NGAL levels also had fewer lesions with PB≥70% (adjusted OR [95%CI]: 0.49 [0.29-0.82]) In patients with impaired kidneys, no differences in high-risk lesions were observed for CysC or NGAL. However, those with higher CysC had higher risk of MACE (hazard ratio(HR): 1.4, 95%CI [1.03–1.92]). This was not the case in patients with normal kidney function. NGAL did not influence risk of MACE.

Conclusions

Mild-to-moderate kidney dysfunction modifies the relationship between CysC and high-risk coronary lesions. This has not been established before, and offers an explanation for the difference in findings between experimental and epidemiologic studies.

INTRODUCTION

Kidney impairment, as assessed by creatinine-based equations of glomerular filtration rate ($eGFR_{Cr}$), is associated with cardiovascular disease independently of established cardiovascular risk factors.[1] In persons with mild kidney dysfunction ($eGFR_{Cr}$ in the range of 60-89 ml/min/1.73m^2), cystatin C (CysC) may outperform $eGFR_{Cr}$ as a predictor of adverse outcome. This is illustrated by the fact that CysC displays a linear association with mortality in patients with such mild GFR reduction, while $eGFR_{Cr}$ has a J-shaped association with mortality, and risk only starts to rise when $eGFR_{Cr}$ falls beneath 60 ml/min/1.73m^2.[2,3] Although some studies have shown linear associations of $eGFR_{Cr}$ with adverse outcome, these associations were linear only in particular ranges of $eGFR_{Cr}$ (specifically, $eGFR_{Cr}$ above 60).[4]

CysC is a cysteine protease inhibitor produced by most nucleated cells, and can be detected in serum or plasma.[5] In in-vitro and animal experiments, a reduction of CysC correlated with increased activity of cysteine proteases cathepsins K and S, which led to breakdown of the elastic lamina in the blood vessel wall.[6] Altered CysC expression has been identified in diseases which progress by extracellular proteolysis, such as atherosclerosis and aortic aneurysms, and metastasis.[7,8] These experiments, pointing towards a favourable role for CysC, do not concur with the positive associations of CysC with adverse outcomes found in epidemiological studies. Studies on the in-vivo association between plasma CysC and coronary atherosclerosis may provide further insight into this discrepancy, but have not yet been performed.

Neutrophil gelatinase-associated lipocalin (NGAL) is a clinically relevant biomarker in acute kidney injury[9] due to its marked increase in plasma and urine after tubulointerstitial kidney damage.[10] Recently, overexpression of plasma NGAL has been found in coronary plaques, where NGAL inhibits elimination of matrix metalloproteinase–9 (MMP-9).[11,12] MMP-9 is involved in extracellular matrix degradation, herewith increasing the risk of plaque rupture.[13] NGAL and NGAL/MMP-9 complex have been shown to predict major adverse cardiovascular events in epidemiological studies.[14,15]

In spite of the above-described associations that have been demonstrated between CysC, NGAL and adverse cardiac events, the presence and shape of a relationship between plasma CysC, NGAL, and coronary atherosclerosis have not yet been investigated in-vivo. To the best of our knowledge, we are the first to perform such an investigation, and to herewith provide a link between fundamental experiments and epidemiological studies. Specifically, our study aimed to investigate whether plasma CysC and NGAL are associated with IVUS-derived characteristics of in-vivo coronary atherosclerosis and 1-year adverse coronary events in patients with normal and mildly-to-moderately impaired kidney function.

MATERIALS AND METHODS

Study population

We have previously described the design of The European Collaborative Project on Inflammation and Vascular Wall Remodeling in Atherosclerosis - Intravascular Ultrasound (ATHEROREMO-IVUS).[16] In this study, we included 581 patients undergoing diagnostic coronary angiography or percutaneous coronary intervention (PCI) for acute coronary syndrome (ACS) or stable angina pectoris (SAP) between 2008 and 2011 in the Erasmus MC, Rotterdam, the Netherlands. Following coronary angiography, intravascular ultrasound (IVUS) of a non-culprit coronary artery was performed. The human research ethics committee of Erasmus MC, Rotterdam, the Netherlands has approved this study. All included patients have signed informed consent, and the study protocol conformed to the Declaration of Helsinki. This study is registered in ClinicalTrials.gov (number: NCT01789411).

Kidney function assessment

Estimated Glomerular Filtration Rate ($eGFR_{Cr}$) was assessed by the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation.[17] Patients were categorized according to eGFR by using the modified definition from the National Kidney Foundation – Kidney Disease Outcome Quality Initiative (K/DOQI) clinical practice guidelines[18]: normal (GFR≥90 ml/min/1.73m^2), mild (GFR 60-89 ml/min/1.73m^2), moderate (GFR 30-59 ml/min/1.73m^2), and severe (GFR 15-29 ml/min/1.73m^2) kidney dysfunction, and kidney failure (GFR<15 ml/min/1.73m^2). No patients with kidney failure were present in this study, and only one patient had $eGFR_{Cr}$ <30 ml/min/1.73m^2. The latter was excluded from further analyses. Patients were stratified into those with normal kidney function and those with mildly-to-moderately impaired kidney function, using an $eGFR_{Cr}$ of 90 ml/min/1.73m^2 as the cut-off value.

Biomarkers

Arterial blood was taken before the procedure and stored at -80 °C within two hours. Samples were available in 570 patients. An immunoturbidimetric high sensitivity assay (Roche Diagnostics Ltd., Rotkreuz, Switzerland) on the Roche Cobas 8000 modular analyser platform was used in the Erasmus MC clinical laboratory to measure the level of C-reactive protein (CRP) in serum samples. The plasma EDTA samples were transported at a temperature of -80 °C to Myriad RBM, Austin, Texas,

USA, where cystatin C and NGAL concentrations were assessed by a validated multiplex assay (Custom Human Map, Myriad RBM, Austin, Texas, USA). As a result of the batch-wise handling of the samples, with an update of the composition of the multiplex assay by the manufacturer in-between two batches, cystatin C was measured in the full cohort of 570 patients, and NGAL in a random subset of 473 patients. Both laboratories were blinded to clinical and imaging data.

Grayscale and radiofrequency intravascular ultrasound (IVUS)

The degree (plaque volume and plaque burden) and composition of the atherosclerotic plaque were assessed. Plaque volume was defined as the total volume of the external elastic membrane occupied by atheroma.[19] Plaque burden was defined as the plaque and media cross-sectional area divided by the external elastic membrane cross-sectional area and is presented as a percentage (Figure 1). A coronary lesion was defined as a segment with a plaque burden of more than 40% in at least three consecutive frames.[16] The composition of the atherosclerotic plaque was characterized into fibrous, fibro-fatty, dense calcium and necrotic core.[20] Subsequently, three types of VH-IVUS high-risk lesions were identified: 1. thin-cap fibroatheroma (TCFA) lesion: a lesion with the presence of >10% confluent necrotic core in direct contact with the lumen; 2. lesion with a plaque burden of ≥70%; 3. lesion with a minimal luminal area (MLA) of ≤4.0mm^2.[21]

Follow-up

Clinical follow-up started at inclusion and lasted one year. The primary clinical endpoint − MACE − was the composite of all-cause mortality, ACS, or unplanned coronary revascularization. ACS was defined as the clinical diagnosis of ST-segment elevation myocardial infarction (STEMI), non-STEMI, or unstable angina pectoris using the guidelines of the European Society of Cardiology.[22,23] Unplanned coronary revascularizations were defined as unplanned coronary artery bypass grafting or repeat percutaneous coronary intervention. The secondary endpoint was the composite of all-cause mortality or ACS. The endpoints were adjudicated by a clinical event committee blinded for biomarker and IVUS data.

