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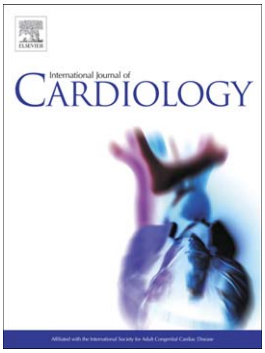

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Original article

Free Light Chains in patients with acute coronary syndromes: Relationships to inflammation and renal function

Running title: Free Light Chains in acute coronary syndromes

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Abstract

Aims: We assessed changes of serum combined free immunoglobulin light chains (cFLC) levels, which are associated with increased all-cause mortality, in ST-elevation myocardial infarction (STEMI) in relation to inflammation and renal function indices.

Methods: cFLC were measured in 48 patients with STEMI on days 1, 3, 7 and 30 with assessment of their relationships with monocyte subsets, high sensitivity C-reactive protein (hsCRP), and cystatin C. Day 1 levels in STEMI patients were compared to 40 patients with stable coronary artery disease, and 37 healthy controls.

Results: There were no significant differences in cFLC levels between the study groups. In STEMI patients, cFLC values peaked on day 7 post-MI and remained elevated on day 30 ($p<0.001$ vs. day 1 for both). hsCRP concentrations peaked on day 3 of STEMI followed by their gradual reduction to the levels seen in the controls ($p<0.001$). In STEMI cFLC correlated with cystatin C ($r=0.55$, $p<0.001$), and negatively correlated with counts of CD14++CD16– monocytes ($r=-0.55$, $p<0.001$). On multivariate Cox regression analysis, cFLC concentrations were associated with increased need for future percutaneous coronary intervention (PCI) ($p=0.019$).

Conclusion: cFLC levels increase during STEMI with peak values on day 7 after presentation and predict the need for future PCI.

Key words: free light chains, heart failure, coronary artery disease, ST-elevation myocardial infarction

Introduction

The biological significance of the adaptive immune system is complex and spreads far beyond just infection control.[1-4] The paramount role of the innate immune system in cardiovascular pathology is very well established with involvement of monocytes/macrophages in atherogenesis and post-injury tissue remodelling being classical examples.[5-7] However, limited data are available on the role of the adaptive immune system in cardiac pathophysiology. Lymphocytes have been found to be involved in atherosclerotic plaque formation, but their low (rather than high) counts are typically associated with poor outcome, for example in heart failure.[5, 8-10] Lymphocyte derived biomarkers, such as immunoglobulin free light chains (FLC) which are produced in excess during antibody production and released into the circulation, may help investigation of the adaptive immune response. [11]

Monoclonal generation of FLC κ and λ is a well-known parameter of plasma cell disorders, such as myeloma.[12] Until recently, limited information was available on polyclonal combined FLC (cFLC, summation of FLC κ and λ) elevations, when there is no obvious predominance of either chain. Polyclonal rise of cFLC predominantly reflects activation and proliferation of B-lymphocytes, although it could be also secondary to their impaired removal by dysfunctional kidneys or reticulo-endothelial system.[13, 14] Elevation of polyclonal cFLC has been reported in inflammatory and autoimmune disorders, diabetes mellitus and chronic kidney disease.[15-18] Also, high cFLC concentrations are associated with activity of autoimmune disorders characterised by B-cell activation, with clearly distinct kinetics for cFLC and a C-reactive protein (CRP), a marker predominantly related to inflammatory responses of innate immunity.[17, 19, 20] cFLC may thus be of clinical value as a biomarker

of the adaptive immunity state. Of note, both the innate and adaptive parts of the immune system work in close interaction.[21] Monocytes and their functional subsets represent a major cellular part of the innate immune system, but limited data are available on their relationship with cFLC.[22]

High cFLC concentrations are highly predictive of mortality in the general population even after adjustment for age, gender and renal function.[23, 24] Raised cFLC were associated with cardiovascular mortality in patients with chronic kidney disease after accounting for CRP levels.[20] Given the pathophysiological relationships discussed above, this biomarker might be related to prognosis in patients with myocardial infarction (MI), there are no data on changes in cFLC in patients with MI at present.

