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Association Between Systemic Neutrophil Gelatinase-Associated Lipocalin and Anemia, Relative Hypochromia, and Inflammation in Chronic Systolic Heart Failure

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Abstract

Neutrophil gelatinase-associated lipocalin (NGAL) is upregulated systemically and by renal tubular cells in response to inflammation and ischemia. Recent interests in NGAL have focused on its ability to predict worsening renal function. However, as an iron-regulatory glycoprotein, the relationship between systemic NGAL levels and indices of anemia has not been examined. In 130 patients with chronic systolic heart failure, we examined the relationship between plasma NGAL levels and indices of anemia independent of underlying renal function and systemic markers of inflammation and oxidant stress. Plasma NGAL levels were significantly elevated in patients with anemia versus without anemia (121 [98, 197] versus 72 [57, 98] ng/mL, $p<0.001$). Plasma NGAL levels were inversely correlated with indices of anemia including red blood cell count ($r= -0.38$, $p<0.0001$), hemoglobin ($r= -0.41$, $p<0.0001$), and red cell distribution width ($r= 0.25$, $p=0.007$), even in those with relatively preserved renal function (estimated glomerular filtration rate ≥60 ml/min/1.73m^2; $n=83$, $p<0.05$ for all). Higher plasma NGAL levels were associated with presence of anemia independent of estimated glomerular filtration rate, plasma high-sensitive C-reactive protein, and myeloperoxidase levels (odds ratio 2.38, 95% confidence interval 1.02 – 6.20, $p=0.045$). Hence, systemic NGAL levels are independently associated with indices of anemia.

Neutrophil gelatinase-associated lipocalin (NGAL) is a small, 25 kDa glycoprotein member of the lipocalin superfamily. In response to diverse cellular stresses, including inflammation and ischemia, NGAL is rapidly released by a variety of cell types, including renal tubular cells, liver hepatocytes, and endothelial and smooth muscle cells [1–7]. Although the clinical utility of NGAL recently has centered on its role as a marker of acute kidney injury and active chronic kidney disease, NGAL was originally identified as a bacteriostatic agent released from secondary granules of activated human neutrophils [8, 9], capable of interfering with bacterial iron uptake by sequestering bacterial ferric siderophores [10, 11]. Subsequently, NGAL has been studied as an iron regulatory glycoprotein involved in iron trafficking and regulation of iron-dependent genes [11–15].

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As part of the non-transferrin bound iron pool, NGAL circulates in both siderophore:iron-associated and siderophore:iron-free forms. Siderophore:iron-associated NGAL delivers iron into cells following 24p3R or megalin receptor-mediated uptake and trafficking into acidic endosomes, where iron is released and subsequently accumulates in the cytoplasm. Delivery of iron is believed to regulate iron-responsive genes involved in promotion of cellular survival, proliferation, and differentiation, including enhanced expression of heme oxygenase-1, ribonuclease reductase, and many cyclin genes and stress-related proteins [13]. In parallel, siderophore:iron-free NGAL is proposed to scavenge excess free intracellular and extracellular iron, limiting labile iron-mediated cytotoxicity [13, 16].

Such pathways point toward an acute compensatory, protective role for NGAL in response to diverse cellular stresses, including inflammatory and oxidative stress [1–4]. However, recent reports have implicated NGAL upregulation as a mechanism contributing to anemia in the setting of chronic inflammation. In experimental models, systemic and medullary NGAL has been demonstrated to induce inhibition of erythropoiesis through induction of apoptosis and arrest of differentiation of erythroid progenitor cells [17–21]. In addition, NGAL has been found to redirect systemic iron to renal proximal tubule cells in response to renal tubular cell injury [13, 22]. In the systemic inflammatory states of both chronic and advanced heart failure, iron deficiency has been described as a common cause of anemia [23, 24].

In the chronic systolic heart failure setting, we test the hypothesis that elevated systemic NGAL levels are associated with systemic indices of anemia after adjusting for underlying renal function and systemic markers of inflammation and oxidant stress.