Statistical analysis

The Kolmogorov-Smirnov test was used to test distributions of continuous variables for normality. CysC and CRP were not normally distributed and were ln-transformed for further analyses. Categorical variables are presented as numbers

and percentages. Continuous variables that were normally distributed are presented as mean±standard deviation (SD); non-normally distributed continuous variables are presented as median and interquartile range (IQR). For reasons of uniformity, all biomarkers are presented as median (IQR).

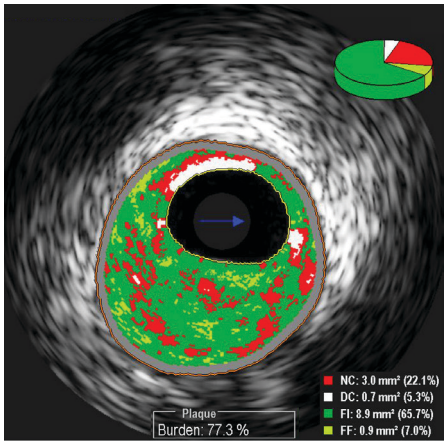
We examined the associations of plasma CysC and NGAL levels with plaque burden, plaque volume, and the presence of high-risk coronary lesions. Plaque volume was normalized for the imaged segment length. We used linear regression and logistic regression analyses with continuous ln-transformed CysC and NGAL concentrations consecutively as independent variables. To assess the effect of kidney function, we included interaction terms (ln-transformed CysC or NGAL, respectively, with dichotomized $eGFR_{Cr}$ (above or below 90 ml/min/1.73m^2)) into the logistic regression models. Subsequently, we stratified all analyses on $eGFR_{Cr}$ of 90 ml/min/1.73m^2. To test whether effect estimates differed between patients with ACS and patients with SAP, Z-tests for heterogeneity were performed.

Cox proportional hazards regression analyses were performed to evaluate the associations between CysC and NGAL and the clinical study endpoints.

Age, gender, indication for coronary angiography, diabetes mellitus, hypertension, and CRP concentration were considered as potential confounders , and were therefore entered into the multivariable linear and logistic regression models. Multivariable adjustment of Cox proportional hazards models was constrained due to the number of clinical endpoints, and was therefore performed in two steps. For MACE, in the first step the adjustment included age, gender, and indication for angiography; in the second step, diabetes mellitus, hypertension and CRP were added.

Finally, we determined the cut-off values of CysC and NGAL that carry the optimal discriminative ability with respect to presence of high-risk coronary lesions and occurrence of MACE. For this purpose, we drew receiver operating characteristic (ROC) curves and calculated the Youden index (highest sum of sensitivity and specificity -1).[24] We considered only statistically significant associations.

All data were analysed with SPSS software (SPSS 20.0; IBM Corp., Armonk, NY). All statistical tests were two tailed, and p values <0.05 were considered statistically significant.



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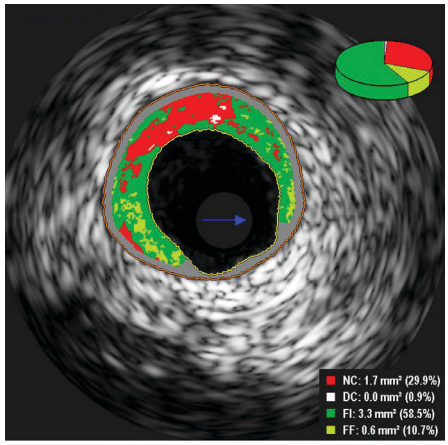

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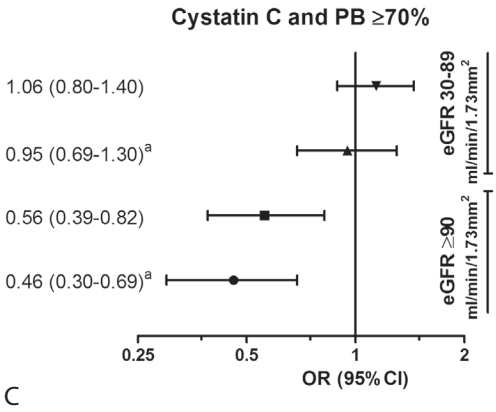

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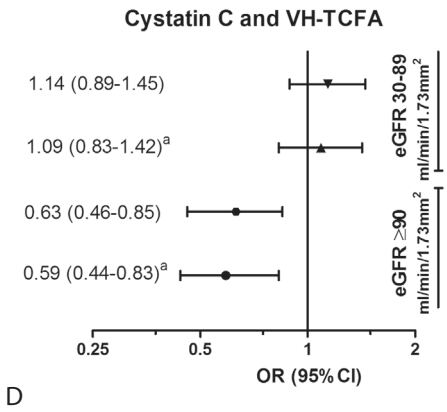

D

FIGURE 1 Plasma cystatin C and presence of VH-IVUS high-risk coronary lesions. A. Lesion with plaque burden (PB) ≥70%. Plaque burden is defined as plaque and media cross-sectional area (i.e., area between yellow contour and red contour) divided by external elastic membrane cross-sectional area (contoured in red); **B.** VH-IVUS derived thin-cap fibroatheroma lesion (VH-TCFA), defined as a lesion (i.e., plaque with a plaque burden >40%) with presence of confluent necrotic core >10% in direct contact with the lumen in at least three frames; **C.** Odds ratio (OR) per standard deviation increase in ln-transformed cystatin C with 95% confidence interval (CI) for lesions with PB ≥70%. **D.** Odds ratio (OR) per standard deviation increase in ln-transformed cystatin C with 95% confidence interval (CI) for VH-TCFA lesions.
FI, fibrous; FF, fibro-fatty; NC, necrotic core, DC, dense calcium.
[a] adjusted for age, gender. diabetes, hypertension, indication for angiography, C-reactive protein.

RESULTS

Baseline characteristics

Mean age was 61.6±11.4 years, 75.7% were men, 54.6% had ACS, and 45.4% had SAP (Table 1). The imaged coronary segment had a median length of 44.3(33.8-55.4)mm. A total of 239 (41.5%) patients had at least one TCFA lesion, 120 (21.0%) had lesions with PB ≥70%, and 175 (30.7%) had lesions with MLA ≤4 mm^2. Median $eGFR_{Cr}$ was 90 (77-98) ml/min/1.73mm^2 in the full cohort with similar values in the subset of ACS patients (91[78-100] ml/min/1.73mm^2) and SAP patients (89 [77-97] ml/min/1.73mm^2). A total of 291 (51.8%) patients had normal kidney function and 271 (48.2%) patients had mild-to-moderate kidney dysfunction. ACS patients exhibited significantly higher NGAL levels compared to patients with SAP, regardless of kidney function, whereas plasma CysC levels were similar in both $eGFR_{Cr}$ groups (Table 1).

TABLE 1 Baseline characteristics.

