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Highlights:

- 1) Plasma combined free light chains (cFLC) are increased in patients with acute heart failure
- 2) In patients with acute heart failure cFLC remain elevated during 3 months after admission
- 3) cFLC are strong independent predictors of poor outcome in patients with acute heart failure

**Free Light Chains In Patients with Acute Heart Failure Secondary to Atherosclerotic
Coronary Artery Disease**

Short title: Free light chains in heart failure

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Disclosures.

ES, BW and GYHL have no conflict of interest. JMF and LKA are employees of The Binding Site Group Ltd, the manufacturers of the assays used to measure FLC in this study. They have not been involved in the statistical analysis of the data.

Abstract

Increased combined free light chains (cFLC) are strongly prognostic of death in general populations and in patients with chronic kidney disease, but scarce data are available on cFLC in heart failure (HF). We aimed to assess the dynamics and prognostic significance of cFLC levels in patients following admission with acute heart failure (AHF). cFLC measurements were compared in 49 patients with AHF, 37 patients with stable HF, 43 patients with stable coronary artery disease and without HF ('disease controls'), and 37 healthy controls. The association of cFLC with death and/or rehospitalisation was assessed. Patients with AHF had significantly elevated cFLC levels, compared to other groups ($p < 0.001$). Patients with stable HF showed higher levels of cFLC than healthy controls. In AHF, cFLC levels correlated with cystatin C (Spearman $r = 0.63$, $p < 0.001$), and creatinine (Spearman $r = 0.47$, $p = 0.002$). During 3 months follow up brain natriuretic peptide (BNP) reduced significantly ($p = 0.017$), but cFLC did not change significantly. In a multivariate Cox regression analysis, the higher quartiles of cFLC were significantly associated with death/readmission (hazard ratio (HR) 8.34 [95% CI 2.38-29.22] $p = 0.0009$) after adjustment for age, gender, BNP and cystatin C levels. Higher quartiles of cFLC were prognostic for death alone (HR 14.0 [95% CI 1.72-113.8], $p = 0.014$). In conclusion, raised serum cFLC concentrations in patients with AHF were independently associated with prognosis. In AHF, elevated cFLC levels persist long after clinical stabilisation, which may reflect immune disturbances and/or the reduced capacity of (perhaps functionally impaired) kidneys and the endothelium to eliminate them.

Key words: free light chains, heart failure, coronary artery disease

Introduction

Heart failure (HF) is a significant contributor to overall mortality in the community and high levels of combined free light chains (cFLC) were associated with increased mortality in the general population.^{1,2} Also cFLC levels have been reported to be associated with unfavourable outcome in chronic kidney disease where cardiovascular disease was the major cause of death; CKD is common finding in patients with HF.³ In the present study, we tested the hypothesis that cFLC levels are abnormal in HF, with the greatest abnormalities seen in acute decompensated HF. Second, we hypothesised that cFLC levels would have prognostic implications for 'death and HF hospitalisation'

Methods

Forty nine consecutive patients admitted to hospital with acute HF (AHF group) of ischemic etiology were recruited and compared to 37 patients with stable chronic HF (SHF group), 43 patients with stable coronary artery disease free from HF (CAD group, 'disease controls'), and 37 healthy controls (HC group). AHF was defined in accordance with the European Society of Cardiology guidelines as the rapid onset/progression of HF symptoms and signs related to reduced cardiac contractility requiring hospital admission (New York Heart Association (NYHA) class symptoms).⁴ All patients had left ventricular ejection fraction (LVEF) of $\leq 40\%$ on echocardiography or left ventriculography. Patients were excluded if the hospital admission was caused by an acute coronary syndrome (chest pain with ST/T wave changes on electrocardiogram \pm positive troponin) or haemodynamically compromising arrhythmia or valvular pathology.