In the present pilot study, we aimed to assess dynamic changes of cFLC levels in STEMI patients over 30 days and their relation to markers of innate immunity (monocyte subsets), inflammation (high sensitivity C-reactive protein [hsCRP]), and an index of renal function, cystatin C, and obtain data on their prognostic significance. cFLC levels in patients with STEMI were compared to levels in patients with stable coronary artery disease (‘disease controls’) and healthy subjects.

Methods

Cross-sectional analysis

cFLC levels were compared between 48 patients with ST-elevation MI (STEMI) and age- and sex-matched control groups: (i) 40 patients with stable coronary artery disease (CAD) and (ii) 37 healthy volunteers. The STEMI was diagnosed according to the European Society of Cardiology definition[25] and treated with primary percutaneous coronary intervention. Median troponin T values were 2.6 [1.30-5.68] μg/l (normal <0.01 μg/l). CAD was confirmed during elective coronary angiography, with no hospital admissions for ≥3 months. Exclusion criteria comprised infectious disease, inflammatory disorders and their treatment [including steroids and non-steroidal anti-inflammatory drugs], cancer, haemodynamically significant valvular heart disease, atrial fibrillation, renal failure and hormone replacement therapy. Additionally, no STEMI patients had a history of previous MI or left ventricular dysfunction.

Longitudinal analysis

In patients with STEMI plasma markers were measured at four time-points: day 1 (during the first 24 h after primary percutaneous coronary intervention (PCI)), day 3, day 7 and day 30. Eighteen patients did not complete follow-up due to withdrawal of consent or death. Thirty patients with cFLC levels available for all time points were included in the longitudinal analysis.

Blood samples were collected from all participants and plasma stored at -70 ºC for batched analysis. Fresh blood was used for haematological analysis and quantification of monocyte subsets as described previously.[26, 27]

All study patients received standard treatment according to current guidelines.[25] The study was performed in accordance with the Helsinki declaration and was approved by the Coventry Research Ethics Committee. All participants provided written informed consent.

Outcome analysis

The prognostic significance of cFLC levels in STEMI was assessed with the primary outcome defined as a composite of 'death, admission for ACS, newly diagnosed HF or HF related hospital admission or new PCI' and the secondary end-point of 'need for new PCI' during follow up.

Flow cytometry

Monocytes and their subsets, as cellular markers of innate immunity and inflammation, were analysed by flow cytometry (BD FACSCalibur™ flow cytometer, Becton Dickinson [BD], Oxford, UK) as previously described.[26, 27] Absolute count of monocyte subsets was established using mouse anti-human monoclonal fluorochrome conjugated antibodies anti-CD16-Alexa Fluor 488 (clone DJ130c; AbD Serotec,Oxford, UK), anti-CD14-PE (cloneM/P9; BD), and anti-CCR2- APC (clone 48607, R&D Systems, Abingdon, UK) in 50 μL of fresh EDTA anticoagulated whole blood in TruCount™ tubes (BD). Monocyte subsets were defined as CD14++CD16CCR2+ (classical), CD14++CD16+CCR2+ (intermediate) and CD14+CD16++ CCR2 (non-classical) in accordance with contemporary nomenclature.

Plasma markers

cFLC concentrations were determined using the Combylite™ assay on the SPAPLUS® turbidimeter (The Binding Site Group Ltd, Birmingham, UK, 95% percentile reference range 9.3-43.3mg/L (determined in serum samples), following the manufacturers recommendations.

[23] Combylite quantifies the combined FLC κ and FLC λ concentration in a single assay.[28] Cystatin C (The Binding Site Group Ltd, reference range 0.56-0.99 mg/L) and hsCRP (Roche/Hitachi Tina-quant® cardiac C-reactive protein high sensitive, Switzerland, reference range 0-3mg/L) concentrations were measured on the BNII^{TM} nephelometer (Siemens, Germany) following the manufacturers recommendations.

Power calculation

As there are no data for FLC, we calculated that minimum number of participants required to achieve 80% power to detect changes of 0.5 standard deviation in non-classical monocytes subsets was n=35 for the cross-sectional study and n=25 for the longitudinal study.

