Original Research

Leukocyte Labile Iron Pool in Patients with Systolic Heart Failure

Panagiotis Korantzopoulos[1], Chara Vlachou[2], Anna Kotsia[1], Kallirroi Kalantzi[1], Alexandra Barbouti[3], Dimitrios Galaris[2], John A. Goudevenos[1]

[1]Department of Cardiology, [2]Laboratory of Biological Chemistry, [3]Department of Anatomy, Histology and Embryology, University of Ioannina Medical School, Ioannina, Greece

Key words: **Left ventricular systolic dysfunction, white blood cells, left ventricular ejection fraction, labile iron pool, redox active iron, oxidative stress.**

Introduction: Data regarding sources of oxidative stress in the failing myocardium are sparse. Leukocytes actively participate in the oxidative damage observed in human heart failure (HF). The intracellular labile iron pool (LIP) represents a source of toxic reactive oxygen species.

Methods: We studied patients with chronic systolic HF who had a left ventricular ejection fraction (LVEF) ≤45%. We examined the LIP status in different populations of leukocytes in HF patients and we investigated its association with clinical and laboratory parameters, including conventional inflammatory markers.

Results: Sixty patients were finally included in the analysis (mean age: 67 ± 11 years, 54 men, 42 with ischemic cardiomyopathy). The multivariate logistic regression analysis showed that only LIP in granulocytes (OR: 0.73; 95% CI: 0.55-0.98; p=0.039) and right ventricular systolic pressure (RVSP) (OR: 0.95; 95% CI: 0.92-0.99; p=0.027) were independently associated with severe LV systolic dysfunction (LVEF≤30%). The correlation analysis revealed that LVEF was inversely associated with LIP in granulocytes (Spearman's rho: -0.39, p=0.002), LIP in monocytes (Spearman's rho: -0.35, p=0.007), and RVSP (Spearman's rho: -0.43, p=0.003). No significant correlation between LVEF and inflammatory indexes was noted.

Conclusions: LIP in granulocytes is independently associated with the severity of LV dysfunction in patients with systolic HF. Intracellular redox active iron may represent a source of leukocyte reactive oxygen species in this setting.

Manuscript received: July 5, 2011;
Accepted: January 16, 2012.

Address: Panagiotis Korantzopoulos

Department of Cardiology University of Ioannina Medical School 451 10 Ioannina, Greece e-mail: p.korantzopoulos@yahoo.gr

The clinical syndrome of heart failure (HF) is an evolving epidemic representing a multifactorial dynamic disorder with different underlying substrates and serious health consequences.[1] An increasing body of evidence suggests that oxidative stress is involved in the pathogenesis and progression of myocardial dysfunction, including chronic HF.[2-4] Several studies have shown that plasma or urinary levels of oxidative stress markers are related to the severity of HF.[4-14] However, data regarding mechanistic insights, such as cytoplasmic sources of oxidative stress in the failing myocardium, are sparse.[15] Interestingly, there are data suggesting that leukocytes actively participate in the oxidative damage observed in human HF.[16-18] On the other hand, the intracellular labile iron pool (LIP) represents the main catalyst for the conversion of weakly reactive oxygen species (ROS), such as hydrogen peroxide and superoxide anion, to extremely reactive free radicals, such as hydroxyl radicals and alcoxyl radicals.[15,19] Thus, the estimation of LIP may contribute to the clarification of the relation between iron status perturbations and pathological conditions associated with oxidative stress.[20-24] We therefore sought to examine the LIP status in different populations of leukocytes collected from systolic HF patients, in order to investigate the potential role of redox active iron in this

setting. We also investigated the association of LIP in leukocytes with clinical and laboratory parameters, including conventional inflammatory markers.

Methods

Study protocol and patients

In this observational study, consecutive patients with systolic HF who were seen in our clinic were screened. We studied patients with chronic systolic HF (ischemic or dilated cardiomyopathy for more than 6 months) who had a left ventricular ejection fraction (LVEF) ≤45%. All patients were on optimal medical treatment, without devices for cardiac resynchronization therapy or LV assist. We excluded patients with acute decompensation, severe valvular dysfunction, recent coronary intervention or cardiac surgery, recent acute coronary syndromes, recent or active infection, chronic rheumatic/inflammatory disease, malignancy, hematologic dyscrasia, bleeding disorder, recent hemorrhagic event, end-stage renal disease, and those who were taking drugs with an anti-inflammatory and/or antioxidant action, apart from statins and blockers of the renin-angiotensin system. We also excluded patients who drank alcohol, or who were smokers.