METHODS

Study Population

We examined the hematologic and inflammatory determinants of systemic NGAL levels in stable, ambulatory patients with chronic systolic heart failure in a single-center, prospective study cohort well-characterized with comprehensive echocardiographic evaluation. All subjects provided informed consent as approved by the Cleveland Clinic Institutional Review Board. Inclusion criteria were as follows: 18 to 75 years of age with a diagnosis of heart failure for at least 3 months, a left ventricular ejection fraction ≤35% at the time of enrollment, New York Heart Association functional class I–IV symptoms. Exclusion criteria included a history of mitral stenosis or mitral valve surgery, severe mitral regurgitation (>3+), or severe aortic stenosis (peak velocity >4 m/s) or aortic regurgitation [25].

Echocardiography

All subjects underwent comprehensive echocardiographic evaluation of cardiac structure as well as systolic and diastolic performance by an experienced sonographer. Comprehensive transthoracic echocardiography was performed using commercially available HDI 5000 (Phillips Medical Systems, N.A., Bothell, Washington) and Acuson Sequoia (Siemens Medical Solutions USA Inc., Malvern, Pennsylvania) machines using previously described techniques[25, 26]. Classification of diastolic stage was determined according to the following modifications of the recommendations set forth by the American Society of Echocardiography [27]. LV mass was calculated according to previously published recommendations [28]. All ventricular volume and mass measurements were indexed to body surface area. Measurements were averaged over three cycles.

Laboratory Testing

All samples were collected into ethylenediaminetetraacetic acid-plasma collecting tubes on ice simultaneously at the time of echocardiographic evaluation, processed and immediately

frozen in aliquots at −80°C until analyzed. All laboratory analyses were performed with investigators blinded to cardio-renal indices and clinical outcomes data. Plasma NGAL levels were measured by a research enzyme-linked immunosorbent assay (Cat. No. KIT 036, BioPorto Diagnostics, Gentofte Denmark). The minimum detection limit of the assay was 20 ng/mL. Intra-assay and inter-assay coefficients of variation were <5% at 65 ng/mL. Plasma high-sensitivity C-reactive protein (hsCRP) levels were determined by the particle enhanced immunonephelometry assay (Dade Behring, Inc., Deerfield IL). The minimum detection limit of the assay was 0.10 ng/mL. Intra-assay and inter-assay CVs were <5%. Plasma myeloperoxidase (MPO) levels were determined by an enzyme-linked immunosorbent assay (CardioMPO II test, Cleveland Heart Labs, Cleveland OH). The minimum detection limit of the assay was 30 pM. Intra-assay and inter-assay CVs were <5%. Plasma amino-terminal pro-B-type natriuretic peptide (NT-proBNP) levels were assayed using a commercially available immunoassay based on electrochemiluminescence technology (Roche Elecsys® NT-proBNP assay, Roche Diagnostics, Indianapolis IN). The minimum detection limit of the assay was 5 pg/mL. Intra-assay and inter-assay CVs were <3%. Complete blood count with differential analysis was performed at the Cleveland Clinic Reference Laboratory utilizing a Sysmex XE-2100 automated hematology analyzer and leukocyte differential counter (Sysmex America, Inc., Mundelein IL). Indices of anemia included hemoglobin, hematocrit, and red blood cell distribution width. Anemia was defined as hemoglobin <12g/dL for men and <11g/dL for women. Estimated glomerular filtration rate (eGFR) was calculated based on serum creatinine levels using the standard 4-variable Modification of Diet in Renal Disease equation [29].