Statistical analysis

Normal data are presented as mean [standard deviation - SD] non-normal data are shown as median [interquartile range, IQR]. Cross-sectional comparisons between the three study populations were made using a chi-square test (for categorical variables), one way analysis of variance (ANOVA) with Tukey post-hoc test (for normal data) or Kruskal Wallis test with Dunn's post-hoc test (for non-normal data). Longitudinal analysis was performed using repeated measures ANOVA with Bonferroni adjustment (normal data) or Friedman test with Dunn's post-hoc test (non-normal data). Only STEMI patients who had blood samples for all time-points were included in the longitudinal analysis. For STEMI patients, correlation coefficients were calculated by Spearman tests (non-normal data). Linear regression analysis was used to establish predictive value of cFLC for LVEF measured 6-weeks post-STEMI. Predictive value of the cFLC for the study outcome parameters in STEMI was assessed using a Cox regression analysis and the Kaplan-Meier log-rank test. In the multivariable Cox regression analysis adjustments were made for parameters showing significant predictive

value (or a strong trends towards significant predictive value) in multivariate analysis. Additionally the multivariable Cox regression analysis included cystatin C (a marker of renal function) and troponin concentrations (a marker of myocardial damage). Data analysis was carried out using SPSS 18.0 (SPSS Inc, Chicago, IL, USA) and a two-sided p-value of <0.05 was considered statistically significant.

Results

The 3 patient groups were well matched for age, gender, current blood pressure level and body mass index, creatinine and estimated glomerular filtration rate (eGFR) (Table 1). Patients with acute STEMI had increased counts of monocytes and neutrophils compared to other groups ($p<0.001$). Healthy controls included a smaller proportion of smokers than other groups ($p<0.001$).

Increased hsCRP and Cystatin C levels were evident in patients with acute STEMI compared to the control groups (Table 1). No significant difference was observed in cFLC values at day 1 between STEMI patients and the two control groups.

In acute STEMI, cFLC correlated with cystatin C (Spearman $r=0.55$, $p=0.00009$), and negatively correlated with counts of 'classical' CD14++CD16– monocyte counts (Spearman $r=-0.55$, $p=0.00005$). There was no correlation between cFLC and other monocyte subsets, troponin T, or CRP. Absolute values of the monocyte subsets in this study population have been reported previously.[27]

Longitudinal analysis

In STEMI patients there was a significant increase in cFLC during the period of observation with highest values seen on day 7 post MI ($p<0.001$ vs. day 1) with increased levels persisting at day 30 ($p<0.001$ vs. day 1) (Table 2 Figure 1). hsCRP concentrations peaked on day 3 of the MI followed by their gradual reduction to the levels seen in controls ($p<0.001$). Cystatin C concentrations were significantly increased (as compared to day 1) values on day 3 ($p=0.006$) and day 30 ($p=0.002$), but not on day 7 ($p>0.05$).

Outcome analysis

Sixteen primary outcome events were recorded during a (median) follow up period of 39 [19-43] months (Table 2). Cox regression analysis, cFLC levels at presentation in acute STEMI were not predictive of the primary outcome (Hazard Ratio 1.03 [0.98-1.09], p=0.29). On Kaplan-Meier analysis, high (>median levels, 24 [20-34] mg/L) cFLC levels were not predictive of the primary outcome (log-rank test p=0.23). Nine secondary outcome events of new PCI occurred during a median follow up of 40 [37-44] months. cFLC concentrations were 23 [19-30] mg/L in patients free from new PCI and 30 [27-41] mg/L in patients who had new PCI [p=0.033, Mann-Whitney test]. On univariate Cox regression analysis, cFLC concentrations were associated with an increased risk for new PCI and remained predictive on multivariate analysis (Table 4). On Kaplan-Meier analysis, there was a trend for high cFLC levels to be related new PCI which did not reach statistical significance (log-rank test, p=0.066) (Figure 2).

Discussion

This pilot study provides the first analysis of cFLC in patients with STEMI. During acute phase of the disease cFLC values were similar to those in stable CAD and healthy individuals. However cFLC levels showed a relatively small but significant increase during the follow up period with peak concentrations determined on day 7 and were still increased on day 30. Secondly, cFLC concentrations measured at presentation were associated with an increased risk for new PCI even on multivariate analysis.