Variable	Total (n=570)	ACS patients (n= 309)	SAP patients (n=261)
Patient characteristics			
Age, years (mean ± SD)	61.5±11.4	59.7±11.9	63.6±10.3
Men, n (%)	430 (75.4)	227 (73.5)	203 (77.8)
Diabetes Mellitus, n (%)	99 (17.4)	40 (12.9)	59 (22.6)
Hypertension, n (%)	295 (51.8)	134 (43.4)	161 (61.7)
Hypercholesterolemia, n (%)	317 (55.6)	137 (44.3)	180 (69.0)
Smoking, n (%)	164 (28.8)	115 (37.2)	49 (18.8)
Positive family history, n (%)	293 (51.5)	140 (45.5)	153 (58.6)
Previous MI, n (%)	184 (32.3)	80 (25.9)	104 (39.8)
Previous PCI, n (%)	185 (32.5)	57 (18.4)	128 (49.0)
Previous CABG, n (%)	18 (3.2)	7 (2.3)	11 (4.2)
Previous stroke, n (%)	23 (4.0)	10 (3.2)	13 (5.0)
Peripheral artery disease, n (%)	36 (6.3)	12 (3.9)	24 (9.2)
History of heart failure, n (%)	19 (3.3)	6 (1.9)	13 (5.0)
Indication for coronary angiography			
Acute coronary syndrome, n (%)	309 (54.2)	309 (100.0)	0 (0.0)
Myocardial infarction, n (%)	159 (27.9)	159 (51.5)	0 (0.0)
Unstable angina pectoris, n (%)	150 (26.3)	150 (48.5)	0 (0.0)
Stable angina pectoris, n (%)	261 (45.8)	0 (0.0)	261 (100.0)

continued

Variable	Total (n=570)	ACS patients (n= 309)	SAP patients (n=261)
Coronary artery disease [a]			
No significant stenosis, n (%)	42 (7.4)	18 (5.8)	24 (9.2)
1-vessel disease, n (%)	301 (52.8)	168 (54.5)	133 (51.0)
2-vessel disease, n (%)	166 (29.1)	88 (28.5)	78 (29.9)
3-vessel disease, n (%)	61 (10.7)	35 (11.3)	26 (10.0)
PCI performed, n (%)	501 (87.9)	287 (92.9)	214 (82.0)
IVUS characteristics			
Segment length (mm), median (IQR)	44.2 (33.7-55.4)	43.9 (32.9-54.1)	44.8 (34.2-57.2)
Plaque burden (%), median (IQR)	39.2 (29.9-46.4)	37.2 (28.0-45.5)	40.1 (31.8-47.7)
Presence of VH-TCFA, n (%)	239 (41.9)	140 (45.5)	99 (37.9)
Presence of PB ≥70%, n (%)	120 (21.0)	56 (18.1)	64 (24.5)
Presence of MLA ≤4 mm^2	175 (30.7)	87 (28.2)	88 (33.7)
Renal function			
eGFR (ml/min/1.73m^2) median (IQR) [b,c]	90 (77-98)	91 (78-100)	89 (77-97)
KDOQI classification[a], n (%)			
GFR ≥90 ml/min/1.73m^2	291 (51.8)	165 (54.3)	126 (48.8)
GFR 60-89 ml/min/1.73m^2	231(41.1)	115 (37.8)	116 (45.0)
GFR 30-59 ml/min/1.73m^2	39 (6.9)	23 (7.6)	16 (6.2)
GFR <30 ml/min/1.73m^2	1 (0.1)	1 (0.3)	0 (0.0)
Serum biomarkers			
NGAL (ng/mL) median (IQR) [d]	197.0 (143.0-254.0)	204.0 (148.2-274.5)	177.0 (141.5-239.0)
$eGFR_{Cr}$ ≥90 ml/min/1.73m^2 [e]	183.0 (143.0-227.0)	193.0 (143.0-243.0)	174.0 (125.0-223.0)
$eGFR_{Cr}$ 30-89 ml/min/1.73m^2 [e]	216.0 (148.0-293.2)	228.5 (149.0-307.0)	197.0 (143.5-257.7)
Cystatin C (ng/ml) median (IQR)	796.0 (691.0-923.0)	791.0 (674.5-915.5)	802.0 (712.5-935.5)
$eGFR_{Cr}$ ≥90 ml/min/1.73m^2	732. 0 (644.0-834.0)	729.0 (637.5-841.5	734.5 (650.7-822.5)
$eGFR_{Cr}$ 30-89 ml/min/1.73m^2	872.0 (775.7-1032.5)	863.0 (745.0-1040.0)	879.0 (781.0-1030.0)
Creatinine (umol/l), median (IQR) [c]	77 (66-86)	77 (65-877)	76 (67-86)
C-reactive protein (mg/l), median (IQR)	2.1 (0.8-5.3)	2.8 (1.1-6.9)	1.4 (0.6-3.1)

ACS, acute coronary syndrome; SAP, stable angina pectoris; CABG, coronary artery bypass grafting; MI, myocardial infarction; PCI, percutaneous coronary intervention; CKD, chronic kidney disease; NGAL, neutrophil gelatinase-associated lipocalin; PB, plaque burden; MLA, minimal luminal area. [a] significant stenosis was defined as a stenosis >50% of the vessel diameter by visual assessment on the coronary angiogram. [b] estimated Glomerular Filtration Rate (eGFRCr) using CKD-EPI equation: $GFR = 141 \times min(Scr/\kappa, 1)^{\alpha} \times max(Scr$

/κ, 1)-1.209 × 0.993Age × 1.018 [if female] × 1.159 [if black] where: Scr is serum creatinine in mg/dL, κ is 0.7 for females and 0.9 for males, α is -0.329 for females and -0.411 for males, min indicates the minimum of Scr /κ or 1, and max indicates the maximum of Scr /κ or 1. [c] Creatinine available in 99%, Total n=562, ACS n=304, SAP n=258; [d] Measurable in sample of total n=473, ACS n=257, SAP n=216. [e] A statistically significant difference in plasma NGAL levels between ACS and SAP patients (for total population, p=0.002; if $eGFR_{Cr}$ ≥90 ml/min/1.73 m^2, p=0.01; if $eGFR_{Cr}$ 30-89 ml/min/1.73 m^2, p=0.03).

Cystatin C, NGAL and degree of atherosclerosis on grayscale IVUS

Numbers of lesions with plaque burden (PB) ≥70% and minimal luminal area (MLA) ≤4mm^2 according to categories of kidney function are depicted in Figure S3. Significant interactions were found between CysC and $eGFR_{Cr}$ in crude (p=0.007) and multivariable (p=0.010) models predicting lesions with PB ≥70%. In patients with normal kidney function, those with higher CysC had lower risk of lesions with PB ≥70% (per SD increase in ln-transformed CysC: OR[95%CI]: 0.56 [0.39-0.82], p=0.002) (Table 2, Figure 1, Figure S1). After multivariable adjustment including CRP levels, risk remained significantly lower (adjusted OR[95%CI]: 0.46 [0.30-0.69], p<0.001). A CysC level of 773.0 ng/ml was the optimal cut-off value to identify patients who did not have lesions with PB≥70% (CysC ≥773.0 ng/ml) (Figure S4). Conversely, in patients with mild-to-moderate kidney dysfunction risk did not differ significantly according to CysC levels (adjusted OR[95%CI]:0.95 [0.69-1.30], p=0.75). Risk of lesions with PB ≥70% displayed a similar pattern in patients with higher NGAL (Table 2). In patients with normal kidney function, an NGAL level of 180.0 ng/ml was the optimal cut-off value to identify patients without lesions with PB ≥70% (NGAL ≥180.0 ng/ml) (Figure S5). Risk of lesions with MLA ≤4mm^2 was not different for patients with higher CysC or NGAL (Table 2).