We carefully recorded demographic, clinical, and laboratory characteristics for every participant. LVEF was calculated using Simpson's method and right ventricular systolic pressure (RVSP) by standard echocardiographic methods. We divided the patient population into 2 groups (severe LV dysfunction, defined as LVEF≤30%, and moderate LV dysfunction, defined as LVEF>30%). We also checked for potential differences between patients in New York Heart Association (NYHA) functional class I-II and patients in NYHA class III-IV.

Laboratory measurements

Laboratory examinations, including complete blood count and biochemical investigations, were performed in the morning hours with the patients in a fasting state. The white blood cell (WBC) count was determined using a Coulter counter. C-reactive protein (CRP) levels were assessed using a high sensitivity immunonephelometric assay (Beckman Coulter/Immage Immunochemistry Systems, Behring Diagnostics Inc., Somerville NJ, USA). The estimation of creatinine clearance was made using the Cockroft-Gault formula.[25] All measurements were made by staff who were blind to the patients' characteristics and treatment.

Human peripheral blood leukocytes were isolated from freshly donated heparinated whole blood after hypo-osmotic lysis of red blood cells, as previously described.[21] Briefly, one volume of blood was mixed with six volumes of lysis buffer, containing 156 mM NH_4Cl, 10 mM $NaHCO_3$ and 97 mM EDTA (ethylenediaminetetraacetic acid), and placed on ice for 20 min in order to allow the lysis of red blood cells. Leukocytes were then collected by centrifugation at 1000 × g for 5 min and washed twice in complete DMEM growth medium (Sigma-Aldrich, St Louis MO, USA). Finally, cells were suspended at a density of 1.5×10^6 cells per ml before being processed for further analysis.

Estimation of LIP in freshly prepared human leukocytes was performed using the metal-sensitive probe calcein (Molecular Probes, Eugene OR, USA) as fluorescent molecule, in combination with flow cytometric analysis. The presence of acetomethoxy esteric groups in the original molecule, calcein-AM, facilitates its diffusion across cell membranes, while the intracellular cleavage of ester bonds by non-specific esterases results in intracellular retention of the negatively charged calcein. The binding of iron, along with other divalent metals, by calcein results in quenching of its fluorescence, while subsequent addition of the strong and specific iron chelator salicylaldehyde isonicotinoyl hydrazone (SIH) (a kind gift from Professor Prem Ponka, McGill University, Montreal, Canada) leads to the removal of iron from calcein and a concomitant increase in fluorescence intensity.[21] In the present study, 1.0 ml leukocyte suspensions (1.5×10^6 cells) were transferred into plastic tubes and analysed by FACScan flow cytometry (PARTEC GmbH, Germany). Light scattering analysis, i.e. forward and side scattering, allowed the gating of leukocytes into three distinct populations based on differences in size and granularity. Subsequently, cells were loaded with calcein by incubating them with 5 nM calcein-AM for 10 min in the dark at 37°C. The intensity of calcein fluorescence was detected as green fluorescence and expressed in histograms against cell counts. Finally, 100 μM of the membrane-permeable, strong and specific iron chelator SIH was added into the cell suspension and calcein fluorescence was detected again 5 min later. The SIH-mediated shift of mean cell-fluorescence in each cell type was expressed in arbitrary units and apparently reflects the level of LIP in the particular cells.

Statistical analysis

Continuous variables are expressed as mean ± SD, or as median (interquartile range) if their values were not normally distributed. The examination of normality was performed using the Kolmogorov-Smirnov test. Comparisons of the continuous variables were performed using the unpaired Student t-test or the non-parametric Mann-Whitney U test as appropriate. Categorical variables are presented as absolute numbers and frequencies and were compared using Fisher's exact test. Correlation analysis between LVEF and different parameters was performed, calculating Pearson's or Spearman's coefficient as appropriate. Multivariate logistic regression analysis was performed in order to examine the association between the candidate parameters and the severity of LV systolic dysfunction (LVEF≤30% vs. LVEF>30%). Variables that demonstrated an association on univariate analysis were inserted into the model. A two-tailed p-value <0.05 was considered significant. All analyses were performed using SPSS software (version 16.0; SPSS Inc., Chicago IL, USA).

