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Cystatin C, but not urinary or serum NGAL, may be associated with contrast induced nephropathy after percutaneous coronary invasive procedures: A single center experience on a limited number of patients

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Key words: Cystatin C ▪ Neutrophil Gelatinase-Associated Lipocalin ▪ Contrast induced nephropathy ▪ Percutaneous coronary invasive procedures.

Objective. This study aimed to test the association of both the baseline values and post-procedural variations of urinary and serum Neutrophil Gelatinase-Associated Lipocalin (NGAL) and Cystatin C (CysC) with contrast induced nephropathy (CIN) occurrence in patients undergoing percutaneous coronary invasive procedures (PCIP), and compare them to serum creatinine and the estimated glomerular filtration rate (eGFR). **Methods.** In 43 patients admitted to our Cardiac Step-Down Unit and submitted to PCIP, we measured serum creatinine and eGFR as the standard markers for CIN diagnosis, and compared them to both serum and urinary NGAL as well as serum CysC, assessed before and 4 hours after PCIP. **Results.** Patients who developed CIN (16%) were older, with significantly higher discharge creatinine values, lower eGFR values at creatinine peak, and higher baseline and post-PCIP CysC values. We did not detect any significant association between baseline serum and urinary NGAL values and their 4 hour variations after contrast medium administration and CIN occurrence. Furthermore, we observed that the baseline values of both serum and urinary NGAL were significantly higher in patients with greater neutrophil count. **Conclusion.** In our population submitted to PCIP, neither baseline serum and urinary NGAL nor their variations after PCIP were related to CIN occurrence, while CysC results were associated with CIN development, earlier than creatinine and eGFR variations.

Introduction

In recent decades, with the increasing use of intravascular iodinate contrast media for diagnostic and therapeutic purposes, contrast induced nephropathy (CIN) has become one of the more common serious complications, in particular in patients undergoing percutaneous coronary invasive procedures (PCIP) (1). In clinical practice, it is important to estimate the individual risk of developing CIN and, with regard to this, a score capable of predicting this complication has been proposed (2). Considering the classical biomarkers of renal function, serum creatinine is unfortunately poorly sensitive, being greatly influenced by many renal and non-renal factors, such as: changes in muscle mass and tubular secretion, race, age, gender, total body volume (3) and therefore it

does not help in distinguishing between the mechanisms of nephrotoxicity. Moreover, variations in creatinine values are not useful for early detection of CIN since increases in this parameter are often observed 48-72 hours after contrast medium administration.

Recent research has been addressed at finding new, more sensitive, biomarkers for the earlier diagnosis of acute kidney injury. Among them, Neutrophil Gelatinase-Associated Lipocalin (NGAL) and Cystatin C (CysC) have been explored, but conflicting results are reported regarding their role in the early detection of CIN (4-7).

The aim of this study was to test the association of both baseline values and post-procedural variations in either urinary and serum NGAL as well as serum CysC with CIN occurrence, after PCIP compared to serum creatinine and the estimated glomerular filtration rate (eGFR).

Materials and methods

From February to August 2010 we prospectively enrolled 43 patients who were admitted to our Cardiac Step-Down Unit and submitted to PCIP, either for stable coronary artery disease such as stable angina and inducible myocardial ischemia, or for acute coronary syndromes. Exclusion criteria were the positivity of the urine culture sample and eGFR $\leq$60 ml/min/1.73 m^2. At admission and during hospitalization, the following laboratory parameters were measured in a fasting blood sample: hemoglobin (g/dL), troponin I (ng/ml), leukocyte count (N/mm^3), neutrophil count (N/mm^3), serum creatinine (mg/dL), serum CysC (ng/ml)(on admission and 4 hours after PCI), fibrinogen (mg/dl), C-reactive protein (CRP) (mg/L).