Overall, no differences could be demonstrated between CysC and NGAL in either plaque burden or normalized plaque volume of the entirely imaged segment (Table 3 and Table S2). Nevertheless, CysC showed a tendency towards lower normalized segment plaque volume (per SD increase in ln-transformed CysC: β [95%CI]: -0.43 [-1.02-0.16], p=0.16) in patients with normal kidney function; whereas no differences were observed in patients with mild-to-moderate kidney dysfunction.

There was no heterogeneity between ACS and SAP patients regarding the differences in IVUS grayscale parameters according to CysC or NGAL levels.

TABLE 2 Plasma cystatin C, NGAL and presence of thin-cap fibroatheroma (VH-TCFA) lesions, lesions with plaque burden (PB) ≥70% and lesions with minimal luminal area (MLA) ≤4mm² stratified according to kidney function.

	Unadjusted model		Multivariable model	
	OR (95% CI)	p-value	OR (95% CI)	p-value
$eGFR_{Cr}$ ≥90 ml/min/1.73m²				
VH-TCFA				
Cystatin C [a]	0.63 (0.46-0.85)	0.002	0.59 (0.44-0.83)	0.002
NGAL [b]	0.77 (0.57-1.04)	0.090	0.72 (0.52-0.98)	0.040
Plaque Burden ≥70%				
Cystatin C [a]	0.56 (0.39-0.82)	0.002	0.46 (0.30-0.69)	<0.001
NGAL [b]	0.56 (0.35-0.89)	0.015	0.49 (0.29-0.82)	0.007
MLA ≤4mm²				
Cystatin C [a]	0.97 (0.72-1.32)	0.88	0.92 (0.67-1.25)	0.59
NGAL [b]	1.03 (0.77-1.37)	0.84	1.07 (0.79-1.45)	0.67
$eGFR_{Cr}$ 30-89 ml/min/1.73m²				
VH-TCFA				
Cystatin C [a]	1.14 (0.89-1.45)	0.30	1.09 (0.83-1.42)	0.55
NGAL [b]	1.01 (0.78-1.29)	0.97	0.96 (0.74-1.24)	0.74
Plaque Burden ≥70%				
Cystatin C [a]	1.06 (0.80-1.40)	0.68	0.95 (0.69-1.30)	0.75
NGAL [b]	1.20 (0.88-1.63)	0.25	1.21 (0.87-1.67)	0.25
MLA ≤4 mm²				
Cystatin C [a]	0.84 (0.64-1.10)	0.19	0.73 (0.53-0.99)	0.042
NGAL [b]	0.98 (0.74-1.29)	0.90	1.05 (0.78-1.41)	0.75

[a] Odds ratio (OR) per standard deviation increase in ln-transformed cystatin C with 95% confidence interval (CI); [b] Odds ratio (OR) per standard deviation increase in NGAL with 95% confidence interval (CI); Multivariable model: adjusted for age, gender, diabetes mellitus, hypertension, indication for angiography, C-reactive protein.

Cystatin C, NGAL and composition of atherosclerosis on radiofrequency VH-IVUS

Absolute numbers of thin-cap fibroatheroma lesions (VH-TCFAs) according to categories of kidney function are depicted in Figure S3. Significant interactions were found between CysC and $eGFR_{Cr}$ in crude (p=0.002) and multivariable (p=0.003) models predicting VH-TCFAs. In patients with normal kidney function, those with

higher CysC levels had lower risk of VH-TCFA lesions (per SD increase in ln-transformed CysC: OR[95%CI]: 0.63 [0.46-0.85], p=0.002) (Table 2, Figure 1, Figure S1). After multivariable adjustment including CRP levels, risk remained significantly lower (adjusted OR[95%CI]: 0.59 [0.44-0.83], p=0.002). CysC of 678.5 ng/ml was the optimal cut-off value to identify patients without VH-TCFA lesions (CysC ≥678.5 ng/ml) (Figure S6). Conversely, in patients with mild-to-moderate kidney dysfunction, risk did not differ significantly according to CysC levels (adjusted OR[95%CI]: 1.09[0.83-1.42], p=0.55). The interaction between NGAL and $eGFR_{Cr}$ was not statistically significant. A tendency towards lower risk of VH-TCFA lesions was observed for higher NGAL, but only in patients with normal kidney function (Table 2). There was no heterogeneity between ACS and SAP patients regarding the difference in VH-TCFA lesions (CysC, p=0.29, NGAL, p=0.57) (Table S1). At the level of the entire segment, no differences were present in radiofrequency VH-tissue types between CysC or NGAL (Table 3 and Table S2).

TABLE 3 Plasma cystatin C, NGAL and segment characteristics (degree of atherosclerosis: plaque volume and plaque burden; composition of coronary atherosclerosis: 4 components) as determined by VH-IVUS stratified according to kidney function.

	Cystatin C [b]		NGAL [c]	
	β coefficient (95% CI)	p-value	β coefficient (95% CI)	p-value
$eGFR_{Cr}$ ≥90 ml/min/1.73m²				
Plaque burden [a]	-0.02 (-0.16 – 0.12)	0.77	-0.05 (-0.18 – 0.09)	0.50
Plaque volume [a]	-0.43 (-1.02 – 0.16)	0.16	-0.19 (-0.77 – 0.38)	0.51
FI (%)	0.52 (-1.11 – 2.15)	0.53	0.60 (-0.98 – 2.19)	0.45
FF (%) [a]	0.03 (-1.10 – 0.17)	0.65	0.12 (-0.02 – 0.25)	0.09
NC (%)	-0.65 (-1.84 – 0.53)	0.28	-0.85 (-2.00– 0.30)	0.15
DC (%) [a]	0.00 (-0.17 – 0.17)	0.99	-0.12 (-0.28 – 0.04)	0.15
$eGFR_{Cr}$ 30-89 ml/min/1.73m²				
Plaque burden [a]	0.00 (-0.11 – 0.12)	0.94	-0.03 (-0.15 – 0.09)	0.66
Plaque volume [a]	0.16 (-0.37 – 0.68)	0.55	-0.04 (-0.59 – 0.51)	0.89
FI (%)	-1.04 (-2.45 – 0.37)	0.15	0.60 (-0.89 – 2.09)	0.42
FF (%) [a]	-0.02 (-0.13 – 0.10)	0.76	-0.01 (-0.12 – 0.11)	0.92
NC (%)	0.44 (-0.47 – 1.35)	0.34	-0.27 (-1.23 – 0.68)	0.57
DC (%) [a]	0.11 (-0.04 – 0.25)	0.15	-0.06 (-0.21 – 0.09)	0.44

FI, fibrous; FF, fibro-fatty; NC, necrotic core, DC, dense calcium. [a] Square root transformed

[b] Unadjusted β coefficient per standard deviation increase in ln-transformed Cystatin C with 95% confidence interval (CI).
[c] Unadjusted β coefficient per standard deviation increase in NGAL with 95% confidence interval (CI).

Cystatin C, NGAL and 1-year MACE

Vital status was acquired for 569 (99.8%) patients. During the 1-year follow-up, 56 patients experienced the primary endpoint (MACE; Figure S3), and 30 patients endured the secondary composite endpoint of all-cause mortality or ACS. In the full cohort, patients with higher CysC had higher risk of MACE (per SD increase in ln-transformed CysC: HR[95% CI]:1.41[1.10-1.79], p=0.006) (Figure 2, Figure S2). After multivariable adjustment, the risk estimate lost statistical significance. For NGAL, significant differences in risk of MACE were not found (Figure 2, and Figure S2).