Statistical Analyses

Continuous variables were summarized as mean ± standard deviation if normally distributed, and as median and interquartile range if non-normally distributed. Normality was assessed by the Shapiro-Wilk W test. Spearman's rank correlation method was used as a nonparametric measure of association for correlations between NGAL levels and clinical and echocardiographic indices. The Wilcoxon rank-sum or Kruskal-Wallis tests were used to compare differences in NGAL levels across categorical variables. Multivariable logistic regression analysis was performed and evaluated by the Likelihood Ratio test to calculate the odds ratios of presence of anemia associated with elevated NGAL levels following adjustment for indices of underlying renal function, inflammation, and oxidant stress. Multivariable linear regression analysis using the Standard Least Squares fitting method was performed to determine the association between NGAL and continuous hemoglobin levels following adjustment for indices of renal function, inflammation and oxidant stress. Natural logarithmic transformations were applied to non-normally distributed variables. All p-values reported are from two-sided tests and a p-value <0.05 was considered statistically significant. Statistical analyses were performed using JMP 9.0 (SAS Institute, Cary, NC).

RESULTS

Table 1 illustrates the baseline characteristics of our chronic systolic heart failure cohort. Mean and median plasma NGAL levels were 93 ± 63 ng/mL and 77 (inter-quartile range 58–108) ng/mL, respectively. Higher systemic NGAL levels were associated with advanced age (r= 0.18, p=0.035), higher plasma NT-proBNP (r= 0.24, p=0.008), hsCRP (r= 0.32, p<0.001), MPO (r= 0.30, p<0.001), absolute neutrophil count (r= 0.42, p<0.0001), and lower eGFR (r= −0.53, p<0.0001), but did not differ according to gender, ethnicity, history of hypertension or diabetes mellitus, or any medication use (p>0.10 for all).

The prevalence of anemia in our chronic systolic heart failure cohort was 12.6% (7.4% in women, 14.1% in men). Plasma NGAL levels were significantly elevated in patients with anemia versus without anemia (121 [inter-quartile range 98–197] versus 72 [inter-quartile

range 57–98] ng/mL, p<0.001; Figure 1). Plasma NGAL levels were inversely correlated with indices of anemia in the entire cohort as well as in those with relatively preserved renal function (estimated glomerular filtration rate ≥60 ml/min/1.73m^2, Table 2).

Higher plasma NGAL levels were directly associated with indices of systemic inflammation, leukocyte activation, and oxidant stress. Specifically, higher plasma NGAL levels modestly correlated with elevated plasma hsCRP (r= 0.32, p<0.001) and plasma MPO levels (r= 0.30, p<0.001) (Figure 2). Plasma NGAL levels were also directly correlated with absolute neutrophil count (r= 0.42, p<0.0001). These relationships were preserved in patients with relatively preserved renal function (p<0.01 for all).

In multivariable logistic regression analysis, higher plasma NGAL levels were associated with presence of anemia even after adjusting for eGFR, and hsCRP, and MPO levels (odds ratio: 2.38, 95% confidence interval 1.02 – 6.20, p=0.045, Table 3). In multivariable linear regression analysis, higher plasma NGAL levels were associated with lower hemoglobin levels following adjustment for age, gender, eGFR, hsCRP, MPO, absolute neutrophil count, and NT-proBNP levels (Std β = −0.35, p=0.012, Table 4).

DISCUSSION

The mechanistic links between heart failure, renal insufficiency and anemia remain poorly defined. Neutrophil gelatinase-associated lipocalin (NGAL) recently has emerged as an important factor in iron homeostasis and erythrocyte growth regulation that may contribute to anemia when chronically elevated. In chronic systolic heart failure patients, we report for the first time an association between elevated systemic NGAL levels and anemia indices that remains following adjustment for underlying renal function and systemic inflammatory markers.

NGAL was originally identified as a product of activated human neutrophils, capable of acting as a bacteriostatic agent by sequestering bacterial ferric siderophores and interfering with bacterial iron uptake [9, 11], and regulated by the redox- and pro-inflammatory cytokine-sensitive transcription factor, nuclear factor-κB (NF-κB) [1, 2, 5]. Therefore, it is consistent to find within our chronic systolic heart failure cohort that systemic NGAL levels are associated with markers of systemic inflammation, including high-sensitivity C-reactive protein, myeloperoxidase, and absolute neutrophil count. These relationships remained present in patients with relatively preserved renal function, which may imply the likely association between systemic NGAL and anemia related to heart failure.