Median cFLC concentrations remained within the normal range even at day 7 post MI; however the analysis matched the data from individual patients and showed a highly statistically significant and consistent increase in most patients, as shown in figure 1. The observed changes can involve several mechanisms. First, they could be reflective of progressive dysfunction of the adaptive immune system soon after MI onset (i.e., B-cell activation and proliferation).[15] For example, Zouggari et al. have shown that mobilization of proinflammatory monocytes following experimental acute MI was mediated by activity of mature B lymphocytes (ie, their CCL7 production).[29] The animal experiments were further supported by evidence from patients with acute MI where high plasma levels of CCL7 and BAFF in patients with acute MI were associated with increased risk of death or recurrent MI.[29] Second, raised cFLC could be due to their delayed removal from circulation. Indeed, our study shows a significant correlation between cFLC and cystatin C, a marker of kidney dysfunction. However it is unlikely that renal impairment is the sole explanation of cFLC elevation, and the changes reflect different dynamics in the biomarkers. For instance, on day 7 of STEMI when highest cFLC levels were noted, cystatin C concentrations on day 7 were not significantly different from those on day 1 of STEMI. No correlation was found between

cFLC and troponin levels suggesting that the extent of myocardial damage may not be of paramount importance for cFLC production. Of note, cystatin C and BNP levels fall by 30 days, whilst cFLC levels remain elevated. This may indicate a relation of cFLC to longer-term processes related to myocardial healing/remodelling.

As expected, there was no significant correlation between cFLC, which is a marker of activity of the adaptive immune system and hsCRP, a liver-derived marker of inflammatory responses related to the innate immune response.[20] However there was a significant negative correlation between cFLC and counts of 'classical' CD14++CD16- monocytes, a cellular marker of innate immunity.

Despite their primary role in innate immune responses, monocytes have diverse functions being involved in numerous inflammatory and reparative responses.[30] FLC can bind directly to human monocytes and can induce mast cell degranulation and neutrophil activation. [31, 32] Monocytes are diverse and include several subsets with specific functions, of which 'classical' monocytes are characterised by high phagocytic activity and proinflammatory phenotype.[22, 26, 33] Although the study does not provide direct insight into the mechanisms of the association between the cFLC and 'classical' monocytes the observed negative correlation between these biomarkers could potentially be related to specific capacity of this monocyte subset to bind cFLC. However it is unclear whether these interactions simply represent a mode of elimination of cFLC from the circulation or they have functional consequences for monocyte activity.

The function and clinical consequences of the cFLC rise post-STEMI remains unclear. Immune abnormalities are known to play an important role in atherosclerosis and cFLC

upregulation post STEMI may reflect arterial processes within unstable coronary artery lesions characteristic of acute coronary syndromes. Of note, cFLC levels are predictive of future need for repeat PCI and thus could be related to accelerated disease progression in ACS patients already in a 'high risk' category (eg, post STEMI). The association remains significant even after adjustment for status of renal function and inflammation as assessed by hsCRP. The mechanistic insight(s) in these associations is still to be provided, but it is likely that cFLC could be implicated in immune responses related to progression of atherosclerosis, particularly in settings post ACS events. These data accord with previous evidence of predictive role of high cFLC concentrations in general population.[23]

Limitations

This pilot study is relatively small and has limited power to establish the prognostic role of cFLC in STEMI, but allows hypothesis generation. Also, our data provide only limited information on possible mechanisms related to changes in cFLC post-MI and specifically designed pathophysiological studies will be required to address the issue.

In conclusion, cFLC levels increase during STEMI with peak values on day 7 after presentation and on multivariate analysis, predict the need for future PCI. The precise pathophysiological and clinical significance of this phenomenon remains unclear but may reflect post-STEMI inflammatory responses or renal reserve changes in these patients, which deserve further exploration.

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Disclosure of interests:

