Serum cystatin C and neutrophil gelatinase-associated lipocalin in predicting the severity of coronary artery disease in diabetic patients

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ABSTRACT

Objective: Cystatin C and neutrophil gelatinase-associated lipocalin (NGAL) are biomarkers of renal functions. We evaluated their roles in predicting the severity of coronary artery disease (CAD).

Methods: Fifty-two consecutive type 2 diabetic patients (32 males, 65.7±8.6 years) who underwent coronary angiography (CAG) for stable CAD were included in this single-center, prospective, cross-sectional study. Patients with an estimated glomerular filtration rate <60 mL/min/1.73 m^2 and with a history of by-pass surgery and/or coronary stent implantation were excluded. The vessel score and Gensini score were calculated to assess the presence and severity of CAD. Mann–Whitney U test, Spearman test, and multiple linear regression analysis were used for the main statistical analyses.

Results: Serum cystatin C levels were higher in patients with multivessel disease than in those with single vessel disease [1260 ng/mL (953–1640) vs. 977 ng/mL (599–1114), p=0.017]. According to the median Gensini score, the higher score group also had higher cystatin C levels than the lower score group [1114 ng/mL (948–1567) vs. 929 ng/mL (569–1156), p=0.009]. However, serum NGAL levels were similar between these subgroups. There was a positive correlation between cystatin C and Gensini score (r=0.334, p=0.016). Multiple linear regression analysis revealed serum cystatin C as an independent predictor of the Gensini score (β=0.360, t=2.311, p=0.026). These results may aid in defining cystatin C as a surrogate marker of the extent of CAD in further clinical trials.

Conclusion: Serum Cystatin C, but not NGAL levels, could predict the severity of CAD in diabetic patients. *(Anatol J Cardiol 2016; 16: 756-61)*

Key words: coronary artery disease, cystatin C, neutrophil gelatinase-associated lipocalin, diabetes mellitus

Introduction

Cardiovascular disease (CVD) is the leading cause of mortality and morbidity in diabetic patients (1). In diabetes mellitus (DM), obstructive coronary artery disease (CAD) is more common, and diabetic patients have more extensive disease than patients with other risk factors for CAD (2). Thus, it has clinical importance to find out simple and robust biomarkers that predict the presence and severity of CAD in this high-risk population.

Neutrophil gelatinase-associated lipocalin (NGAL) belongs to the lipocalin superfamily of proteins that accumulate in granules of neutrophils (3). It has been demonstrated to be released in case of renal tubular damage due to ischemia and/or nephrotoxins and suggested as an early marker of acute kidney injury (4). Furthermore, NGAL is expressed in endothelial cells and macrophages in atherosclerotic plaques (5). It enhances the activity of matrix metalloproteinase-9, which plays important role in plaque instability (6). NGAL modulates inflammation and

promotes development and progression of atherosclerosis (3–6). Previously, serum NGAL levels have been shown to be associated with the extent of stable CAD (7) and to be elevated in acute coronary syndromes (8). However, despite the putative role of inflammation in pathogenesis of type 2 DM and CVD (9, 10), NGAL has not yet been elucidated in predicting the severity of CAD in diabetic patients.

Cystatin C is a low-molecular-weight (13kD) protein belonging to the cystatin super- family of cysteine proteinase inhibitors. It is synthesized by all nucleated cells, filtered from glomeruli, and is almost completely reabsorbed by proximal renal tubules (11). When compared to serum creatinine, it is less influenced by age, gender, race, muscle mass, exercise, and diet. Therefore, serum cystatin C is accepted as a superior blood parameter than creatinine clearance for estimating renal functions (12). Cystatin C has been shown to be related with increased coronary atherosclerotic burden (13, 14), probably due to contribution to inflammatory processes. Inflammatory cytokines stimulate the production of lysosomal cathepsins,

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which degrade extracellular matrix proteins; and concentration of cystatin C, a cathepsin inhibitor, is elevated to counterbalance this elastolytic overactivity (15). In metabolic syndrome, cystatin C has been found to be associated with severity of CAD (16). However, there are limited data evaluating this association in diabetic population.