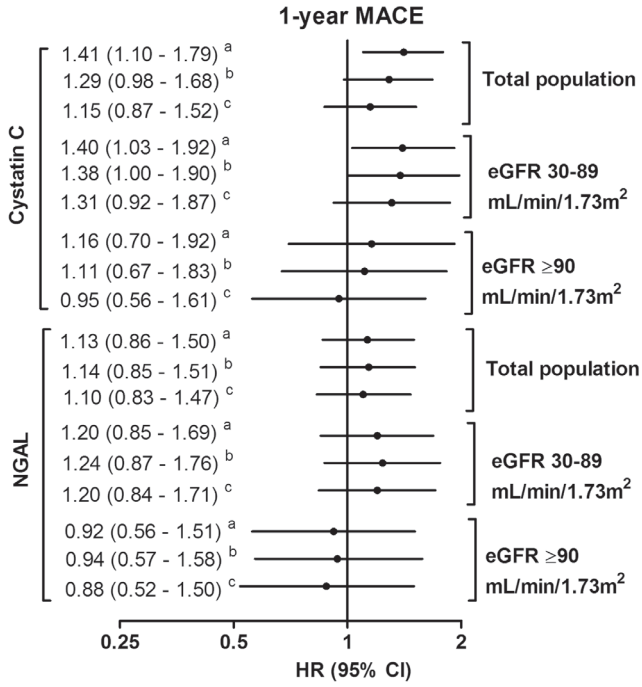

FIGURE 2 Plasma cystatin C, NGAL and occurrence of the 1-year MACE. MACE, major adverse coronary event; Hazard ratio (HR) per standard deviation increase in ln-transformed cystatin C and per standard deviation increase in NGAL with 95% confidence interval (CI). [a] unadjusted model; [b] adjusted for age, gender, indication for angiography; [c] adjusted for age, gender, indication for angiography, diabetes mellitus, hypertension, C-reactive protein; Multivariable adjustment was constrained by the limited number of clinical endpoint.

In patients with normal kidney function, those with higher CysC levels did not have higher risk of MACE (Figure 2, Figure S2). In patients with mild-to-moderate kidney dysfunction, those with higher CysC levels had higher risk of MACE in univariable analysis (HR[95%CI]:1.40[1.03-1.92], p=0.03) (Figure 2, Figure S2). In multivariable analysis, the HR lost statistical significance, but did not materially change (HR[95%CI]:1.31 [0.92-1.87], p=0.12).

Both in the total population and in patients with mild-to-moderate kidney dysfunction, a CysC of 849.0 ng/ml was the optimal cut-off value to identify patients who developed MACE (CysC ≥849.0 ng/ml) (Figure S7). Patterns of risk of the secondary endpoint (all-cause mortality and ACS) according to CysC and NGAL levels were similar to those of MACE (Table S3). Finally, stratification on the indication for angiography confirmed the risk patterns which were found in the full cohort (Table S4).

DISCUSSION

We found that in patients with normal kidney function, those with higher CysC levels had fewer high-risk coronary lesions (VH-TCFA and lesions with PB ≥70%), while risk of MACE was not different. Conversely, when kidney function was mildly-to-moderately impaired, no differences in high-risk lesions were observed, but those with higher CysC levels had higher risk of MACE. Therefore, with regard to prediction of cardiovascular risk, CysC appears to carry potential only when eGFR$_{Cr}$ is below 90 ml/min/1.73m^2. Furthermore, patients with higher NGAL levels had fewer lesions with PB≥70%, but only when they had normal kidney function. No differences in MACE were found for NGAL, and thus its use for cardiovascular risk prediction could not be substantiated. Altogether, our results on CysC suggest novel pathophysiological insights, because they offer an explanation for the difference in findings observed in experimental and epidemiologic studies so far, and imply that the association between CysC and cardiovascular disease may not be solely explained through its correlation with GFR.

Higher CysC levels have been associated with occurrence of cardiovascular events in various epidemiological studies.[25] Conversely, animal experiments suggest that higher CysC may be favourable. Atherosclerotic mice deficient in CysC display increased plaque size and macrophage content, increased elastic lamina degradation and accumulation of smooth muscle cells.[26,6] Studies in humans have also found reduced CysC in atherosclerotic and aneurysmatic aortic lesions.[7] Xu et al. have demonstrated that immune cells (CD8+ dendritic cells (DC) and macrophages), which are involved in atherosclerotic processes, are major contributors to

the circulating CysC pool.[27,28] However, besides a correlation with GFR, the mechanisms that may explain the link between CysC and cardiovascular disease are still unclear. Our study provides additional insights. We found that in patients with normal kidney function, those with higher CysC levels had fewer high-risk coronary lesions, and did not have higher risk of MACE. This is in accordance with a potential 'athero-protective' effect.

Conversely, in patients with mild-to-moderate kidney dysfunction, differences in high-risk lesions according to CysC level were not present. This could possibly be explained by the changes in CysC physiology that occur in impaired kidneys. When kidney function deteriorates, circulating plasma CysC increases and oxidative stress advances, both of which stimulate Cys to form homodimers.[28,29] When CysC forms homodimers, it cannot inhibit cysteine proteases, because the inhibitory region is hidden within the dimer interface. Thus, it may no longer be able to exhibit 'athero-protective' properties.[30] Although these hypotheses are compelling, additional clinical and experimental studies are necessary to further substantiate the effect modification by kidney function that we observed.

Our findings suggest that NGAL may act on coronary artery disease through a different mechanism than currently investigated. A potential lack of predictive precision due to a limited number of MACE may explain the difference between the current results and previous studies.[15,31] On the other hand, a recent meta-analysis that investigated NGAL as a predictor of cardiovascular disease concluded that strong evidence for independent predictive value of NGAL is still lacking.[32] Notably, we found higher plasma NGAL levels in ACS patients compared to SAP patients, independently of kidney function. This could possibly be explained by neutrophilia as a consequence of more severe cardiac damage in ACS patients compared to SAP patients.[33] However, no heterogeneity between ACS and SAP patients was observed in the relationship between NGAL and IVUS-features of coronary atherosclerosis.

Study limitations

Some limitations of this study merit consideration. This study is currently the largest cohort in which the associations between IVUS plaque characteristics, CysC and NGAL were investigated. Yet, we cannot exclude the possibility of a chance finding with regard to effect modification by kidney function. However, both the cut-off value (based on K/DOQI guidelines) and the study population (no kidney failure/eGFR<30) were chosen a priori. Still, our findings should be considered hypothesis-generating and warrant external validation. Second, kidney function

was determined by the creatinine-based CKD-EPI formula, without direct measurement of GFR. Although the CKD-EPI formula has displayed better performance than the Modification of Diet in Renal Disease (MDRD) equation,[17] it is still possible that a few patients are misclassified. Third, VH-IVUS imaging was limited to a pre-specified target segment of a non-culprit coronary artery. This study design was chosen based on the hypothesis that such a non-stenotic segment reflects coronary wall pathophysiology of the larger coronary tree.[34,35] This hypothesis, on its part, was based on ex-vivo, as well as in-vivo studies using IVUS in patients with myocardial infarction. These studies have demonstrated the presence of TCFAs in places other than the culprit lesion or even culprit artery.[16,36] In fact, we were subsequently able to confirm this hypothesis, by demonstrating that imaging characteristics of the non-culprit artery are associated with increased risk of MACE within the current study population.[34] Therefore, this study design allows us to investigate whether the patient's burden and vulnerability of atherosclerotic disease – as reflected by the phenotype of a non-culprit artery segment – is associated with blood biomarkers.[16] Finally, although the spatial resolution of IVUS-VH is formally too low to detect thin caps, we have demonstrated that VH-IVUS derived TCFA lesions strongly and independently predict the occurrence of MACE within the current study population.[34]