We also measured urinary and serum NGAL (ng/ml) (on admission and 4 hours after PCI); values of urinary NGAL were also adjusted for urinary creatinine values by calculating the urinary NGAL to urine creatinine ratio. For NGAL assays fresh urine and EDTA samples were collected from all patients. EDTA samples were immediately centrifuged and aliquoted. All samples were kept at -80°C until assayed in the following weeks. Serum NGAL was measured with the Triage NGAL Test (Biosite-Inverness Medical, Waltham, MA), which is a point-of-care-immunoassay for the quantitative determination of NGAL in EDTA anticoagulated whole blood or serum specimen. Urine NGAL was assessed with an automated immunoassay (Abbott Park, IL) on the Architect platform. The Architect NGAL assay is a non-competitive two-site sandwich immunoassay that utilizes two mouse antibodies, recognizing distinct NGAL epitopes. For CysC measurement, serum blood samples were kept at -20°C until assayed in the following weeks. Serum CysC was assayed by means of a nephelometric method (nephelometry Dako; IMMAGE® 800, Beckman Coulter, Inc. Fullerton, CA). All tests were performed by a well trained laboratory technician according to the manufacturers' instructions. We calculated eGFR (ml/min/1.73 m^2) with the MDRD formula. On admission, a urine culture was taken for each patient.

In agreement with previous studies, CIN was defined as an increase in serum creatinine by $\geq$25% or $\geq$0.5 mg/dl over baseline values, 48 hours after PCIP (2). The study protocol was in accordance with the Helsinki Declaration of 1975, as revised in 1983. Informed consent was obtained from all patients before enrollment. Our study had a prospective design. All data were prospectively collected in a dedicated database and analyzed.

Statistical analysis

Statistical analysis was performed with SPSS (Statistic Package for Social Sciences,

Chicago, USA) for Windows (Version 17). Categorical variables were expressed as frequencies and percentages. Continuous variables were expressed as mean values ± SD, when normally distributed, or otherwise median (range), when appropriate. Normal distribution of variables was checked by assessing the skewness and kurtosis z values, with results between -1.96 and +1.96 indicating normality, by the Shapiro-Wilk test, with a p value > 0.05, and by visual inspection of their histograms, normal Q-Q plots and box plots (data not shown). Fisher's exact test was used to compare categorical variables between CIN- and CIN+ patients. The non-parametric Mann-Whitney *U* test for unpaired data was used to compare variables between CIN- and CIN+ patients; this latter test was also used to analyze urinary and serum values of NGAL as well as the urinary NGAL/creatinine ratio either before and after PCIP in relation to the presence of inflammation, defined as neutrophil count higher than 4200/mm^3; this cutoff value for neutrophils showed association with higher leukocyte count and higher values of erythrocyte sedimentation rate or C reactive protein, confirming an inflammatory pattern. This has been previously reported in the literature to be associated with a higher prevalence of respiratory symptoms, such as bronchitis and persistent cough (8). To evaluate differences of variables analyzed between baseline and post-contrast medium administration values, the nonparametric Wilcoxon rank sum test for paired data was used. Correlations among biomarkers of renal damage and other clinical and laboratory parameters were assessed using the Pearson correlation test, when analyzing variables normally distributed, or the Spearman rank correlation test for variables not normally distributed.

To determine the best baseline CysC threshold associated with CIN occurrence, receiving operating characteristics (ROC) analysis was performed and the area under the ROC curves (AUROC) was calculated; the optimal cut off point was chosen among the coordinates of the ROC curves at the point that gave a balanced weight between sensitivity and specificity. A p value <0.05 was considered statistically significant.

Results

Our population was composed of 59 patients, of which 16 were excluded due to the positivity of their urine culture sample, or for eGFR ≤60 ml/min/1.73 m^2. In Table 1 the main clinical and angiographic characteristics of the 43 patients included in our analysis are presented. Out of them, 7 patients (16%) developed CIN. Patients who developed CIN were significantly older than those who did not.

In Table 2 laboratory data of the patients analyzed in relation to CIN occurrence are reported. Patients who developed CIN had significantly higher discharge serum creatinine values, lower eGFR values at serum peak creatinine and higher serum CysC values, either before and after PCIP. There was no significant difference in serum CysC between the values measured before and those assessed after PCIP in either of the two subgroups analyzed. Moreover, there was no significant difference between the two groups of patients in serum NGAL before and after PICP, or in urinary NGAL before and after PCIP, even after adjustment for urinary creatinine values. Patients who developed CIN underwent in a significantly higher percentage bare-metal stent implantation. In patients who developed CIN no significant variations between baseline and post-PCIP values in serum CysC, urinary and serum NGAL, or urinary NGAL/creatinine ratio were observed.