We argued that these biomarkers could reflect the increased inflammatory status in diabetic coronary atherosclerosis. Accordingly, in the present study, we aimed to determine serum cystatin C and NGAL levels for predicting the severity of CAD in diabetic patients.

Methods

Study population

This was a single-center, prospective, cross-sectional study. Power analysis of the study was performed using the program of G*Power power-and-sample size calculation Version 3.1.9.2. Düsseldorf Universität, Germany (17). Accordingly, with an alpha error value of 0.05 and Cohen's effect size of 0.8 and power of 0.80, the projected sample size was found to be 52 subjects for the inferential statistics (t tests-means: difference between two independent groups). The study was conducted in accordance with the guidelines proposed in the Helsinki Declaration and approved by Başkent University Institutional Review Board and supported by Başkent University Scientific Research Fund. All the participants gave written informed consent before enrollment.

The subjects were consecutive type 2 diabetic patients who underwent elective CAG due to suspected diagnosis of stable CAD in our institute. Patients with a serum creatinine of >1.4 mg/dL for men and >1.2 mg/dL for women and/or with an estimated glomerular filtration rate (eGFR) <60 mL/min/1.73 m^2, active or chronic liver diseases, active infectious conditions, chronic inflammatory or rheumatological diseases, and malignancies were not included. Also, patients with unstable angina, congestive heart failure, severe valvular disorders, and history of prior percutaneous coronary intervention /coronary artery by-pass graft operation were not included. Fifty-two eligible type 2 diabetic patients were analyzed.

Weight and height of the patients were measured without heavy outer garments and shoes, after a 12-hour fasting period. Office blood pressure was measured two times on the right arm in the sitting position, with a 5-min interval, and the average value was used. Hypertension was accepted as systolic blood pressure ≥140 mm Hg; diastolic blood pressure, ≥90 mm Hg, or being treated with anti-hypertensive medication. Diabetes mellitus was defined as being treated with anti-diabetic medications (oral anti-diabetics / insulin). Smoking was defined as the regular use of tobacco. Hyperlipidemia was defined as having total cholesterol level >200 mg/dL or use of hypolipidemic medications. eGFR was calculated by Modification of Diet in Renal Disease (MDRD) equation (18).

Laboratory measurements

The venous blood samples for routine laboratory measurements were taken after 12-hours overnight fasting within 72 hours prior to CAG and analyzed in 1 hour. For determination of cystatin C and NGAL levels, the venous blood samples were drawn on the morning of CAG and stored at –20°C until analysis time, and were detected with sandwich enzyme immunoassay method using commercial Human Cystatin C kits (Biovendor Laboratory Medicine Inc., Brno, Czech Republic) and Human Lipocalin-2/NGAL kits (Biovendor Laboratory Medicine Inc.), respectively, by a microelisa analyser device (DSX Model; Dynex Technologies, Chantilly, VA, USA).

Coronary angiography

The coronary angiographies were performed using the standard Judkins technique. The angiograms were reviewed by two independent experienced invasive cardiologists for the presence and severity of CAD. Coronary vessel score was ranged between 0 and 3 according to the number of the main coronary arteries—left anterior descending (LAD), left circumflex (Cx), and right coronary artery (RCA)—with a ≥50% stenosis. Significant CAD was defined as a vessel score ≥1. Gensini score (19) was calculated for evaluation of the CAD severity in each patient. This system scores the stenosis in the epicardial coronary arteries (1 point for 1–25% stenosis, 2 for 26–50% stenosis, 4 for 51–75% stenosis, 8 for 76–90% stenosis, 16 for 91–99% stenosis, and 32 for total occlusion) and multiplies this number by a constant number in term of the anatomical position of the lesion. Multipliers are 5 for the left main coronary artery; 2.5 for the proximal segment of the LAD and Cx; 1.5 for the mid-segment of the LAD; 1 for the RCA, the distal segment of the LAD, the posterolateral artery, and the obtuse marginal artery; and 0.5 for other segments. The score in every segment is calculated, and the total score gives the Gensini score. The patients who have history of coronary revascularization (percutaneous coronary intervention or coronary artery bypass graft operation) were excluded from the study since the original Gensini's scoring system is technically inappropriate for such cases. The median Gensini score was 23 (5–51, IQR: 46). The patients were divided into groups of severe and non-severe CAD according to this median score.