CONCLUSION

This study provides new insights into the role of plasma CysC and NGAL in coronary atherosclerosis. Most importantly, it shows that in patients with normal kidney function, those with higher CysC levels have fewer high-risk coronary lesions, while in patients with impaired kidneys, those with higher CysC have higher risk of MACE. Thus, this study implies that mild-to-moderate kidney dysfunction modifies the relationship between plasma CysC and coronary artery disease. This has not been established before, and it offers an explanation for the difference in findings observed in experimental and epidemiologic studies.With regard to cardiovascular risk prediction, CysC showed predictive capacities when $eGFR_{Cr}$ was below 90 ml/min/1.73m^2, whereas NGAL levels were not predictive of MACE.

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SUPPLEMETARY INFORMATION

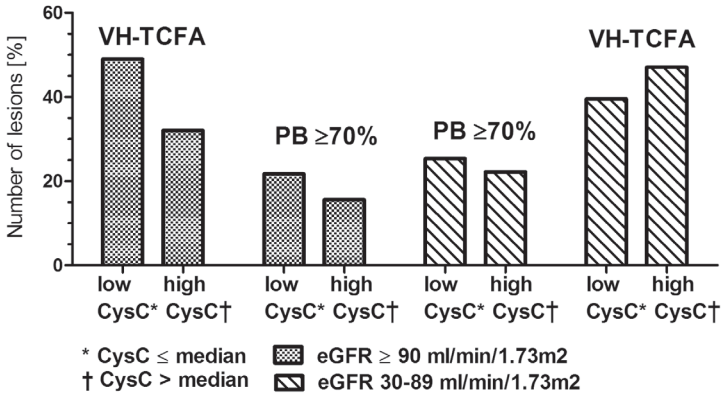

FIGURE S1 Relative number of thin-cap fibroatheroma (VH-TCFA) lesions and lesions with plaque burden (PB)≥70% per strata of kidney function ($eGFR_{cr}$) and plasma cystatin C (CysC) levels above and below median.

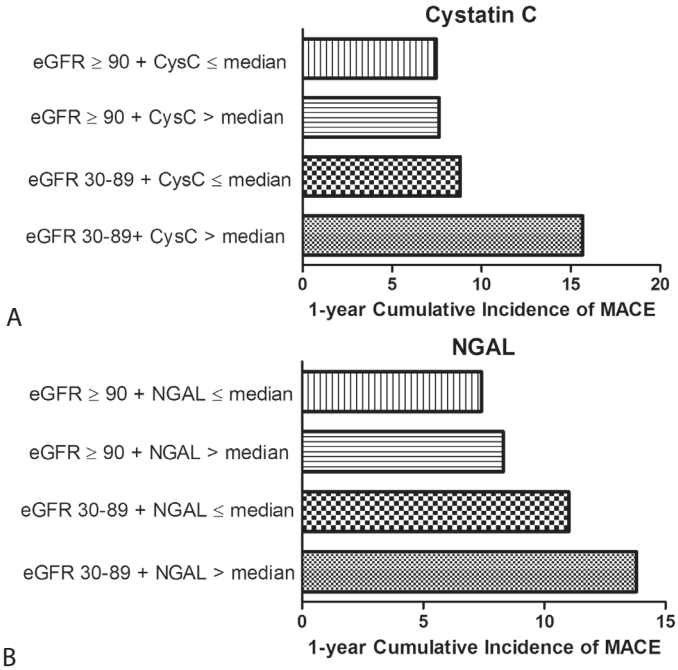

FIGURE S2 1-year cumulative incidence of major adverse coronary events (MACE). A. 1-year MACE per strata of kidney function ($eGFR_{cr}$) and plasma cystatin C (CysC) levels above and below median. **B.** 1-year MACE per strata of kidney function ($eGFR_{cr}$) and plasma NGAL levels above and below median.

TABLE S1 Plasma cystatin C, NGAL and presence of thin-cap fibroatheroma (VH-TCFA) lesions, lesions with plaque burden (PB)≥70%, and lesions with minimal luminal area (MLA) ≤4 mm².

	Unadjusted		Multivariable model	
	OR (95% CI)	p-value	OR (95% CI)	p-value
Total population				
VH-TCFA				
Cystatin C[a]	0.93 (0.78-1.10)	0.38	0.89 (0.74-1.08)	0.25
NGAL [b]	0.92 (0.76-1.10)	0.36	0.88 (0.72-1.07)	0.19
Plaque Burden ≥70%				
Cystatin C [a]	0.93 (0.76-1.14)	0.50	0.75 (0.59-0.95)	0.018
NGAL [b]	0.95 (0.74-1.21)	0.67	0.92 (0.71-1.19)	0.51
MLA ≤4mm²				
Cystatin C [a]	0.90 (0.75 - 1.08)	0.27	0.79 (0.65-0.98)	0.028
NGAL [b]	1.00 (0.82 - 1.21)	0.95	1.01 (0.82-1.24)	0.90
ACS patients				
VH-TCFA				
Cystatin C [a]	0.86 (0.69-1.07)	0.18	0.80 (0.62-1.03)	0.085
NGAL [b]	0.86 (0.67-1.09)	0.21	0.85 (0.66-1.08)	0.18
Plaque Burden ≥70%				
Cystatin C [a]	0.81 (0.61-1.09)	0.17	0.57 (0.40-0.81)	0.002
NGAL [b]	0.87 (0.62-1.24)	0.45	0.81 (0.56-1.17)	0.26
MLA ≤4mm²				
Cystatin C [a]	1.04 (0.79-1.28)	0.97	0.86 (0.65-1.14)	0.30
NGAL [b]	1.11 (0.85-1.44)	0.44	1.10 (0.84-1.44)	0.50
SAP patients				
VH-TCFA				
Cystatin C [a]	1.05 (0.81-1.37)	0.70	1.06 (0.79-1.42)	0.70
NGAL [b]	0.96 (0.70-1.30)	0.78	0.95 (0.69-1.31)	0.75
Plaque Burden ≥70%				
Cystatin C [a]	1.04 (0.78-1.40)	0.77	1.00 (0.72-1.38)	0.97
NGAL [b]	1.01 (0.77-1.58)	0.60	1.05 (0.73-1.52)	0.79
MLA ≤4mm²				
Cystatin C [a]	0.77 (0.58-1.02)	0.071	0.73 (0.53-0.99)	0.044
NGAL [b]	0.91 (0.67-1.24)	0.55	0.90 (0.65-1.24)	0.51

ACS, acute coronary syndrome; SAP, stable angina pectoris. Multivariable model: adjusted for age, gender, diabetes mellitus, hypertension, indications for angiography, C-reactive protein. [a] Odds ratio (OR) per standard deviation increase ln-transformed cystatin C with 95% confidence interval (CI). [b] Odds ratio (OR) per standard deviation increase in NGAL with 95% confidence interval (CI).