As depicted in Table 3, patients (n=14) with a positive inflammatory pattern (neutrophil count higher than 4200/mm^3)

Table 1 Clinical characteristics and angiographic data of patients enrolled

Characteristics	All patients (n=43)	CIN - (n=36)	CIN + (n=7)	p value
Age, years ($\bar{x}$ ± SD)	67.3±9.6	65.4 ±9.0	77.1±6.6	0.002
Male/Female	31/12	26/10	5/2	0.644
Overweight (BMI > 25 kg/m^2), n (%)	25 (58.1)	20 (55.6)	5 (71.4)	0.366
Diabetes mellitus, n (%)	9 (20.9)	6 (16.7)	3 (42.9)	0.147
Smoker, n (%)	24 (55.8)	22 (61.1)	2 (28.6)	0.121
Hypertension, n (%)	36 (83.7)	30 (83.3)	6 (85.7)	0.682
Previous CAD, n (%)	21 (48.8)	17 (47.2)	4 (57.1)	0.473
Previous PCI, n (%)	16 (37.2)	13 (36.1)	3 (42.9)	0.525
Previous CABG, n (%)	3 (7.0)	3 (8.3)	0 (0)	0.579
Heart Failure, n (%)	5 (11.6)	4 (11.1)	1 (14.3)	0.608
Dyslipidemia, n (%)	30 (69.8)	24 (66.7)	6 (85.7)	0.303
Family History of CAD, n (%)	15 (34.9)	13 (36.1)	2 (28.6)	0.532
Stable CAD, n (%)	17 (39.5)	14 (38.9)	3 (42.9)	0.581
NSTE-ACS, n (%)	26 (60.5)	22 (61.1)	4 (57.1)	0.581
LVEF, n (%)	55 (50-60)	55 (55-60)	55 (45-55)	0.120
Coronary artery disease extension				
No disease, n (%)	7 (16.3)	6 (16.7)	1 (14.3)	0.681
1 vessel, n (%)	6 (14.0)	5 (13.9)	1 (14.3)	0.681
2 vessels, n (%)	9 (20.9)	9 (25)	0 (0)	NA
3 vessels, n (%)	21 (48.8)	16 (44.4)	5 (71.4)	0.152
Contrast medium amount, ml ($\bar{x}$ ± SD)	264.49±136.13	266.44±140.89	254.43±117.38	0.910
PCI, n (%)	28 (65.1)	23 (63.8)	5 (71.4)	0.532
POBA, n (%)	2 (4.6)	1 (2.7)	1 (14.3)	0.302
BMS, n (%)	5 (11.6)	2 (5.5)	3 (42.8)	0.024
DES, n (%)	21 (48.8)	20 (55.5)	1 (14.3)	0.054
IABP use, *n*	0 (0)	0 (0)	0 (0)	NA
Hemodynamic instability	3 (7.0)	2 (5.6)	1 (14.3)	0.421

BMI=Body mass index; BMS=Bare metal stent; CABG=Coronary artery by-pass graft; CAD=Coronary artery disease; DES=Drug eluting stent; IABP=Intra-aortic balloon pump; LVEF=Left ventricular ejection fraction; NSTE-ACS=Non-ST-elevation Acute Coronary Syndrome; PCI=Percutaneous coronary intervention; POBA=Percutaneous only balloon angioplasty.

had significantly higher values of urinary NGAL, either pre-PCIP and post-PCIP, even when adjusted for urinary creatinine values, as well as higher values of pre-PCI serum NGAL. Moreover, using the Pearson correlation test, a significant correlation was found between age and both serum CysC pre-PCIP (r=0.62; p=0.000) and serum CysC post-PCIP (r=0.58; p=0.000), between eGFR and both serum creatinine at admission (r=-0.71; p=0.000) and serum CysC pre-PCIP (r=-0.45; p=0.003), as well as between serum CysC pre-PCIP and creatinine at the peak (r=0.47; p=0.001) and at discharge (r=0.39; p=0.01).