Statistical analysis

The normality of distribution was tested using Kolmogorov–Smirnov test. Continuous variables were presented as mean±SD if normally distributed otherwise as median with 25th and 75th percentiles. Categorical variables were presented as numbers and percentages and were compared using chi-square test (Fisher's exact test if needed). Student's t-test or Mann–Whitney U test was used for comparison of continuous variables where appropriate. Correlation analysis was performed using Spearman test. A multiple linear regression analysis model was generated in order to investigate the independent predictors of the Gensini score. Receiver operating characteristic (ROC) analysis was
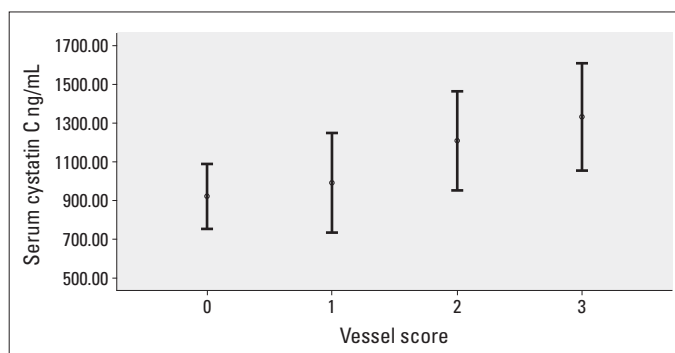
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Table 1. Demographics and baseline clinical parameters of the patients

	All patients	Severe CAD (n=26)	Non-severe CAD (n=26)	*P*
Age, years	65.7±8.6	64.3±8.4	66.5±9.6	0.08
Gender, male/female	32/20	19 / 7	13/13	0.15
BMI, kg/ m2	29.2±3.9	29.2±4.2	29.3±3.6	0.91
Smoking, %	21.2	30.8	11.5	0.17
Hypertension, %	88.5	88.5	88.5	1.00
Hyperlipidemia, %	69.2	73.1	65.4	0.76
Gensini score	23 (5–51)	51 (29–69)	4.75 (1–10)	<0.001
Laboratory findings				
HbA1c, %	7.41±1.45	7.66±1.47	7.18±1.43	0.24
BUN, mg/dL	18.63±6.86	19.92±8.28	17.34±4.89	0.18
Scr, mg/dL	0.86±0.17	0.92±0.15	0.80±0.17	0.006
eGFR, mL/min /1.73 m2	88.32±18.70	82.96±19.50	93.69±18.75	0.049
Hemoglobin, g/dL	13.76±1.40	13.81±1.56	13.72±1.25	0.80
Total-C, mg/dL	188.81±35.36	190.66±31.80	186.52±40.22	0.73
LDL-C, mg/dL	112.00±30.55	116.44±31.10	107.17±29.86	0.30
HDL-C, mg/dL	40.89±9.64	41.15±10.59	40.61±8.76	0.86
Triglycerides, mg/dL	147 (119–196)	150 (132–192)	129 (99–197)	0.20
Medications, %				
Insulin	23.1	34.6	11.5	0.10
Oral antihypoglycemic agents	86.5	80.8	92.3	0.42
ACEI	28.8	23.1	34.6	0.54
ARB	36.8	42.3	30.8	0.56
CCB	32.7	38.5	26.9	0.55
β-blockers	53.8	61.5	46.2	0.40
Statins	57.7	53.8	61.5	0.78
Acetylsalicylic acid	59.6	65.4	53.8	0.57
Warfarin	3.8	3.8	3.8	–
Nitrates	11.8	15.4	7.7	0.67