TABLE S2 Plasma cystatin C, NGAL and segment plaque volume, burden and VH-tissue types as determined by VH-IVUS, in the total population and stratified by indication for angiography.

	Cystatin C [a]		NGAL [b]	
	β coefficient (95% CI)	p-value	β coefficient (95% CI)	p-value
Total population				
Plaque burden [c]	0.03 (-0.05 – 0.11)	0.44	-0.02 (-0.11 – 0.07)	0.67
Plaque volume [c]	0.04 (-0.31 – 0.39)	0.83	-0.05 (-0.44 – 0.34)	0.79
FI (%)	-0.46 (-1.42 – 0.50)	0.35	0.52 (-0.54 – 1.58)	0.33
FF (%) [c]	0.01 (-0.07 – 0.09)	0.86	0.05 (-0.04 – 0.13)	0.29
NC (%)	-0.17 (-0.83 – 0.50)	0.62	-0.56 (-1.29– 0.17)	0.13
DC (%) [c]	0.09 (-0.01 – 1.19)	0.072	-0.07 (-0.18 – 0.04)	0.22
ACS patients				
Plaque burden [c]	0.01 (-0.10 – 0.12)	0.89	-0.05 (-0.16 – 0.07)	0.43
Plaque volume [c]	-0.22 (-0.68 – 0.24)	0.35	-0.27 (-0.77 – 0.23)	0.29
FI (%)	-0.20 (-1.50 – 1.10)	0.76	0.54 (-0.87 – 1.95)	0.45
FF (%) [c]	-0.05 (-0.16 – 0.06)	0.39	0.08 (-0.04 – 0.20)	0.18
NC (%)	-0.19 (-1.12– 0.74)	0.69	-0.98 (-1.98 – 0.02)	0.055
DC (%) [c]	0.12 (-0.01 – 0.24)	0.079	-0.06 (-0.20 – 0.08)	0.39
SAP patients				
Plaque burden [c]	0.05 (-0.07 – 0.16)	0.41	0.07 (-0.06 – 0.21)	0.29
Plaque volume [c]	0.35 (-0.20 – 0.90)	0.21	0.39 (-0.25 – 1.02)	0.23
FI (%)	-0.66 (-2.09 – 0.76)	0.36	0.03 (-1.61 – 1.67)	0.97
FF (%) [c]	0.71 (-0.04 – 0.18)	0.22	0.03 (-0.10 – 0.16)	0.64
NC (%)	-0.08 (-1.03 – 0.86)	0.86	-0.09 (-1.18 – 1.00)	0.87
DC (%) [c]	0.04 (-0.12 – 0.19)	0.64	-0.02 (-0.20 – 0.16)	0.85

ACS, acute coronary syndrome; SAP, stable angina pectoris. FI, fibrous, FF; fibro fatty; NC, necrotic core; DC, dense calcium. [a] Unadjusted β per standard deviation increase in ln-transformed cystatin C with 95% confidence interval (CI) [b] Unadjusted β per standard deviation increase in NGAL with 95% confidence interval (CI). [c] Square root transformed.

TABLE S3 Plasma cystatin C, NGAL and composite endpoint of all-cause mortality/acute coronary syndrome(ACS) in total population and stratified by kidney function ($eGFR_{Cr}$).

All-cause mortality/ ACS	Unadjusted		Model 1		Model 2	
	HR (95%CI)	p-value	HR (95%CI)	p-value	HR (95%CI)	p-value
Total population						
Cystatin C [a]	1.67(1.24-2.27)	<0.001	1.51 (1.08-2.10)	0.015	1.24 (0.88-1.77)	0.19
NGAL [b]	1.20 (0.85-1.71)	0.30	1.17 (0.81-1.70)	0.40	1.11 (0.77-1.61)	0.56
$eGFR_{Cr}$ ≥90 ml/min/1.73m²						
Cystatin C [a]	1.39 (0.72 – 2.65)	0.32	1.33 (0.72 – 2.48)	0.36	1.11 (0.54-2.25)	0.78
NGAL [b]	1.04 (0.57-1.89)	0.89	1.12 (0.63-1.98)	0.69	1.08 (0.56-2.11)	0.81
$eGFR_{Cr}$ 30-89 mL/min/1.73 m²						
Cystatin C [a]	1.81 (1.23-2.66)	0.003	1.73 (1.17-2.55)	0.006	1.59 (1.01-2.50)	0.04
NGAL [b]	1.26 (0.80-1.98)	0.31	1.22 (0.77-1.94)	0.39	1.18 (0.74-1.87)	0.48

Model 1: adjusted for the age, gender, indication for angiography; **Model 2**: model 1 + diabetes mellitus, hypertension, C-reactive protein. Multivariable adjustment was constrained by the limited number of clinical endpoints.

[a] Hazard ratio (HR) per standard deviation increase in ln-transformed cystatin C with 95% confidence interval (CI).

[b] Hazard ratio (HR) per standard deviation increase in NGAL with 95% confidence interval (CI).

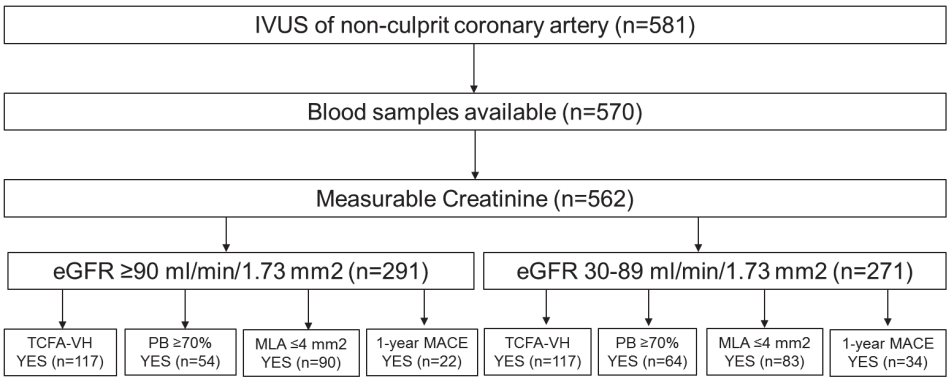

FIGURE S3 Absolute numbers of thin-cap fibroatheroma (VH-TCFA) lesions, lesions with plaque burden (PB)≥70%, lesions with minimal luminal area (MLA)≤4 $mm^{2,}$ and 1-year major adverse coronary events (MACE) per strata of kidney function ($eGFR_{Cr}$).

TABLE S4 Plasma cystatin C, NGAL and major adverse coronary events (MACE) and the composite of all-cause mortality/acute coronary syndrome (ACS), stratified by indication for angiography.