Results

Sixty patients with systolic heart failure were finally included in the analysis (mean age 67 ± 11 years, 54 men, 42 with ischemic cardiomyopathy). The demographic, clinical, and laboratory characteristics of the 2 groups, namely patients with severe LV dysfunction (LVEF≤30%, n=28) and patients with moderate LV dysfunction (LVEF>30%, n=32) are presented in Table 1. Most of these parameters were comparable between the 2 groups. The following parameters, which were demonstrated to differ notably between the 2 goups (p≤0.10) were entered into the multivariate

Table 1. Demographic, clinical, and laboratory characteristics of the patients.

	LVEF≤30% (N=28)	LVEF>30% (N=32)	p
Age (years)	67.6 ± 9.5	66.9 ± 12.7	0.82
Sex (males)	26 (93%)	28 (88%)	0.67
BMI (kg/m^2)	25.4 (9.7)	24.7 (5.0)	0.59
NYHA class III-IV	14 (50%)	9 (28%)	0.10
Atrial fibrillation	12 (43%)	9 (28%)	0.28
Heart rate (beats/min)	70 (17)	73 (11)	0.09
Systolic blood pressure (mmHg)	120 (20)	130 (22)	0.81
RVSP (mmHg)	42.6 ± 19.6	28.3 ± 15.9	0.01
Ischemic cardiomyopathy	21 (75%)	21 (65%)	0.57
Diabetes	7 (25%)	10 (32%)	0.77
Hypertension	16 (57%)	20 (62%)	0.79
Drug therapy:			
ACE-I/ARBs	25 (89%)	31 (96%)	0.33
B-blockers	25 (89%)	26 (81%)	0.48
Aldosterone antagonists	21 (75%)	18 (56%)	0.17
Statins	19 (67%)	25 (78%)	0.39
Laboratory parameters:			
Hemoglobin (gr/dl)	13.5 (3.8)	13.2 (3.6)	0.37
WBC count (per mm^3)	7340 (2150)	7400 (1295)	0.89
Creatinine clearance (ml/min)	53.4 ± 24.6	62.1 ± 29.6	0.26
K+ (mEq/l)	4.2 ± 0.5	4.4 ± 0.4	0.23
Na+ (mEq/l)	136 (3)	138 (3)	0.36
Uric acid (mg/dl)	8.5 ± 2.5	7.6 ± 2.3	0.21
hsCRP (mg/l)	9.2 (7.8)	8.67 (8.8)	0.43
Ferritin (ng/ml)	112 (143)	140 (202)	0.62
Fe (μg/dl)	68 ± 41	76 ± 32	0.42
TIBC (μg/dl)	311 ± 87	315 ± 46	0.83
LIP in granulocytes (arbitrary units)	16.21 (4.92)	14.12 (3.31)	0.01
LIP in monocytes (arbitrary units)	26.03 (5.99)	23.01 (5.91)	0.05
LIP in lymphocytes (arbitrary units)	10.63 (3.17)	9.95 (2.00)	0.22

Data are expressed as mean ± SD, or as median (IQR), or as number (percentage). LVEF – left ventricular ejection fraction; BMI – body mass index; NYHA – New York Heart Association; RVSP – right ventricular systolic pressure; ACE-I – angiotensin converting enzyme inhibitors; ARBs – angiotensin receptor blockers; WBC – white blood cell; hsCRP – high sensitivity C-reactive protein; Fe – iron; TIBC – total iron binding capacity; LIP – labile iron pool.

model: LIP in granulocytes, LIP in monocytes, heart rate, RVSP, and NYHA class I-II vs. III-IV. The multivariate logistic regression analysis showed that only LIP in granulocytes (odds ratio, OR: 0.73; 95% confidence interval, CI: 0.55-0.98; p=0.039) and RVSP (OR: 0.95; 95% CI: 0.92-0.99; p=0.027) were independently associated with severe LV systolic dysfunction. The correlation analysis revealed that the LVEF was inversely associated with LIP in granulocytes (Spearman's rho: -0.39; p=0.002), LIP in monocytes (Spearman's rho: -0.35; p=0.007), and RVSP (Spearman's rho: -0.43; p=0.003). No significant correlation of LVEF with inflammatory indexes was noted.