Data are presented as mean±standard deviation, median with 25th and 75th percentiles, or number and frequencies where indicated
ACEI - angiotensin converting enzyme inhibitor; ARB - angiotensin receptor blocker; BMI - body mass index; BUN - blood urea nitrogen; C - cholesterol; CAD - coronary artery disease; CCB - calcium channel blocker; eGFR - estimated glomerular filtration rate; HbA1c - hemoglobin A1c; HDL-C - high-density lipoprotein cholesterol; LDL-C - low-density lipoprotein cholesterol; Scr - serum creatinine
Student's t-test or Mann–Whitney U Test and chi-square test were used for statistical analyses

Figure 1. Comparison of cystatin C levels according to the vessel score. Serum cystatin C concentrations were increased across the vessel score (*P*=0.03). Posthoc tests revealed significant differences in cystatin C levels between no-vessel and three-vessel disease (*P*=0.016) and between one-vessel and three-vessel disease (*P*=0.020)

The patients were divided into subgroups of severe and non-severe CAD. Patients with higher Gensini score had higher serum creatinine and lower eGFR values compared with those having lower Gensini score. Other parameters were similar in regard to groups arranged with Gensini score (Table 1).

There was a trend toward higher cystatin C levels in patients with significant CAD (n=34) than in those without (n=18) [1101 (908–1378) vs. 915 (569–1162), respectively, p=0.09]. Regarding the patients with significant CAD, serum cystatin C levels were found to be higher in patients with multivessel disease (n=18) than in those with single vessel disease (n=16) [1260 ng/mL (953–1640) vs. 977 ng/mL (599–1114), respectively, p=0.017]. Serum cystatin C concentrations were increased across the vessel score [0 vessel, n=18: 915 ng/mL (569–1162); single vessel, n=16: 977 ng/mL (599–1114); two vessels, n=8: 1199 ng/mL (915–1476); and three vessels, n=10: 1393ng/mL (967–1724); p=0.03] (Fig. 1). The patients with higher Gensini score had also higher cystatin C levels than those with lower Gensini score [1114 ng/mL (948–1567) vs. 929 ng/mL (569–1156), respectively, p=0.009]. In all of the above-mentioned subgroups, there were no differences in serum NGAL levels (Table 2 summarizes cystatin C and NGAL levels compared between the groups).

Cystatin C levels were positively correlated with Gensini score (r=0.334, p=0.016), and serum creatinine levels (r=0.501, p<0.001), and negatively with eGFR value (r=0.479, p<0.001). Multiple linear regression analysis revealed that serum cystatin C (=0.360, t=2.311, p=0.026) and hemoglobin A1c (=0.271, t=2.143, p=0.038) were the independent predictors of the Gensini score (Table 3). ROC curve analysis (Fig. 2) revealed that serum cystatin C level of 1111 ng/mL has a 67% sensitivity and 75% specificity for estimating multivessel CAD [p=0.017, area under the curve (AUC): 0.740, 95% CI: 0.568–0.911].

used to predict the cut off points for cystatin C in determination of multi-vessel disease. Then, sensitivity and specificity were calculated. Statistical analysis was performed by SPSS software (version 17; Statistical Package for Social Sciences, Chicago, IL). A p value of <0.05 was considered statistically significant.

Results

The mean age of the study population (20 females and 32 males) was 65.7±8.6 years. The clinical data and demographic features of the entire study population are presented in Table 1.

Discussion

In the present study, we investigated the diagnostic value of serum cystatin C and NGAL levels for the prediction of CAD severity in type 2 diabetic patients. Our results showed that serum

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Table 2. Cystatin C and NGAL concentrations according to extent and severity of coronary artery disease

	Vessel score			Gensini score		
	Single-vessel	Multi-vessel	P	Lower gensini score	Higher gensini score	P
Serum cystatin C, ng/mL	977 (599–1114)	1260 (953–1640)	0.017	929 (569–1156)	1114 (948–1567)	0.009
Serum NGAL, ng/mL	92 (60–133)	95 (65–152)	0.64	101 (63–139)	92 (62–143)	0.69
NGAL - neutrophil gelatinase-associated lipocalin. Mann–Whitney U test was used for statistical analyses						

cystatin C levels were higher in multivessel CAD and strongly predicted the disease severity. However, we did not observe any association between serum NGAL levels and the severity of CAD. To our best of knowledge, this is the first study investigating cystatin C and NGAL levels simultaneously in regard to coronary atherosclerotic burden in type 2 diabetic patients.