	Unadjusted		Model 1		Model 2	
	HR (95%CI)	p-value	HR (95%CI)	p-value	HR (95%CI)	p-value
ACS patients						
MACE						
Cystatin C [a]	1.41 (1.01-1.98)	0.047	1.24 (0.85-1.81)	0.27	1.10 (0.73 – 1.65)	0.66
NGAL [b]	1.17 (0.80 – 1.70)	0.43	1.17 (0.80 – 1.72)	0.42	1.14 (0.76 – 1.69)	0.53
All-cause mortality/ACS						
Cystatin C*	1.61 (1.15 – 2.40)	0.007	1.47 (0.98 – 2.20)	0.06	1.23 (0.79 – 1.91)	0.37
NGAL [b]	1.33 (0.89 – 1.98)	0.16	1.35 (0.89 – 2.03)	0.15	1.33 (0.86 – 2.06)	0.20
SAP patients						
MACE						
Cystatin C [a]	1.39 (0.98 – 1.97)	0.07	1.35 (0.92-1.98)	0.12	1.25 (0.85-1.84)	0.26
NGAL [b]	1.18 (0.77 – 1.80)	0.44	1.10 (0.72 – 1.69)	0.66	1.08 (0.70 – 1.64)	0.73
All-cause mortality/ACS						
Cystatin C [a]	1.71 (1.02 – 2.88)	0.042	1.61 (0.90 – 2.87)	0.11	1.40 (0.76 – 2.59)	0.28
NGAL [b]	0.84 (0.42 – 1.70)	0.64	0.79 (0.39 – 1.61)	0.52	0.78 (0.38 – 1.58)	0.49

ACS, acute coronary syndrome; SAP, stable angina pectoris. **Model 1**: adjusted for the age, gender; **Model 2**: model 1 + diabetes mellitus, hypertension, C-reactive protein.
[a] Hazard ratio (HR) per standard deviation increase in ln-transformed cystatin C with 95% confidence interval (CI).
[b] Hazard ratio (HR) per standard deviation increase in NGAL with 95% confidence interval (CI).

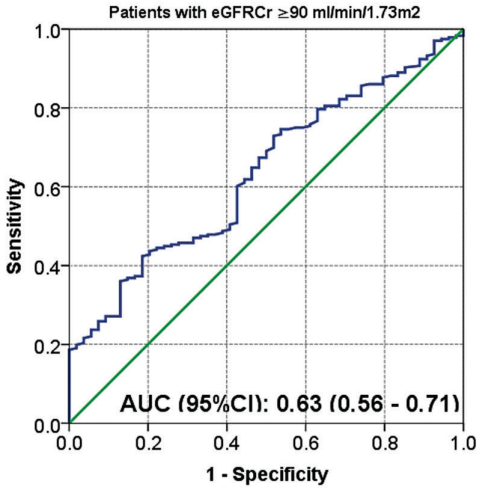

FIGURE S4 Receiver operator characteristic (ROC) curve of plasma cystatin C (CysC) for the prediction of absence of lesion with plaque burden (PB)≥70% in patients with $eGFR_{Cr}$ ≥90 ml/min/1.73m². CysC of 773.0 ng/ml is optimal cut-off value, based on Youden index (highest sum of sensitivity and specificity -1), discriminating between patients who did not have lesion with PB ≥70% (CysC ≥773.0 ng/ml), and those who had (CysC <773.0 ng/ml). AUC (95%CI), area under the ROC curve with corresponding 95% confidence interval.

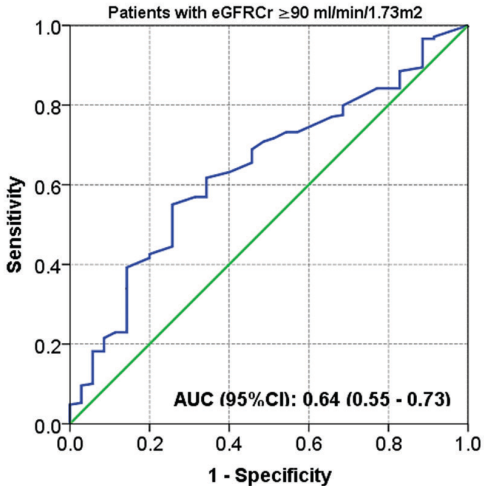

FIGURE S5 Receiver operator characteristic (ROC) curve of plasma NGAL for the prediction of absence of lesion with plaque burden (PB)≥70% in patients with $eGFR_{Cr}$ ≥90 ml/min/1.73m². NGAL of 180.0 ng/ml is optimal cut-off value, based on Youden index (highest sum of sensitivity and specificity -1), discriminating between patients who did not have lesion with PB≥70% (NGAL ≥180.0 ng/ml) and those who had (NGAL <180.0 ng/ml). AUC (95%CI), area under the ROC curve with corresponding 95% confidence interval.

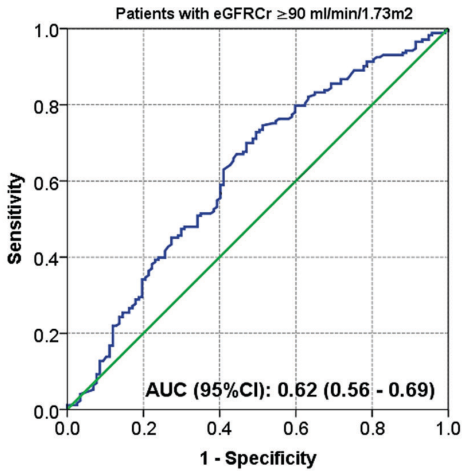

FIGURE S6 Receiver operator characteristic (ROC) curve of plasma cystatin C (CysC) for the prediction of absence of thin-cap fibroatheroma (VH-TCFA) lesion in patients with $eGFR_{Cr}$ ≥90 ml/min/1.73m². CysC of 678.5 ng/ml is optimal cut-off value, based on Youden index (highest sum of sensitivity and specificity -1), discriminating between patients who did not have VH-TCFA lesion (CysC ≥678.5 ng/ml), and those who had (CysC <678.5 ng/ml). AUC (95%CI), area under the ROC curve with corresponding 95% confidence interval.

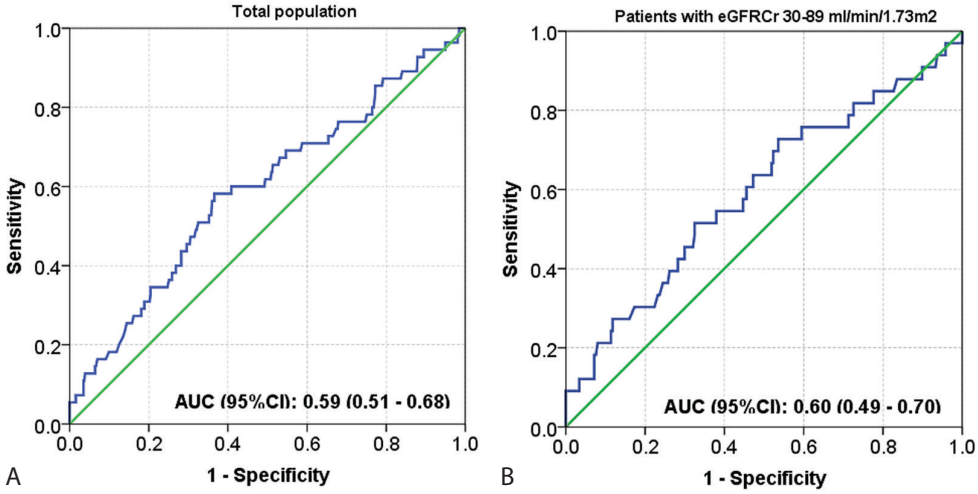

FIGURE S7 Receiver operator characteristic (ROC) curve for plasma cystatin C (CysC) for the prediction of the occurrence of major adverse coronary events (MACE) in total population and in patients with $eGFR_{Cr}$ 30-89 ml/min/1.73m². CysC of 849.0 ng/ml is optimal cut-off value, based on Youden index (highest sum of sensitivity and specificity -1), discriminating between patients who developed MACE (CysC ≥849.0 ng/ml) and those who did not (CysC <849.0 ng/ml). AUC (95%CI), area under the ROC curve with corresponding 95% confidence interval.