The SHF group included patients with LVEF $\leq 40\%$ and no deterioration in clinical condition, hospital admission or change in medication for the preceding six months. All SHF patients had an ischaemic etiology of HF. In this group, 10 (27%) patients had NYHA I class symptoms, 24 (65%) patients had NYHA II class symptoms, and 3 (8%) had NYHA III class symptoms. For CAD 'disease control' group eligibility, criteria included history of myocardial infarction >6 months previously and/or angiographically documented stenosis $>50\%$ in ≥ 1 coronary artery and LVEF $>50\%$. Patients with SHF and CAD were recruited from outpatient clinics at Sandwell and West Birmingham Hospitals NHS Trust. The study was performed in accordance with the Helsinki declaration and was approved by the Warwickshire Research Ethics Committee. All participants provided written informed consent.

To minimise potential confounders, CAD was the aetiology of HF in all patients. This inclusion criterion enabled both AHF and SHF patients to be compared to the CAD group as disease controls with a similar profile of cardiovascular risk factors and co-morbidities (e.g. diabetes, hypertension) and medications used. For all study groups, exclusion criteria included infectious and inflammatory disorders, cancer, haemodynamically significant valvular heart disease, creatinine $>200 \mu\text{mol/l}$, steroids and hormone replacement therapy.

At baseline all participants had a full medical history and clinical examination performed by a cardiologist. Non-fasting peripheral venous blood samples were analyzed by flow cytometry within 60 minutes of collection and plasma was stored at -70°C for batch analysis of biomarkers, including brain natriuretic peptide (BNP), high sensitivity C-reactive protein (hsCRP), cFLC and cystatin C.

In order to assess the dynamics of cFLC in AHF over time, blood samples were

analyzed at the following time-points: (i) during the first 24 hours after admission, (ii) on the day of hospital discharge and (iii) 3 months following hospital admission. Recruitment began on 30 October 2009 and all patients were followed until 30 July 2011 to register any cases of death or re-hospitalisation. The primary endpoint was defined as a combination of the first occurrence of re-hospitalization or death; the secondary endpoint was any-cause of death alone.

Cystatin C and cFLC (Combylite™ assay, The Binding Site Group Ltd, Birmingham, UK) were measured on the SPAPLUS® turbidimeter (The Binding Site Group Ltd) following the manufacturer's recommendations. The reference ranges were 0.56-0.99mg/L and 9.3-43.3mg/L (determined in serum samples),¹ respectively. Combylite quantifies the combined FLC κ and FLC λ concentration in a single assay.⁵ There was no evidence of monoclonal expansions, which could potentially influence FLC concentrations, in any sample either by positive serum protein electrophoresis or abnormal FLC κ/λ ratio (Freelite®, The Binding Site Group Ltd).⁶ hsCRP (Siemens, Germany) concentrations were measured on the BNII™ nephelometer (Siemens, Germany).

Plasma levels of interleukin-6 were measured by cytometric bead array technology. The BD FACSCalibur flow cytometer was used for data acquisition, with FCAP Array v2.0.2 software (Burnsville, Minnesota, USA) for data analysis. Commercially available Human interleukin-6 Flex (Becton Dickenson, Oxford, UK) was used according to the manufacturer's recommendations. The lower limit of detection of interleukin-6 was 1.0pg/ml. BNP was measured using a commercially available enzyme immunoassay set (human BNP-32, Peninsula Laboratories, LLC, CA, USA) according to manufacturer's specifications.^{7,8} The inter- and intra- assay coefficients of variation for interleukin-6 and BNP assays were <5%.

Normally distributed data are presented as mean \pm standard deviation (SD) and non-normally distributed data are shown as median (interquartile range, IQR). Cross-sectional comparisons between the four 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 AHF patients who completed 3 months follow-up were included in the longitudinal analysis. For AHF patients, correlation coefficients were calculated by Pearson and Spearman tests for normal and non-normal data, respectively. Cox regression analysis was used to establish predictive value of the study for parameters for the study outcome.