The Spearman rank correlation test showed a significant correlation between the serum CysC pre-PCIP and urinary

Table 2 Laboratory data in the overall study population and in relation to CIN development

Laboratory data	All patients (n=43)	CIN - (n=36)	CIN + (n=7)	p value
Admission serum creatinine, mg/dl	0.85±0.17	0.85±0.17	0.87± 0.20	0.910
Peak serum creatinine, mg/dl	0.95±0.20	0.91±0.16	1.19±0.21	0.002
Discharge serum creatinine, mg/dl	0.88±0.21	0.83±0.17	1.12±0.25	0.004
Admission eGFR, ml/min/1.73m^2	86.7±20.4	87.5± 20.4	82.7±21.9	0.356
eGFR at serum creatinine peak, ml/min/1.73m^2	77.2±19.5	81.2±17.8	56.7±15.5	<0.001
Peak TnI, ng/ml	0.21 (0.07-2.60)	0.21 (0.07-2.60)	1.80 (0.02-2.60)	0.89
Cystatin C pre-PCIP, ng/ml	1.10±0.26	1.05±0.24	1.34±0.16	0.001
Cystatin C post-PCIP, ng/ml	1.07±0.23	1.02±0.22	1.32±0.14	0.001
Urinary NGAL pre-PCIP, ng/ml	8.30 (5.50-18.60)	8.25 (5.53-17.18)	9.30 (4.60-27.70)	0.784
Urinary NGAL post-PCIP, ng/ml	8.60 (4.50-15.90)	8.25 (4.58-15.88)	9.20 (3.80-18.50)	0.735
Adjusted Urinary NGAL pre-PCIP*, ng/ml	10.92 (6.81-20.30)	10.78 (6.42-19.96)	15.87 (7.44-27.06)	0.664
Adjusted Urinary NGAL post-PCIP*, ng/ml	13.22 (8.19-30.99)	13.10 (8.28-26.63)	15.63 (7.93-38.21)	0.784
Serum NGAL pre-PCIP, ng/ml	76 (60-184)	76 (60-172)	94 (60-272)	0.640
Serum NGAL post-PCIP, ng/ml	104 (60-181)	107 (60-172)	97 (60-209)	0.936
Admission Hb, g/dl	13.5 (12.6-14.5)	13.5 (12.7-14.5)	13.5 (10.8-15.0)	0.987
Nadir Hb, g/dl	12.05±1.69	12.09±1.62	11.81±2.12	0.488
Admission Leucocytes, N/mm^3	7290 (5850-8920)	7090 (5730-8770)	7560 (6780-10400)	0.356
Peak Leucocytes, N/mm^3	8100 (6360-9710)	8040 (6295-9700)	9190 (7920-10600)	0.176
Neutrophils, N/mm^3	3360 (2350-4890)	3180 (2300-4390)	4510 (3100-7520)	0.147
Fibrinogen peak, mg/dl	483.19±98.15	468.33±91.92	559.57±100.11	0.024
ESR, mm/h	13 (7-23)	13 (7-21)	13 (6-30)	0.984

eGFR=Estimated glomerular filtration rate; ESR Erythrocyte sedimentation rate; Hb=Hemoglobin; NGAL=Neutrophil Gelatinase-Associated Lipocalin; PCIP=Percutaneous coronary invasive procedures; TnI=Troponin I. *Adjusted for urinary creatinine values. Values are presented as mean ± standard deviation when normally distributed or, otherwise, as median (interquartile range).

Table 3 Comparison between inflammatory and non-inflammatory states in relation to the timing of samples collection with respect to PCIP

Parameters	Inflammation* (n=14)	No Inflammation (n=29)	p value
Urinary NGAL pre-PCIP, ng/ml	16.60 (9.73-28.80)	7.80 (5.10-14.15)	0.005
Urinary NGAL post-PCIP, ng/ml	16.05 (9.05-20.00)	6.10 (3.80-10.65)	0.002
Urinary NGAL pre-PCIP/creatinine, ng/ml	18.11 (15.03-27.93)	8.49 (4.97-14.20)	0.002
Urinary NGAL post-PCIP/creatinine, ng/ml	20.30 (11.60-39.71)	12.43 (7.76-22.42)	0.046
Serum NGAL pre-PCIP, ng/ml	126 (86-216)	68 (60-121)	0.034
Serum NGAL post-PCIP, ng/ml	125 (102-188)	68 (60-170)	0.10

*Defined as neutrophil count higher than 4200/mm^3; NGAL=Neutrophil Gelatinase-Associated Lipocalin; PCIP=Percutaneous coronary invasive procedures. All values are reported as median (interquartile range).

NGAL post-PCIP/creatinine ratio (r=0.32; p=0.035), and urinary NGAL post-PCIP (r=0.43; p=0.004) in the overall population of patients, and a mild but significant correlation between serum creatinine at discharge and NGAL post-PCIP/creatinine ratio (r=-0.30; p=0.048).