ES, LDT, GYHL – no conflict of interest

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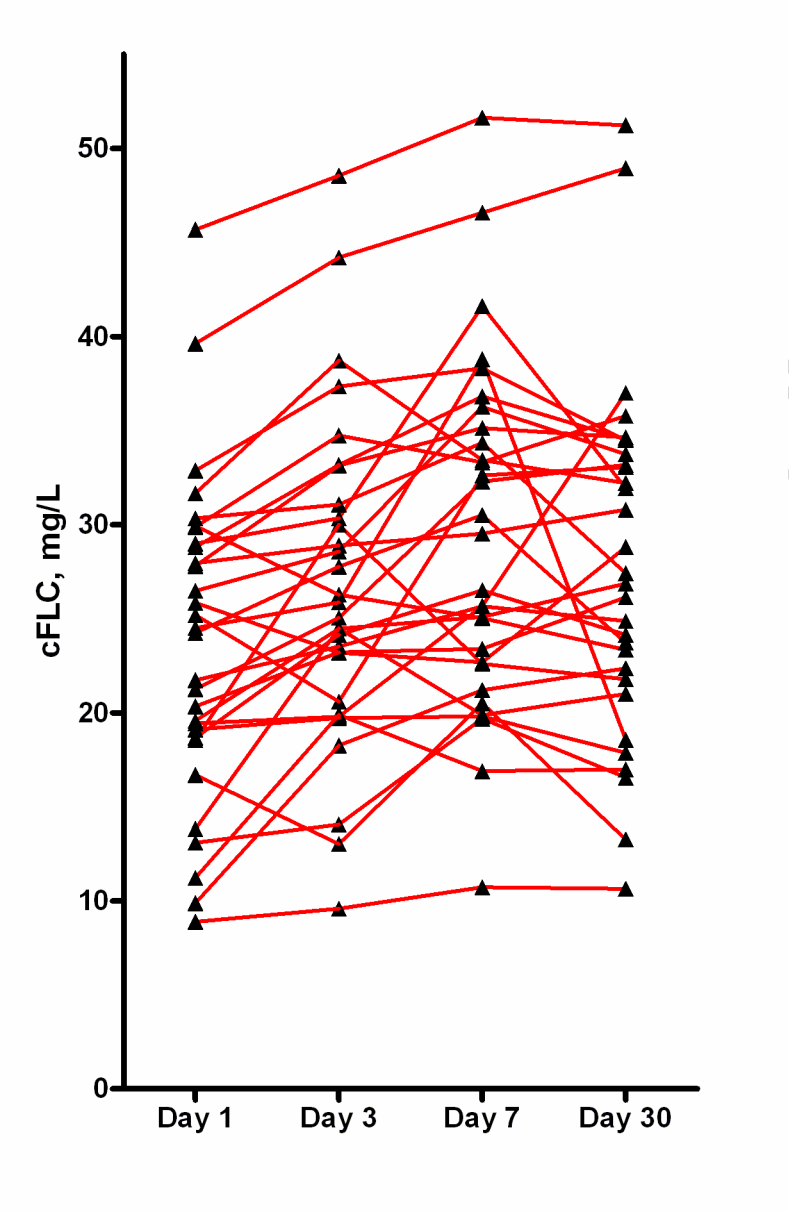

Figure 1. Dynamics of the cFLC in STEMI

cFLC, combined free light chains.