Kaplan Meier estimates for the distribution of time from index admission to the primary end-point were computed and log-rank analysis was performed to compare event free survival for patients with values of cFLC, BNP, hsCRP and estimated glomerular filtration rate (eGFR) above and below the median value at admission. 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 (i.e. AHF, SHF, CAD) were comparable for age, gender, blood pressure and body mass index (Table 1). Patients with AHF had higher BNP and creatinine levels and lower eGFR vs SHF. As expected, patients with AHF more often received loop diuretics and less beta-blockers than participants from all other groups. Patients with AHF

had increased counts of monocytes and neutrophils and lower haemoglobin concentrations compared to other groups ($p<0.001$).

Patients with AHF had a highly significant increase in levels of cFLC, cystatin C, hsCRP, interleukin-6, monocytes and neutrophils compared to other groups ($p<0.001$) (Table 1, Figure 1 for cFLC). Also patients with SHF showed higher levels cFLC and cystatin C compared with 'healthy controls' ($p<0.05$ and $p=0.008$, respectively). There were no significant differences in hsCRP, interleukin-6 and leukocyte counts between the patients with SHF and control groups free from HF.

In AHF cFLC correlated with cystatin C (Spearman $r=0.63$, $p=0.000001$), creatinine (Spearman $r=0.47$, $p=0.002$) and inversely with eGFR (Spearman $r=-0.39$, $p=0.01$). Cystatin C correlated with creatinine levels (Pearson $r=0.70$, $p=0.0000004$) and inversely with eGFR (Pearson $r=-0.64$, $p=0.000001$). hsCRP correlated with interleukin-6 levels (Spearman $r=0.69$, $p=0.0000001$), and neutrophil counts (Spearman $r=0.51$, $p=0.004$). There was no significant association between concentrations of cFLC, cystatin C, hsCRP and LVEF or BNP values.

Thirty-two AHF patients (65%) completed all 3 blood test time-points. The median length of hospital stay was 9 days (IQR 5-13). Nineteen patients (39%) reached the primary end point of death or re-hospitalization, with a median duration of follow up of 221 days (IQR 140-453). Fourteen patients (29%) reached the secondary end point of death. Twelve patients died of HF, 1 died from a ruptured abdominal aortic aneurysm and the cause of death in one patient was unknown.

During the 3 month follow up period, there was a significant reduction in BNP concentrations ($p=0.017$), but no significant change in cFLC or hsCRP levels (Table 2). A non-significant trend was observed towards a further increase in cystatin C ($p=0.072$).

cFLC concentrations were significantly higher in patients reaching the primary endpoint of death or re-hospitalization (72 [86-118] mg/L) compared to those who remained free of events (58 [39-64] mg/L). In a univariate Cox regression analysis, cFLC concentrations were highly significantly associated with the primary end-point and remained associated with the outcome after adjustment for age, gender, BNP, cystatin C levels and LVEF (Table 3). On univariate Cox regression analysis, cFLC concentrations were significantly predictive of the occurrence of the secondary end-point (death alone) and remained associated with the outcome after adjustment for age, gender, BNP and cystatin C levels and LVEF. Levels of hsCRP, interleukin-6 and leukocyte counts were not significantly associated with the primary or secondary outcomes (Table 3).

To further establish the predictive significance of cFLC in AHF, its values were analysed as quartiles. On univariate Cox regression analysis, quartiles of cFLC were significantly associated with the primary end-point (hazard ratio 2.6 [95% confidence interval 1.6-4.3], $p=0.0002$) and remained associated with the outcome after adjustment for age, gender, BNP and cystatin C levels (hazard ratio 9.4 [95% confidence interval 2.7-32], $p=0.0004$) and after additional adjustment for LVEF (hazard ratio 12.5 [95% confidence interval 2.9-54], $p=0.001$). On a univariate Cox regression analysis, quartiles of cFLC were significantly predictive of the occurrence of the secondary end-point (death alone, (hazard ratio 2.3 [95% confidence interval 1.3-4.1], $p=0.003$) and still remained associated with the outcome after adjustment for age, gender, BNP and cystatin C levels (hazard ratio 8.5 [95% confidence interval 1.8-40], $p=0.007$).