The results from the ROC curve showed that the optimal cut-off value of serum CysC for CIN diagnosis was 1.18 ng/ml. For this value, sensitivity was 0.857 and specificity 0.778 (AUROC=0.863; p=0.003). Finally, by univariate analysis using this latter cut-off, CysC values higher than 1.18 ng/mL resulted in a significant association with CIN occurrence (p=0.003).

Discussion

The main finding of our investigation was that in patients undergoing PCIP, high values of serum CysC, even at baseline, may be considered a useful marker associated with CIN occurrence, earlier than creatinine and eGFR variations. Furthermore, in our series, neither serum nor urinary NGAL showed any significant association with the development of CIN. Data about these novel biomarkers as predictors of acute kidney injury after PCIP are inconsistent and contradictory.

It is well known that CysC, a protease inhibitor, is freely filtered in normal circumstances by the glomeruli and completely reabsorbed in the proximal tubule. In the absence of tubular dysfunction, its serum levels reflect glomerular filtration; therefore it could be used as a convenient measure of glomerular filtration rate (9). CysC is not affected by storage conditions or interfering substances: some limitations of the serum creatinine level, including the effects of gender, body muscle mass and diet, do not significantly influence serum CysC levels. Elevated urine CysC levels may also indicate tubular epithelial damage, and have been proposed as an additional urine biomarker for acute kidney injury (10). In particular, in intensive care settings, serum CysC has been able to detect acute kidney injury earlier and has been more sensitive than serum creatinine in detecting minor glomerular filtration rate reductions (11), even in patients with sepsis (12).

This early increase in serum CysC following a kidney injury is also reported in studies that investigated renal toxicity after contrast medium administration (4-6, 13-18). In all these investigations the peak of serum CysC was significantly associated with the development of CIN occurring 24-48 hours after PCIP. Moreover, in agreement with previous studies (12, 19, 20), our investigation demonstrated that high baseline values of CysC are associated with CIN occurrence.

On the other hand, in the study by Rubichini and colleagues (7), variations in serum creatinine from the baseline offered better diagnostic accuracy for predicting CIN at an earlier stage than similar changes in CysC in patients with known mild or moderate chronic renal disease, undergoing PCI. However, in our investigation we evaluated patients with eGFR $\geq$60 ml/min/1.73 m^2.

Although our study strengthens the role of baseline CysC as a biomarker early associated with CIN occurrence in the setting of PCIP, as already reported (20), no significant variation in CysC in the subgroup of patients developing CIN was observed after contrast medium administration. Interestingly, patients who developed CIN were significantly older and a significant correlation was found between CysC and patient age. Moreover, CysC pre-PCIP results were significantly correlated with values of urinary NGAL assessed after PCIP, even after adjustment for urinary creatinine values, as well as with creatinine at the peak and at discharge, confirming that even values of CysC at baseline may be associated with CIN since they correlate with later changes in creatinine, as

well as in other markers of acute kidney injury, assessed after contrast medium administration.

Furthermore, in our series we also evaluated both urinary and serum NGAL values as possible helpful markers for CIN development. NGAL, a small protein of the lipocalin superfamily, expressed by a wide variety of tissues, is primarily synthesized in the ascending loop of Henle and in the collecting ducts. It acts as a growth and differentiating factor in renal epithelium, by limiting apoptosis and maintaining the tubular structure. Raised NGAL concentrations might represent a physiological or adaptative response to injury (21), since it is has a nephroprotective effect in the proximal tubule. NGAL is rapidly upregulated in kidneys after an ischemic or nephrotoxic insult, and limits parenchymal damage (22). For these reasons NGAL has been explored as a biomarker of acute and chronic kidney injury. Both serum and urinary NGAL concentrations have been shown to be good predictors of CIN development in previous several studies (4, 23). Bachorzewska-Gajewska and colleagues (4) demonstrated in patients with stable angina that NGAL levels were significantly higher in patients with CIN from 2 hours after PCI (serum, $p<0.005$) and 4 hours (urinary, $p<0.005$), respectively. In another study (23), a significant increase in both serum and urinary NGAL occurred in patients developing CIN two hours after contrast medium administration. However, although NGAL is considered a promising biomarker for early diagnosis of acute tubular necrosis (24), serum NGAL is already elevated in patients with chronic renal disease, and it rises 6-times less than urinary NGAL in cases of renal injury (7).