Comparison of patients with NYHA class I-II (n=34) and patients with NYHA III-IV (n=26) showed that there was a significant difference in the LIP of granulocytes (13.69[3.78] vs. 15.40[4.16], p=0.03), in creatinine clearance (65[28.7] ml/min vs. 42[40.3] ml/min, p=0.04), in RVSP (26.8 ± 15.1 mmHg vs. 46.1 ± 18.6 mmHg, p=0.003), in LVEF (p=0.002), and a marginally significant difference in uric acid levels (7.1 ± 2.5 mg/dl vs. 8.9 ± 1.9 mg/dl, p=0.054). However, multivariate logistic regression analysis showed that only RVSP was independently associated with advanced NYHA class (OR: 1.07, 95% CI: 1.01-1.12, p=0.015).

Discussion

The results of the present study demonstrate that the LIP in circulating granulocytes is indepedently associated with the severity of LV dysfunction in patients with systolic HF. More specifically, there was a correlation between LVEF and LIP in granulocytes or LIP in monocytes, while LIP in granulocytes was independently associated with severe systolic HF (LVEF≤30%). Although we studied several other clinical and laboratory parameters, including conventional inflammatory markers (hsCRP, WBC count, and uric acid), only LIP in granulocytes and RVSP were independently associated with severe LV systolic dysfunction.

Previous studies that investigated the association between oxidative stress markers and the severity of HF have mainly focused on functional capacity according to the NYHA classification.[7,8,10,12,13] Although LVEF may be affected by the loading conditions, it represents a more objective index of LV systolic dysfunction compared to NYHA class. In our study, the LIP in granulocytes was increased in patients with advanced NYHA class (III-IV) but after multivariate analysis this association was not significant.

Substantial evidence suggests that oxidative stress is implicated in the pathophysiology of HF and may contribute to the disease progression. Indeed, various parameters, such as cardiomyocyte apoptosis and necrosis, ventricular remodeling, mechanoelectric uncoupling, and endothelial dysfunction, have been shown to be induced by oxidative stress.[2-4] The clinical utility of oxidative stress biomarkers as prognostic indexes in HF is limited and controversial, but they may elucidate novel pathways and point to novel targets for potential interventions.[6] On the other hand, we have to acknowledge that serum or urinary markers of oxidative stress represent indirect indexes of oxidative damage and do not provide mechanistic insights regarding ROS production. Moreover, patients with HF have concomitant comorbidities that may interfere with the pathophysiological alterations and oxidative modifications observed in this setting. Additionally, despite the substantial evidence for the pathogenetic role of oxidative stress in experimental models of HF,[2-4,15] the association of clinical HF with serum oxidative stress markers has not been clearly proved to be a causal relationship.

Inflammation and immune activation increases oxidative stress and may be implicated in the pathogenesis and progression of HF.[4,5,26,27] Interestingly, there are data showing amplification of oxidative stress in circulating leukocytes of patients with HF, implying a contribution to the oxidative damage.[16-18] Specifically, it has been demonstrated that neutrophil superoxide generating capacity (via the NADPH oxidase) is enhanced in patients with congestive HF compared to control subjects.[16] Similarly, Kong et al showed increased intracellular oxidative stress in 3 subsets (neutrophils, monocytes, lymphocytes) of circulating leukocytes in patients with acute decompensated HF.[17] The highest levels of intracellular oxidants were observed in monocytes.[17] These alterations in leukocytes were associated with a marked reduction in mitochondrial transmembrane potential, and an increased propensity for apoptosis.[17] Notably, in a more recent study it was shown that cytosolic oxidative stress in circulating leukocytes and platelets is increased in patients with worsening HF.[18] Also, higher levels of protein nitrosylation were detected in the leukocytes of HF patients, implying that intracellular ROS cause meaningful protein damage.[18] These findings were attributed to the deficient pulmonary capillary clearance of ROS-positive leukocytes and

platelets, as well as to increased production of ROS by mitochondria.[18]