In this study, we utilized surrogate measures of iron deficiency, such as low mean corpuscular hemoglobin concentration (representing a relative deficiency of hemoglobin incorporation into the erythrocytes) and red cell distribution width (representing enhanced erythropoiesis) [30]. In the setting of both chronic and advanced heart failure, iron deficiency has been described as a common cause of anemia [23, 24]. In an effort to shuttle systemic iron to renal tubular cells to support cell proliferation and prevent apoptosis, elevated systemic NGAL levels may transiently contribute to iron deficiency anemia. In animal models, NGAL has been demonstrated to be able to strip iron from transferrin and redirect iron from the liver and spleen to the proximal tubule of the kidney [13, 22]. With recent interests in intravenous iron therapy as potential treatment modality for heart failure[31], further investigations regarding the relative contributions of NGAL in the development of relative iron deficiency anemia in heart failure are warranted.

Based on our understanding of the mechanistic role of NGAL, it is also conceivable that elevated systemic NGAL levels may also contribute to anemia as a direct inhibitor of erythrocyte maturation [17]. Local synthesis of NGAL by immature medullary erythroid stem

cell progenitors constitutes an autocrine regulatory pathway promoted by interleukin-1 that induces inhibition of erythropoiesis through induction of apoptosis and arrest of differentiation [18, 19]. In addition, in studies examining exogenous administration of recombinant NGAL in mice, systemic NGAL has been shown to traffic to receptors on the surface of marrow cells where it acts to inhibit the recovery of suppressed hematopoietic function [19–21]. In this fashion, chronic elevation of systemic NGAL levels, as a compensatory defense against systemic inflammatory and oxidative stress, may deleteriously act to suppress medullary hematopoietic function. Along with the ability of NGAL to enhance and prolong the proteolytic activity of matrix metalloproteinase-9, these findings appear to imply a natural history in which NGAL levels are beneficial when elevated acutely, but potentially deleterious when elevated chronically. Recent reports have even suggested that chronic elevations of NGAL may not simply be a marker of, but also a contributor to, progressive renal dysfunction in chronic kidney disease through aberrant tubular cell proliferation[32]. In addition, although acutely beneficial in ischemia-reperfusion models, NGAL contributes to worsening of antibody-induced nephritis via promotion of inflammation and apoptosis[33]. NGAL injection was found to nephritis and blockade has been hypothesized as a novel therapeutic approach[33]. The effects of acutely and chronically elevated systemic and local NGAL levels need to be further investigated.

LIMITATIONS

There are several limitations of our current analysis. We did not collect serial systemic NGAL levels or serial indices of anemia to assess the relationship between baseline and changes in levels of systemic NGAL with changes in indices of anemia. We also did not have measures of functional iron deficiency (such as serum iron, total iron binding capacity, or ferritin) to confirm the presence of underlying functional iron deficiency, despite the lack of a gold standard for such condition. The cross-sectional nature of our study limits any direct demonstration of a cause-and-effect for different mechanistic pathways that may underlie the link between NGAL and anemia.

Despite these potential limitations, we have demonstrated the association between NGAL and anemia to exist after adjustment for underlying renal function and systemic markers of inflammation and oxidative stress. While additional studies need to performed to determine whether NGAL plays a causal role in the pathogenesis of anemia in heart failure, it is interesting to hypothesize that systemic and local NGAL upregulation may contribute to anemia through iron sequestration and erythrocyte growth regulation. NGAL blockade consequently may offer a novel therapeutic approach. In addition, systemic NGAL levels may help identify patient subpopulations who will benefit most from intravenous iron therapy. Further studies are warranted to better understand the role of NGAL in predisposing to anemia in the setting of disease progression of chronic heart failure.

CONCLUSION

Systemic NGAL levels are associated with indices of anemia independent of underlying renal function and systemic markers of inflammation and oxidant stress in chronic systolic heart failure.