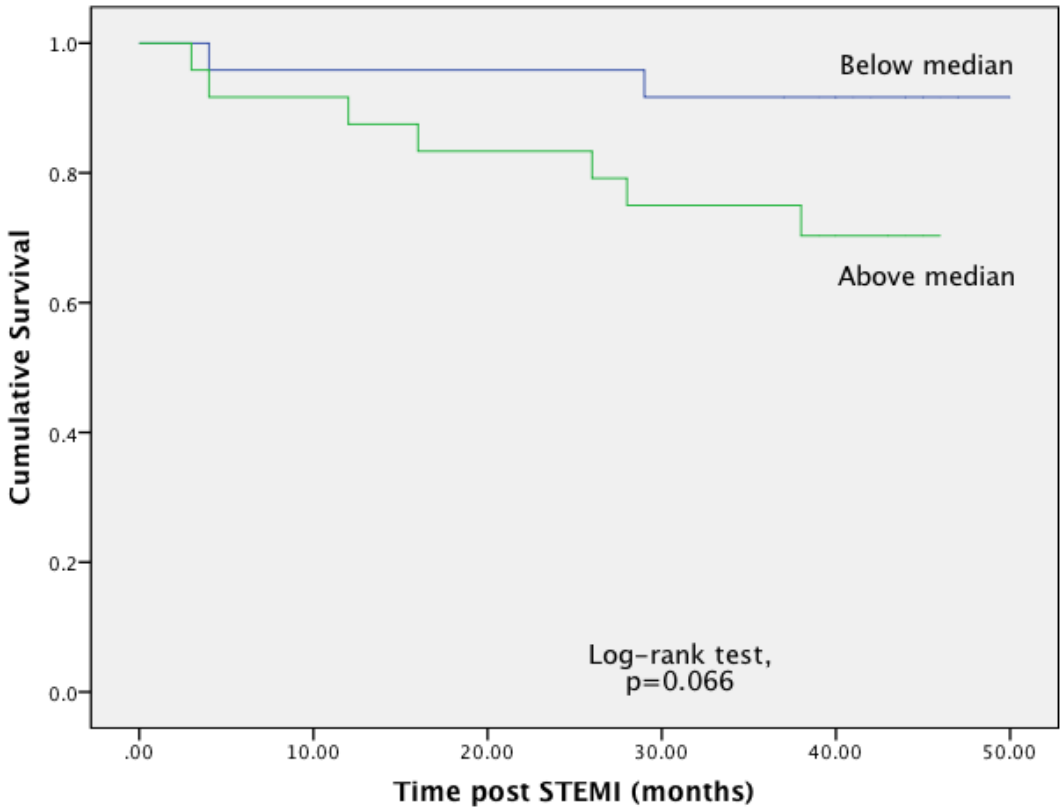

Figure 2. Predictive value of the above median cFLC levels for the secondary outcome (Kaplan-Meier analysis)

Table 1. Characteristics of the study groups

	STEMI (n=48)	Stable CAD (n=40)	Healthy (n=37)	p
Demographic and clinical characteristics				
Age, years	58 [12]	60 [11]	60 [14]	0.54
Male gender, n [%]	41 [85]	33 [83]	31 [84]	0.93
Systolic BP, mmHg	132 [18]	133 [15]	128 [17]	0.74
BMI, kg/m^2	30 [6]	29 [5]	27 [4]	0.30
LVEF, %	54 [14]	67 [8]	-	0.24
Creatinine, μmol/L	92 [19]	90 [18]	78 [17]	0.26
eGFR, $ml/min/1.73m^2$	75 [15]	73 [14]	83 [10]	0.34
Hypertension, n [%]	23 [48]	20 [50]	5 [14]	0.001
Diabetes, n [%]	16 [33]	8 [20]	0	0.001
COPD, n [%]	3 [6]	3 [8]	0	0.26
Smoking, n [%]	28 [58]	17 [43]	1 [3]	<0.001
Medications				
Aspirin, n [%]	48 [100]	36 [90]	5 [14]	<0.001
Clopidogrel, n [%]	48 [100]	20 [50]	0	<0.001
B-blockers, n [%]	33 [69]	27 [68]	0	<0.001
ACEI/ARA, n [%]	43 [90]	29 [73]	4 [11]	<0.001
Statins, n [%]	46 [96]	34 [85]	5 [14]	<0.001
Blood leucocytes				
WBC, 10^3 per μL	10.8 [3.1]*†	6.6 [1.8]	5.9 [1.3]	<0.001
Neutrophils, 10^3 per μL	8.1 [2.9]*†	3.9 [1.2]	3.6 [1.1]	<0.001

Lymphocytes, 10^3 per μL	1.7 [0.7]	2.0 [0.6]	1.8 [0.7]	0.24
Total monocytes, per μL	952 [375]*†	599 [165]	514 [196]	<0.001
CD14++CD16- monocytes, per μL	765 [322]*†	494 [146]	422 [169]	<0.001
CD14++CD16+ monocytes, per μL	98 [30-161]	35 [22-50]	27 [9-54]	<0.001
CD14+CD16++ monocytes, per μL	61 [40-89]	56 [42-82]	48 [35-62]	0.069
Platelets, 10^3 per μL	266 [98]	226 [69]	254 [67]	0.094
Plasma biomarkers				
cFLC, mg/L	26 [19-32]	24 [20-34]	25 [21-34]	0.70
Cystatin C, mg/L	1.06 [0.25]†	1.12 [0.29]	0.95 [0.15]	0.008
hsCRP, mg/L	8.3 [1.9-17]*†	1.5 [0.8-3.3]	1.0 [0.6-2.1]	<0.001

*p<0.05 vs. stable coronary artery disease; †p<0.05 vs. healthy controls; ACEI, angiotensin converting enzyme inhibitor; ARA, angiotensin receptor antagonists; BMI, body mass index, BNP, brain natriuretic peptide; CAD, coronary artery disease; COPD, chronic obstructive pulmonary disease; eGFR, estimated glomerular filtration rate; cFLC, combined free light chains; hsCRP, high sensitivity C-reactive protein; LVEF, left ventricular ejection fraction; WBC, whole blood count. Data are presented as mean [SD] for normally distributed variables and median [IQR] for non-normally distributed variables.