Cardiovascular diseases (CVDs) are the leading causes of mortality and morbidity in DM, and diabetic patients have more extensive disease than patients having other risk factors for CAD (1, 2). The increased prevalence of accompanying disorders such as hypertension and dyslipidemia also contribute to the higher CVD event rates in diabetes (20). The increased CVD risk in type 2 DM can be explained by a complex combination of various traditional and nontraditional risk factors. The pathogenesis of CVD in diabetes includes epigenetic, intracellular, and metabolic changes resulting in oxidative stress, inflammation, and endothelial dysfunction (9). In type 2 DM, chronic toxicity of glucose and free fatty acids is related with the induction of inflammatory processes within various tissues, particularly in vascular wall (10). Moreover, a low-grade inflammation plays an important role in the pathogenesis of the insulin resistance (21). Due to this strict relation, different biochemical markers reflecting inflammatory processes are used for screening CVD risk in diabetics.

It has been shown that impaired renal functions are related with increased all-cause and cardiovascular mortality, adverse cardiovascular events, and hospitalization rate in a large, community-based population (22). NGAL and cystatin C have been proven as biomarkers of renal injury and functions and accepted being accurate parameters for estimating renal functions (4, 12). These biologic substances also have important contributions to inflammatory processes involved in atherosclerosis as discussed earlier (3–6, 13–15).

In recent clinical trials, cystatin C has been found to be related with severity of CAD and a predictor of mortality and adverse cardiovascular events in patients with acute coronary events (23–25). Niccoli et al. (13) evaluated 70 patients with normal renal functions and stable CAD and reported a relation between increased cystatin C and coronary atherosclerotic burden. However, in the study of Doğaner et al. (26), which included 88 non-diabetic patients with an eGFR of >60 mL/min/1.73 m², cystatin C levels were lower in patients with CAD, and they showed an inverse association between number of involved coronary artery and cystatin C levels. They speculated that higher levels of cystatin C might indicate the presence of vulnerable plaques. That study, however, did not show the plaque morphology. They

Table 3. Multivariate linear regression analysis model to investigate independent predictors of Gensini score

Variable	β	T	P	95% CI
Age	0.230	1.500	0.141	(-0.264–1.799)
Gender	0.155	1.154	0.255	(-6.784–24.920)
Smoking	0.104	0.684	0.498	(-14.188–28.745)
Hypertension	-0.136	-1.074	0.289	(-34.878–10.639)
Hyperlipidemia	0.083	0.655	0.516	(-10.710–21.005)
HbA1c	0.271	2.143	0.038	(0.316–10.370)
Cystatin C	0.360	2.311	0.026	(0.002–0.330)
eGFR	0.005	0.032	0.974	(-0.412–0.425)
CI - confidence interval; eGFR - estimated glomerular filtration rate; HbA1c - hemoglobin A1c				
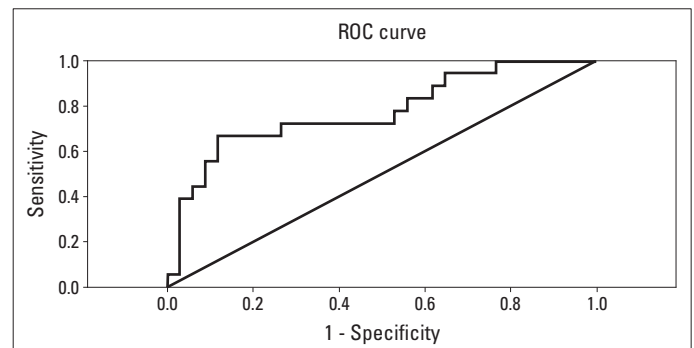
Figure 2. Receiver operating characteristics curve analysis to find out predictive values of cystatin C levels in estimating multivessel CAD (*P*=0.017, AUC: 0.740, 95% CI: 0.568–0.911)