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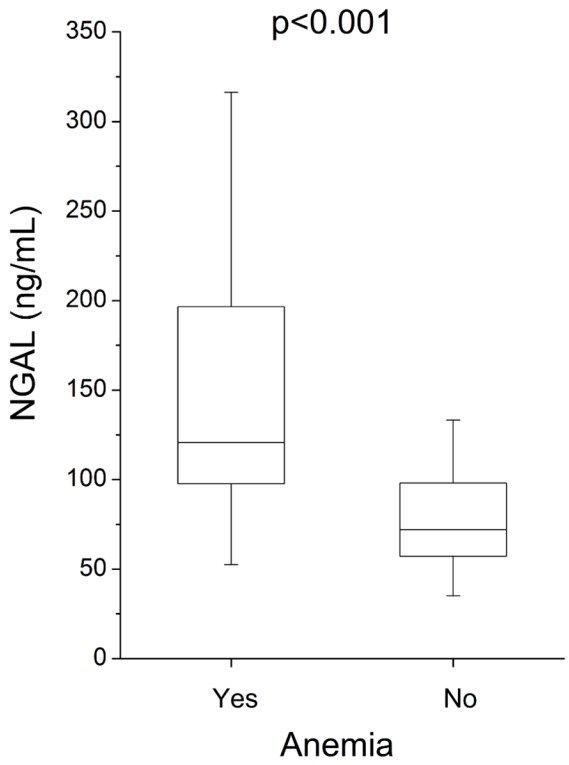

Figure 1.
Plasma NGAL Levels Stratified According to Presence of Anemia for Chronic Systolic Heart Failure Subjects (n=130). *Abbreviations:* NGAL, neutrophil gelatinase-associated lipocalin.

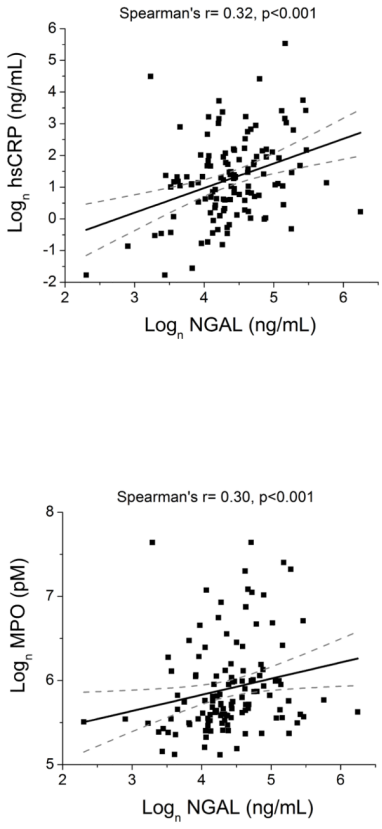

Figure 2.

Correlations between Plasma NGAL and Indices of Systemic Inflammation, Leukocyte Activation and Oxidant Stress for Chronic Systolic Heart Failure Subjects (n=130).

Abbreviations: NGAL, neutrophil gelatinase-associated lipocalin; hsCRP, high-sensitivity C-reactive protein; MPO, myeloperoxidase.

Table 1

Baseline Subject Characteristics (n=130).

Variable	Value
Demographics:	
Age (years)	57 ± 13
Male gender, n (%)	98 (75%)
Body mass index (kg/m^2)	28 ± 5
African American, n (%)	22 (17%)
Caucasian, n (%)	108 (83%)
Heart failure history:	
Ischemic etiology, n (%)	57 (44%)
NYHA class III or IV, n (%)	43 (33%)
Co-morbidities:	
Hypertension, n (%)	70 (55%)
Diabetes mellitus, n (%)	37 (29%)
Echocardiographic indices:	
LV mass index (g/m^2)	157 ± 46
LV end-diastolic volume index (mL/m^2)	110 ± 35
LV ejection fraction (%)	26 ± 6
Diastolic stage III, n (%)	45 (35%)
Medications:	
Angiotensin converting enzyme inhibitors or angiotensin receptor blockers, n (%)	119 (94%)
Beta-blockers, n (%)	83 (65%)
Spironolactone, n (%)	32 (27%)
Loop diuretics, n (%)	98 (77%)
Digoxin, n (%)	74 (62%)
Anemia Indices:	
Red blood cell (M/uL)	4.5 ± 0.6
Hemoglobin (g/dL)	13.7 ± 1.7
Hematocrit (%)	40.7 ± 4.8
Red cell distribution width (%)	14.7 ± 2.3
Laboratory data:	
eGFR (mL/min/1.73m^2)	72 ± 25
NT-proBNP (pg/mL)	1241 [540–3292]
MPO (pM)	307 [256–430]
hsCRP (ng/mL)	3.3 [1.5–7.3]
NGAL (ng/mL)	77 [58–108]