For a Kaplan-Meier analysis, patients were dichotomized using the median cFLC value (62 mg/L). Patients with high cFLC (above median levels) had significantly worse outcomes (primary endpoint) compared to those below the median levels (log rank, $p=0.00004$) (Figure 2). Patients with high cFLC also had a worse outcome for the secondary endpoint (death alone) (log rank, $p=0.002$) (Figure 2). Kaplan-Meier analyses were performed for BNP, hsCRP and eGFR levels and no significant predictive values were found for these parameters (Supplementary figure 1).

Discussion

In this study, we show *for the first time* a highly significant rise in cFLC in patients with acute decompensated HF. The cFLC levels remained elevated for at least 3 months after HF exacerbation and their high concentrations were strongly associated with poor intermediate term outcomes. Elevated cFLC have been associated with increased all-cause mortality, with circulatory disease being a predominant cause of death; however this is the first report linking cFLC to a specific cardiovascular outcome.^{1,2}

Several mechanisms can be responsible for increased cFLC in HF, which could be simplified to their increased production due to stimulation of the adaptive immune system (i.e., B-cell activation and proliferation)⁹⁻¹¹ and impaired elimination due to dysfunction of the kidneys or reticulo-endothelial system.^{12,13} A combination of all these mechanisms is likely to take place in the setting of AHF. Indeed, patients with AHF typically show some deterioration of renal function and in our study there was a strong association between cFLC levels and parameters of renal impairment, particularly cystatin C levels.¹⁴ Abnormal cFLC

removal from the circulation could also be due to diminished pinocytic activity of capillary endothelial cells, which is consistent with endothelial dysfunction that is evident in HF.¹⁵⁻¹⁷

Although high total lymphocyte counts are generally linked to a better prognosis in HF, sparse clinical data are available on the functionality of B-cell subsets in this condition and their role in immune disarrangement in this disorder. Consequently cFLC may potentially represent a useful prognostic biomarker in HF patients independent of parameters of high intracardiac pressure (i.e. BNP) and inflammation related to innate immunity (i.e., hsCRP). Indeed, in our study hsCRP correlated with interleukin-6 concentrations and neutrophil counts, but showed no significant correlation with cFLC, in agreement with previous data.¹⁸ Importantly, cFLC preserved its prognostic significance even after accounting for renal status indicating the cFLC rise is not solely because of renal dysfunction and has incremental prognostic significance in this regard. However, investigation into the exact mechanisms of cFLC upregulation in HF was beyond the scope of this pilot study. Also, it remains unclear whether the prognostic power of cFLC simply reflects the background abnormalities leading to their increase or it could also be attributable to direct detrimental effects of high cFLC concentrations. FLC can cause mast cell degranulation, alter neutrophil chemotaxis and exhibit direct nephrotoxic effects.^{19,20} Indeed, symptomatic improvement in acute HF treated by levosimendan was partly attributable to a reduction in cFLC concentration.²¹

This pilot study is relatively small in size and the findings need to be confirmed in larger studies, which will also allow comparison to other biomarkers. Our work also provides only limited insights into mechanisms responsible to cFLC-related abnormalities in HF and does not answer the question of whether high cFLC contribute to HF progression or they are secondary to other processes, which also cause HF deterioration. These questions need to be tested in specifically designed studies.

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Figure 1. Combined free light chains in the study groups

AHF, acute heart failure; CAD, coronary artery disease. cFLC, combined free light chains; SHF, stable heart failure. Median and interquartile ranges are indicated by the solid lines.