Neutrophils and monocytes have phagocytic capabilities and are involved in the respiratory burst. The NADPH oxidase complex, which is arranged vectorially in the cell membranes, produces superoxide anions in the extracellular space, as well as in the phagocytic vacuoles. This complex is much less active in other types of leukocytes, such as B-lymphocytes. The activation of leukocyte NADPH oxidase and myeloperoxidase has been implicated in the systemic oxidative stress observed in HF.[2,3,10,16] In the aforementioned studies regarding HF and leukocyte oxidative stress, the exact source of increased intracellular ROS production was not elucidated in depth.

The existence of an intermediate and transitory form of intracellular iron that can participate in redox reactions has been proposed and is variably termed as "LIP", "redox-active iron", "free iron", and more.[19-22] The redox active intracellular iron induces intracellular oxidative damage by catalyzing Fenton-type reactions,[20] which have been implicated in the pathophysiology of the failing myocardium and other cardiovascular disorders.[15,24] There is also evidence that LIP toxicity plays a key role in cardiopulmonary bypass-associated acute kidney injury.[23] Even though anemia and iron deficiency have been described in HF populations,[24,28] the LIP pool status has not been investigated. Recently, we developed a methodology for LIP estimation in human leukocytes using flow cytometry. This method has been validated and we have previously reported a positive association of leukocyte LIP with aging.[21] This is the first study to investigate the leukocyte LIP in patients with systolic HF. Bearing in mind the aforementioned considerations, LIP may represent the main source of leukocyte ROS in HF and a potential target for interventions with specific iron-chelating compounds.

Limitations

Several limitations are apparent. First, we have to acknowledge that this was a small pilot study, while there was a predominance of men in our population. However, the patients were carefully selected, since strict inclusion and exclusion criteria were applied. Second, although we recorded several clinical and laboratory parameters we do not have data on levels of brain natriuretic peptide. Third, we did not measure other non-specific plasma oxidative stress markers, since our principal aim was to evaluate the redox iron status of leukocytes by exploring specific sources of ROS. In addition, we did not include healthy subjects, since there are substantial differences in many clinical and laboratory variables, including drugs with antioxidant and anti-inflammatory actions, between healthy people and HF patients. Therefore, the study of independent associations using multivariate analysis would be infeasible. Furthermore, our principal aim was to investigate the potential association between leukocyte LIP and the severity of systolic HF. Finally, we did not evaluate the potential prognostic role of the studied parameters with respect to future untoward events. This is a subject of future research.

References

1. McMurray JJ. Clinical practice. Systolic heart failure. N Engl J Med. 2010; 362: 228-238.
2. Korantzopoulos P, Galaris D, Papaioannides D, Siogas K. The possible role of oxidative stress in heart failure and the potential of antioxidant intervention. Med Sci Monit. 2003; 9: RA120-125.
3. Giordano FJ. Oxygen, oxidative stress, hypoxia, and heart failure. J Clin Invest. 2005; 115: 500-508.
4. Eleuteri E, Magno F, Gnemmi I, et al. Role of oxidative and nitrosative stress biomarkers in chronic heart failure. Front Biosci. 2009; 14: 2230-2237.
5. Braunwald E. Biomarkers in heart failure. N Engl J Med. 2008; 358: 2148-2159.
6. Trachtenberg BH, Hare JM. Biomarkers of oxidative stress in heart failure. Heart Fail Clin. 2009; 5: 561-577.
7. Keith M, Geranmayegan A, Sole MJ, et al. Increased oxidative stress in patients with congestive heart failure. J Am Coll Cardiol. 1998; 31: 1352-1356.
8. Hokamaki J, Kawano H, Yoshimura M, et al. Urinary biopyrrins levels are elevated in relation to severity of heart failure. J Am Coll Cardiol. 2004; 43: 1880-1885.
9. Polidori MC, Praticó D, Savino K, Rokach J, Stahl W, Mecocci P. Increased F2 isoprostane plasma levels in patients with congestive heart failure are correlated with antioxidant status and disease severity. J Card Fail. 2004; 10: 334-338.
10. Tang WH, Brennan ML, Philip K, et al. Plasma myeloperoxidase levels in patients with chronic heart failure. Am J Cardiol. 2006; 98: 796-799.
11. White M, Ducharme A, Ibrahim R, et al. Increased systemic inflammation and oxidative stress in patients with worsening congestive heart failure: improvement after short-term inotropic support. Clin Sci (Lond). 2006; 110: 483-489.
12. Radovanovic S, Krotin M, Simic DV, et al. Markers of oxidative damage in chronic heart failure: role in disease progression. Redox Rep. 2008; 13: 109-116.
13. Nagayoshi Y, Kawano H, Hokamaki J, et al. Differences in oxidative stress markers based on the aetiology of heart failure: comparison of oxidative stress in patients with and without coronary artery disease. Free Radic Res. 2009; 43: 1159-1166.
14. Amir O, Paz H, Rogowski O, et al. Serum oxidative stress level correlates with clinical parameters in chronic systolic