Abbreviation: NYHA, New York Heart Association; LV, left ventricular; eGFR, estimated glomerular filtration rate; NT-proBNP, aminoterminal pro-B-type natriuretic peptide; MPO, myeloperoxidase; hsCRP, high-sensitivity C-reactive protein; NGAL, neutrophil gelatinase-associated lipocalin.

Table 2

Univariate Correlation between Plasma Neutrophil Gelatinase-Associated Lipocalin (NGAL) and Anemia Indices for the Overall Chronic Systolic Heart Failure cohort (n=130) and Subjects with Relatively Preserved Renal Function (estimated glomerular filtration rate ≥60 ml/min/1.73m^2; n=83).

	Overall (n=130)		Estimated glomerular filtration rate ≥60 ml/min/1.73 m^2 (n=83)	
Variable	**Spearman's r**	**p-value**	**Spearman's r**	**p-value**
Red blood cell (M/uL)	−0.38	<0.0001	−0.16	0.152
Hemoglobin (g/dL)	−0.41	<0.0001	−0.34	0.002
Hematocrit (%)	−0.37	<0.0001	−0.26	0.022
Red cell distribution width (%)	0.25	0.007	0.24	0.030

Table 3

Multivariable Logistic Regression Analysis for Presence of Anemia within the Overall Chronic Systolic Heart Failure Cohort (n=130).

Neutrophil Gelatinase-Associated Lipocalin*	**Odds ratio (95% confidence interval)**	**p-value**
Unadjusted	3.33 (1.78 – 6.97)	<0.0001
Adjusted for Age, Gender, eGFR	2.24 (1.05 – 5.32)	0.037
Adjusted for Age, Gender, hsCRP	3.60 (1.78 – 8.27)	<0.001
Adjusted for Age, Gender, MPO	3.80 (1.90 – 8.79)	<0.0001
Adjusted for Age, Gender, NT-proBNP	2.19 (1.09 – 5.07)	0.026
Adjusted for Age, Gender, hsCRP, MPO and NT-proBNP	2.92 (1.29 – 7.79)	0.008
Adjusted for Age, Gender, eGFR, hsCRP, and MPO	2.67 (1.11 – 7.47)	0.028

Abbreviations: eGFR, estimated glomerular filtration rate; hsCRP, high-sensitivity C-reactive protein; MPO, myeloperoxidase; NT-proBNP, aminoterminal pro-B-type natriuretic peptide.

* Odds ratio per 1 standard deviation increment (1 standard deviation of Ln NGAL = 0.58 ng/mL)

Table 4

Multivariable Linear Regression Analysis for the Relationship between Hemoglobin Levels and Clinical Predictors within the Overall Chronic Systolic Heart Failure Cohort (n=130).

Variable	Standard β	p-value
NGAL (ng/mL)	−0.35	0.012
Age (years)	−0.17	0.133
Gender (male)	0.13	0.243
eGFR (mL/min/1.73m^2)	0.22	0.093
hsCRP (ng/mL)	−0.12	0.300
MPO (pM)	−0.06	0.637
Absolute neutrophil count (K/μL)	0.22	0.074
NT-proBNP (pg/mL)	−0.16	0.217

Abbreviations: NGAL, neutrophil gelatinase-associated lipocalin; eGFR, estimated glomerular filtration rate; hsCRP, high-sensitivity C-reactive protein; MPO, myeloperoxidase; NT-proBNP, aminoterminal pro-B-type natriuretic peptide.