also found no correlation between cystatin C and eGFR in their study population. Subsequently, in two clinical trials including both diabetic and non-diabetic stable CAD patients without renal dysfunction (eGFR of >60 mL/min/1.73 m²), cystatin C was correlated with severity of CAD quantified by Gensini score (14, 27). Qing et al. (16) provided that cystatin C levels were elevated in asymptomatic CAD in metabolic syndrome patients. They demonstrated positive correlations between cystatin C and number of diseased vessel and the Gensini score, and they found cystatin C to be an independent predictor of CAD severity calculated by Gensini score. In this point, our results are in agreement with those studies demonstrating a close relation between cystatin C and severity of CAD. We showed that patients with multivessel disease and those with higher Gensini score had also increased serum cystatin C levels. In our study, there was a positive and modest correlation between cystatin C levels and the Gensini

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score and linear regression analysis revealed cystatin C as an independent predictor of the Gensini score. Furthermore, in our study, AUC of ROC analysis was 0.740. It was reported as 0.622 by Qing et al. (16) and 0.588 by Wang et al. (27). This indicates that cystatin C as a sole marker had a higher predictive value in our study. Nevertheless, our AUC value indicates that there could be some additional unknown predictors affecting atherosclerotic burden in this cohort, supporting the idea that atherosclerosis is a multifactorial and complex disease. When evaluating renal functions of the diabetic patients, the cut-off value of 1111 ng/mL can be an acceptable level to effectively identify severe CAD.

Serum NGAL has been shown to be elevated in a variety of CAD such as coronary ectasia (28), coronary slow flow phenomenon (29), and unstable coronary syndromes, including acute myocardial infarction (30, 31). However, the role of NGAL in predicting severity of stable CAD has not yet been clearly elucidated. Zografos et al. (7) evaluated 73 stable CAD patients (21 with diabetes) and reported that serum NGAL level was modestly correlated (r=0.356) with Gensini score. In our study, however, serum NGAL levels were not related with presence or severity of stable CAD in diabetic patients.

To our knowledge, there have been no data regarding simultaneous measurements of cystatin C and NGAL for the evaluation of stable CAD in either diabetic or non-diabetic population. Still, possible mechanisms underlying the link between cystatin C and CAD need to be described. It can simply be argued that renal mechanisms are responsible for this relation. However, in our diabetic cohort, mean serum creatinine values were within the normal range, but mean eGFR value was relatively low; this suggested slightly impaired kidney functions. Patients with severe CAD had higher serum creatinine, lower eGFR values, and higher cystatin C levels. Importantly, cystatin C remained as a predictor for CAD severity, even after adjustment for eGFR value. Hence, we cannot exactly explain the association between cystatin C and CAD severity via solely impaired kidney dysfunction, which predicts severe cardiovascular outcomes. Based on previous clinical trials demonstrating positive correlations between serum cystatin C and highly sensitive C reactive protein and fibrinogen (14, 27), it could be speculated that cystatin C participates in inflammatory processes that are involved in coronary atherosclerosis. Further clinical investigations with larger cohorts are needed to explain exact mechanisms.

Study limitations

Ours was a single-center and relatively small-sized study. We did not determine relations between CAD and serum NGAL and cystatin C levels after graduating the patients into different stages of nephropathy. Use of complementary biomarker(s) of inflammation and considering the duration of DM which may affect levels of the markers could render our results more contributory. Ultimately, in biomarker assays racial/ethnic differences should be regarded, and our results may not generalize to the all diabetic population.

Conclusion

Our findings showed that serum cystatin C levels, but not NGAL levels, were significantly associated with severity of stable CAD in type 2 diabetic patients. Serum cystatin C levels could be a marker of coronary atherosclerosis independent of kidney function. We believe that our study may inspire further trials to investigate cystatin C in this regard.

Conflict of interest: None declared.

Peer-review: Externally peer-reviewed.

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