Figure 2. Survival analysis.

Kaplan-Meier analysis of predictive value of combined free light chains concentrations in acute heart failure for the primary outcome of death or re-hospitalisation (left) and the secondary outcome of death alone (right).

Supplementary figure 1. Kaplan-Meier analysis of predictive value of the high sensitivity C-reactive protein, brain natriuretic peptide and estimated glomerular filtration rate in acute heart failure for the primary outcome (top row) and the secondary outcome (bottom row)

Table 1. Characteristics of the study groups

Variable	Acute HF (n=49)	Stable HF (n=37)	CAD (n=43)	Healthy (n=37)	p
Demographic and clinical characteristics					
Age (years)	71±11	70±10	68±9	67±7	0.23
Men	34 (69%)	30 (81%)	29 (67%)	23(62%)	0.34
Systolic blood pressure (mmHg)	139±28	124±24	135±18	143±16	0.07
Body mass index (kg/m ²)	28±5	29±4	28±4	26±3	0.50
Brain natriuretic peptide (pg/L)	522 (239- 1142)	71 (35-256)	-	-	<0.001
Left ventricular ejection fraction (%)	29±9†	29±10†	58±7	-	<0.001
Creatinine (µmol/L)	127±37*†‡	103±24†	87±20	76±15	<0.001
Estimated glomerular filtration rate (ml/min/1.73m ²)	50±17*†‡	62±16‡	72±15	84±9	<0.001
Hypertension	27 (55%)‡	17 (46%)	27 (63%)	6 (16%)	0.006
Diabetes mellitus	18 (37%)‡	8 (22%)‡	8 (19%)‡	0	0.003
Chronic obstructive pulmonary disease	7 (14%)‡	2 (5%)	3 (7%)	0	0.14
Smoker	25 (51%)‡	20 (54%)‡	14 (33%)‡	2(5%)	<0.001
Haematological parameters					

Haemoglobin (g/dL)	12.2±2.0*†‡	13.8±1.6	13.4±1.7	14.0±1.0	<0.001
Neutrophils (10 ³ per μL)	5.66±2.43*†‡	4.10±1.18	3.70±1.16	3.58±1.06	<0.001
Lymphocytes (10 ³ per μL)	1.33±0.69	1.70±0.71	1.63±0.55	1.77±0.60	0.058
Monocytes (per μL)	856±301*†‡	630±164	544±140	513±193	<0.001
Combined free light chains (mg/L)	62 (45-92)*†‡	38 (27-44)‡	25 (20-42)	24 (20-36)	<0.001
Cystatin C (mg/L)	2.08±0.66*†‡	1.35±0.36‡	1.21±0.51	0.99±0.17	<0.001
High sensitivity C- reactive protein (mg/L)	12.6 (4.7- 29.7)*†‡	1.9 (1.1-4.8)	1.5 (0.8-3.5)	1.3 (0.7-3.3)	<0.001
Interleukin-6 (pg/mL)	11 (7-16)*†‡	2.6 (1-4)	1.9 (1-3)	1.7 (0.5-3.0)	<0.001
Medications					
Aspirin	36 (73%)‡	31 (84%)‡	37 (86%)‡	5 (14%)	<0.001
ACEI/ARA	39 (80%)‡	31 (84%)‡	33 (77%)‡	4 (11%)	<0.001
Loop diuretics	48 (98%)*†‡	31 (84%)†‡	2 (5%)‡	0	<0.001
Statins	41 (84%)‡	33 (89%)‡	38 (88%)‡	4 (11%)	<0.001
Beta-blockers	20 (41%)*†‡	28 (76%)‡	33 (77%)‡	0	<0.001

Data are presented as mean±SD for normally distributed variables and median (IQR) for non-normally distributed variables. *p<0.05 vs. stable heart failure; †p<0.05 vs. stable coronary artery disease; ‡p<0.05 vs. healthy controls; ACEI, angiotensin converting enzyme inhibitor; ARA, angiotensin receptor antagonists.