Furthermore, urinary NGAL remains low in patients with pre-renal azotemia or normal renal function (25). Urinary NGAL is of interest after cardiac surgery; in this setting its values resulted in a significant association for early diagnosis of acute kidney injury (26, 27). Haase et al. (28) analyzed data from 191 patients in 3 studies and found that when urinary NGAL is measured within 6 hours after contrast medium administration, it has better sensitivity than serum creatinine alone for acute kidney injury detection. Moreover, recent studies and a meta- analysis have demonstrated the role of serum and urinary NGAL in the prediction of CIN (15, 16, 29-36) and, although this biomarker has also been reported to have a low sensitivity in predicting CIN in specific settings, such as ACS (37), a previous study also suggested that it can also predict the severity of CIN (38).

Despite the positive results of these biomarkers in predicting CIN in the above mentioned studies, in our investigation we did not detect any significant association between baseline serum and urinary NGAL values and their 4 hour variations after contrast medium administration and CIN occurrence. Our results may have several explanations: first of all, our analysis included patients with acute coronary syndromes, a condition in which inflammation is present, so that in these patients raised serum NGAL levels may reflect the degree of inflammatory status (39) due to the acute cardiac disease more than that of acute kidney injury. Accordingly, in our series, we observed that baseline values of either serum and urinary NGAL were significantly higher in patients with a higher neutrophil count, making the post-PCIP variations less significant. On the contrary, we believe that this biomarker could be useful for CIN detection in patients with stable coronary artery disease, as previously reported (4, 23), without other causes of acute inflammation, such as urinary tract infections or acute exacerbations of obstructive pulmonary disease, which is not the typical population of Cardiac Step Down or Intensive Care Units.

Though strong evidence has been provided for the use of NGAL in a variety of settings, its route into clinical management protocols is still under evaluation (40). Moreover, our results are in agreement with those of a recent review reporting the "rise and fall" of NGAL in acute kidney injury (41). In fact, authors conclude that since a rise in NGAL was first suggested more than a decade ago as a potential biomarker of early acute injury, the idea of this marker as a troponin for the kidneys has been relinquished because it also increases unpredictably during other chronic and acute inflammatory conditions frequently encountered in intensive care settings (41). The availability of assays measuring kidney-specific NGAL will improve the chance of detecting patients at risk of acute kidney injury.

Limitations of study

Our study has also several limitations: first of all, a limited number of patients investigated; and second, the heterogeneity of coronary artery disease patients, for whom PCIP were performed.

Conclusion

In conclusion, our investigation demonstrates that in a population of patients admitted to a Cardiac Step-Down Unit and submitted to PCIP, neither baseline serum and urinary NGAL nor their variations after PCIP are useful for an early diagnosis of CIN. In particular, the use of this biomarker to detect CIN is limited in ACS patients, due to its inability at discriminating between inflammatory injury associated with CIN development or with the acute cardiac disease *per se*. On the contrary, in our observation, CysC resulted in an association with CIN occurrence earlier than creatinine and eGFR variations, even though further research in this area is needed, given the limitations of this study.

What is already known on this topic

Contrast induced nephropathy (CIN) is a common and serious complication in patients undergoing percutaneous coronary invasive procedures (PCIP). Serum creatinine is poorly sensitive for CIN detection and its variations are not useful for an early diagnosis of this complication. Recent research has been addressed at finding novel, more sensitive, biomarkers for an earlier diagnosis of CIN; among them, Neutrophil Gelatinase-Associated Lipocalin (NGAL) and Cystatin C (CysC) have been explored but with conflicting results.

What this study adds

In this study, neither baseline serum and urinary NGAL nor their variations after PCIP were related to CIN occurrence, while CysC results were associated with CIN development, earlier than creatinine and estimated glomerular filtration rate variations. Moreover, NGAL values results were significantly associated with markers of acute inflammation, suggesting that this biomarker could be useful for CIN detection in patients with stable coronary artery disease, or when no other causes of acute inflammation are present.

Authors' contributions: Conception and design: EC and CG; Acquisition, analysis and interpretation of data: GA and AT; Drafting the article: MGD; Revising it critically for important intellectual content: AC and EG; Approved final version of the manuscript: EC, CG and AC.

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