Table 2. Longitudinal analysis of the study parameters.

N=30	Day1	Day3	Day7	Day 30	P value
cFLC, mg/L	24 [19-29]*†‡	25 [20-32]	28 [22-35]	27 [22-34]	0.00000008
Cystatin C, mg/L	1.01 [0.22]*‡	1.08 [0.17]	1.06 [0.18]	1.09 [0.22]	0.001
hsCRP, mg/L	5.9 [1.7-12]*‡	16.9 [5.3-41]†‡	4.4 [1.6-11]‡	1.3 [0.6-2.7]	0.0000000003
'Classical' CD14++CD16- monocytes, per μL	810 [58]‡	785 [59]‡	712 [63]	557 [31]*	< 0.0001

*p<0.05 vs. day 3, †p<0.05 vs. day 7; ‡p<0.05 vs. day 30. cFLC, combined free light chains; hsCRP, high sensitivity C-reactive protein.

Table 3. Characteristics of patients with and without the primary outcome event

	Primary outcome event occurred		p
	Yes (n=16)	No (n=32)	
Demographic and clinical characteristics			
Age, years	64 [11]	54 [11]	0.006
Male gender, n [%]	15 [94]	26 [81]	0.25
Systolic BP, mmHg	128 [15]	135 [20]	0.48
BMI, kg/m^2	26 [4]	32 [6]	0.049
LVEF, %	46 [12]	56 [14]	0.039
Creatinine, μmol/L	100 [23]	88 [15]	0.062
eGFR, ml/min/1.73m^2	70 [17]	77 [14]	0.16
Hypertension, n [%]	9 [56]	14 [44]	0.41
Diabetes, n [%]	7 [44]	9 [28]	0.28
COPD, n [%]	0	3 [9]	0.21
Smoking, n [%]	6 [38]	22 [69]	0.04
Medications			
Aspirin, n [%]	16 [100]	32 [100]	1.00
Clopidogrel, n [%]	8 [50]	15 [47]	0.84
B-blockers, n [%]	11 [69]	22 [69]	1.00
ACEI/ARA, n [%]	13 [81]	30 [94]	0.18
Statins, n [%]	15 [94]	31 [97]	0.61
Plasma biomarkers			
cFLC, mg/L	26 [21-38]	25 [19-31]	0.42

Cystatin C, mg/L	1.05 [0.89-1.15]	0.96 [0.86-1.16]	0.33
hsCRP, mg/L	4.6 [1.2-9.6]	9.1 [2.6-21.3]	0.14

ACEI, angiotensin converting enzyme inhibitor; ARA, angiotensin receptor antagonists; BMI, body mass index, BNP, brain natriuretic peptide; CAD, coronary artery disease; COPD, chronic obstructive pulmonary disease; eGFR, estimated glomerular filtration rate; cFLC, combined free light chains; hsCRP, high sensitivity C-reactive protein; LVEF, left ventricular ejection fraction; WBC, whole blood count. Data are presented as mean [SD] for normally distributed variables and median [IQR] for non-normally distributed variables.

Table 4. Cox regression analysis of the predictive value of cFLC for new PCI.

Parameter	Hazard ratio [95% confidence interval]	p value
Univariate analysis		
cFLC, mg/L	1.10 [1.02-1.19]	0.018
Cystatin C, mg/L	3.65 [0.30-43.7]	0.31
hsCRP, mg/L	0.86 [0.73-1.01]	0.065
Age, years	1.01 [0.95-1.07]	0.77
Gender	0.04 [0.00-88.7]	0.41
Troponin, ng/mL	1.06 [0.88-1.27]	0.53
Multivariate analysis		
cFLC, mg/L*	1.10 [1.02-1.19]	0.019
cFLC, mg/L†	1.25 [1.02-1.52]	0.031
cFLC, mg/L‡	1.76 [1.02-3.02]	0.041

cFLC, combined free light chains; hsCRP, high sensitivity C-reactive protein.

*Adjusted for age, cystatin C and hsCRP;

†Adjusted for age, cystatin C, hsCRP and troponin.

‡ Adjusted for age, cystatin C, hsCRP, troponin and left ventricular ejection fraction