Table 2. Longitudinal analysis of the study parameters.

N=32	Admission	Discharge	Follow up	p
Combined free light chains (mg/L)	61 (45-77)	62 (48-83)	63 (48-86)	0.14
Cystatin C (mg/L)	1.90±0.49	1.95±0.53	2.04±0.56	0.072
High sensitivity C-reactive protein (mg/L)	13.1 (5.0- 29.9)	9.4 (2.9-27.1)	6.9 (2.7-17.5)	0.42
Brain natriuretic peptide (pg/L)	480 (230-900)*	253 (183-581)	218 (74-342)	0.017
Interleukin-6 (pg/mL)	12 (6–16)	8 (4–18)	6 (3–14)	0.60

*p<0.05 vs. 3 months follow up.

Table 3. Cox regression analysis for predictors of the primary outcome (combination of death from any cause and cardiology related hospital admissions) and the secondary outcome (death from any cause alone).

	Primary outcome		Secondary outcome	
	Hazard ratio	p	Hazard ratio	p value
	[95.0% CI]	value	[95.0% CI]	
Univariate analysis				
Age	1.01 [0.97-1.06]	0.63	1.03 [0.98-1.09]	0.27
Sex	1.09 [0.41-2.86]	0.87	1.25 [0.42-3.72]	0.69
Previous myocardial infarction	1.13 [0.44-2.88]	0.80	1.16 [0.39-3.46]	0.79
Hypertension	1.13 [0.45-2.81]	0.80	1.10 [0.38-3.18]	0.85
Diabetes	0.95 [0.37-2.45]	0.92	0.62 [0.19-2.00]	0.42
Smoking	1.96 [0.77-5.00]	0.16	2.90 [0.91-9.26]	0.07
Systolic blood pressure	1.00 [0.97-1.02]	0.69	1.00 [0.97-1.03]	0.96
Body mass index	0.84 [0.70-1.01]	0.06	0.79 [0.63-1.01]	0.06
Brain natriuretic peptide	1.00 [1.00-1.00]	0.07	1.00 [1.00-1.00]	0.11
Left ventricular ejection fraction	0.95 [0.91-1.00]	0.07	0.93 [0.87-0.99]	0.017
Creatinine	1.01 [1.00-1.03]	0.07	1.02 [1.00-1.04]	0.026
Estimated glomerular filtration rate	0.98 [0.94-1.01]	0.20	0.96 [0.91-1.01]	0.09
Haemoglobin	0.99 [0.75-1.30]	0.93	0.86 [0.60-1.26]	0.45
Neutrophils	0.87 [0.65-1.16]	0.33	0.91 [0.67-1.25]	0.58
Lymphocytes	0.99 [0.37-2.67]	0.99	0.70 [0.23-2.15]	0.53
Monocytes	1.00 [0.99-1.00]	0.09	1.00 [0.99-1.00]	0.45

Combined free light chains	1.02 [1.01-1.03]	0.0001	1.02 [1.01-1.04]	<0.001
Cystatin C	1.47 [0.80-2.69]	0.22	1.82 [0.93-3.56]	0.08
C-reactive protein	1.00 [0.98-1.01]	0.75	1.00 [0.99-1.02]	0.97
Interleukin-6	0.99 [0.96-1.02]	0.50	0.99 [0.96-1.03]	0.58
Multivariate analysis				
Combined free light chains*	1.08 [1.04-1.13]	<0.001	1.10 [1.03-1.18]	0.003
		1		
Combined free light chains**	1.10 [1.04-1.16]	0.001	1.16 [1.03-1.30]	0.011

*Adjusted of age, gender, brain natriuretic peptide and cystatin C levels; **Adjusted of age, gender, brain natriuretic peptide, cystatin C levels and ejection fraction. CI, confidence interval