heart failure patients. Clin Cardiol. 2009; 32: 199-203.
15. Ide T, Tsutsui H, Kinugawa S, et al. Direct evidence for increased hydroxyl radicals originating from superoxide in the failing myocardium. Circ Res. 2000; 86: 152-157.
16. Ellis GR, Anderson RA, Lang D, et al. Neutrophil superoxide anion—generating capacity, endothelial function and oxidative stress in chronic heart failure: effects of short- and long-term vitamin C therapy. J Am Coll Cardiol. 2000; 36: 1474-1482.
17. Kong CW, Hsu TG, Lu FJ, Chan WL, Tsai K. Leukocyte mitochondria depolarization and apoptosis in advanced heart failure: clinical correlations and effect of therapy. J Am Coll Cardiol. 2001; 38: 1693-1700.
18. Ijsselmuiden AJ, Musters RJ, de Ruiter G, et al. Circulating white blood cells and platelets amplify oxidative stress in heart failure. Nat Clin Pract Cardiovasc Med. 2008; 5: 811-820.
19. Barbouti A, Doulias PT, Zhu BZ, Frei B, Galaris D. Intracellular iron, but not copper, plays a critical role in hydrogen peroxide-induced DNA damage. Free Radic Biol Med. 2001; 31: 490-498.
20. Galaris D, Pantopoulos K. Oxidative stress and iron homeostasis: mechanistic and health aspects. Crit Rev Clin Lab Sci. 2008; 45: 1-23.
21. Doulias PT, Vlachou C, Boudouri C, Kanavaros P, Siamopoulos KC, Galaris D. Flow cytometric estimation of 'labile iron pool' in human white blood cells reveals a positive association with ageing. Free Radic Res. 2008; 42: 253-259.
22. Galaris D, Skiada V, Barbouti A. Redox signaling and cancer: the role of "labile" iron. Cancer Lett. 2008; 266: 21-29.
23. Haase M, Bellomo R, Haase-Fielitz A. Novel biomarkers, oxidative stress, and the role of labile iron toxicity in cardiopulmonary bypass-associated acute kidney injury. J Am Coll Cardiol. 2010; 55: 2024-2033.
24. Kell DB. Iron behaving badly: inappropriate iron chelation as a major contributor to the aetiology of vascular and other progressive inflammatory and degenerative diseases. BMC Med Genomics. 2009; 2: 2.
25. Scrutinio D, Passantino A, Santoro D, Cacciapaglia E, Farinola G. Prognostic value of formulas estimating excretory renal function in the elderly with systolic heart failure. Age Ageing. 2009; 38: 296-301.
26. Celis R, Torre-Martinez G, Torre-Amione G. Evidence for activation of immune system in heart failure: is there a role for anti-inflammatory therapy? Curr Opin Cardiol. 2008; 23: 254-260.
27. Oikonomou E, Tousoulis D, Siasos G, Zaromitidou M, Papavassiliou AG, Stefanadis C. The role of inflammation in heart failure: new therapeutic approaches. Hellenic J Cardiol. 2011; 52: 30-40.
28. Nanas JN, Matsouka C, Karageorgopoulos D, et al. Etiology of anemia in patients with advanced heart failure. J Am Coll Cardiol. 2006; 48: 2485-2489.