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γ-Butyrobetaine Is A Proatherogenic Intermediate in Gut Microbial Metabolism of L-Carnitine to TMAO

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Usefulness of Relative Hypochromia in Risk Stratification for Nonanemic Patients With Chronic Heart Failure

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Anemia has been reported to affect up to 20% to 30% of patients with heart failure (HF) and has consistently been associated with increased morbidity and mortality.[1–5] Multiple mechanisms are involved in the development of anemia in the setting of HF, including nutritional deficiencies (such as malabsorption, impaired metabolism), acute or chronic blood loss (such as gastrointestinal bleeding),[3] intrinsic renal disease leading to insufficient erythropoietin production,[3] hemodilution from volume expansion,[6] increased levels of circulating inflammatory cytokines (as in anemia of chronic inflammation),[7,8] and medication use or hematinic abnormalities.[4] The presence of anemia has become increasingly recognized as an important factor in the development and progression of HF.[5] As one of the most common causes of anemia, iron deficiency is associated with impaired functional capacity, worse cardiac function, and adverse cardiac outcome.[4,9,10] However, the definition of iron deficiency is largely restricted to quantifying levels of circulating iron and its binding proteins. Mean corpuscular hemoglobin concentration (MCHC), the index of hemoglobin (Hb) in blood cells, reported in complete blood cell count, reflects amount of iron incorporated into circulating erythrocytes.[11–13] We have previously reported the prognostic implications of relative hypochromia (defined as low MCHC in the setting of normal Hb) in a small observational series of ambulatory patients with significant left ventricular impairment.[14] Here, we investigate the effect of hypochromia on a larger and broader patient population of stable patients with heart failure especially regarding the impact of changes in MCHC levels over time on long-term survival. We also investigate the association between relative hypochromia and severity of HF assessed by biochemical, physiological, and functional measures and the association between relative hypochromia and various inflammatory and oxidative stress markers in the setting of HF.

Methods

The Cleveland Clinic GeneBank study is a large, prospective cohort study from 2001 to 2007 with clinical and longitudinal outcomes data composed of consenting subjects who underwent elective diagnostic cardiac catheterization procedure. All participants gave written informed

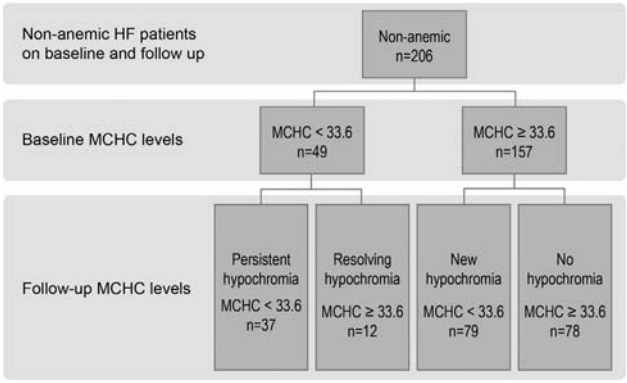

Figure 1. Change in MCHC levels in nonanemic patients over time.

consent approved by the Cleveland Clinic Institutional Review Board. History of HF was detected by (a) directly asking patient by research personnel, (b) reviewing medical records for confirmation (all patients were seen by cardiologist at Cleveland Clinic before the left heart catheterization), and (c) ICD codes and adjudication by research personnel. This analysis included 1,579 subjects with HF with the New York Heart Association functional class II to IV without evidence of myocardial infarction (cardiac troponin I <0.03 ng/ml) with hematologic data available for analysis. Five-year survival was ascertained for all subjects after enrollment through a combination of prospective patient contact, electronic medical record review, and confirmed by the Social Security Death Index.

Complete blood cell count was performed on blood samples collected at the day of enrollment using ADVIA 120 hematology analyzer (Siemens, New York, New York). Anemia was defined as Hb <13 g/dl for men and <12 g/dl for women.[15] Indexes of anemia included Hb, hematocrit, MCHC (ratio of Hb to hematocrit), and mean corpuscular volume (MCV). The normal range of MCHC is 33 to 36 g/dl.[16] Nonanemic patients were grouped into quartiles based on MCHC levels.

Follow-up levels of Hb and MCHC levels were obtained from reviewing the electronic medical records when these tests were measured for clinical purposes. To study change of MCHC levels over time, a fixed cutoff of 180 ± 90 days was used, and level closest to day 180 was selected. Only nonanemic patients on baseline and follow-up were analyzed to assess effect of MCHC change over time on patient's outcome. To study the effect of MCHC change over time on outcomes, patients were grouped using cutoff of quartile 1 for MCHC; thus, 4 groups were identified: persistent hypochromia, resolving hypochromia, new hypochromia, and no hypochromia (Figure 1).

HF severity was assessed based on plasma B type natriuretic peptide levels (BNPs; using Abbott Architect ci8200; Abbott Laboratories, Abbott Park, Illinois) and Duke Activity Score Index (DASI)[9,17] on day of enrollment. Left ventricular ejection fraction (LVEF) was collected for all patients. High-sensitivity C-reactive protein (hsCRP), ceruloplasmin, and serum arylesterase activity were all measured at the time of enrollment in a randomly selected sample of 790 patients. Ceruloplasmin and hsCRP were measured by Abbott Architect ci8200 (Abbott Laboratories).[18,19] Serum arylesterase activity level was determined using a modification of a spectrophotometry-based assay as previously described.[20]

The Student's *t* test, Wilcoxon rank-sum test, or Kruskal-Wallis test for continuous variable and the chi-square test for categorical variables were used to examine the difference between the groups. Kaplan-Meier analysis and Cox proportional hazards regression were used for time-to-event analysis to determine hazard ratio and 95% confidence intervals (CIs) for 5-year survival. Levels of MCHC were adjusted for traditional cardiac risk factors in a multivariate model, including age, gender, diabetes mellitus, systolic blood pressure, hyperlipidemia, creatinine clearance, smoking, coronary artery disease, LVEF, body mass index, and medications (angiotensin-converting enzyme inhibitors/angiotensin receptor blockers, β blockers, and aspirin). Analyses were repeated after adjusting for Hb, MCV, and DASI. All statistical analyses were performed using R 2.15.1(Vienna, Austria).

Results

Table 1 lists the baseline characteristics of 1,579 patients with stable HF enrolled in this study. Mean Hb and MCHC were 12.6 ± 1.6 g/dl and 33.9 ± 1.2 g/dl, respectively (all normally distributed). About half of the patients (n = 785 [49.7%]) were nonanemic. Among this nonanemic group, the median MCHC was 34.3 g/dl (interquartile range of 33.6 to 35 g/dl). Patients with low levels of MCHC were more likely to be women, have lower creatinine clearance, and have less prevalence of smoking and coronary artery disease (Table 1).

Anemic patients with HF showed increased risk of 5-year mortality (hazard ratio 2.2, 95% CI 1.8 to 2.7, p <0.001). Overall, patients with lower MCHC had increased mortality risk (quartiles 1 vs 4, hazard ratio 2.5, 95% CI 2.0 to 3.3, p <0.001). Among patients with normal Hb levels, patients with decreased MCHC levels have increased mortality risk in a graded pattern (Figure 2, Table 2). All results remained significant even after multivariate adjustments with traditional coronary risk factors and known prognostic cardiorenal, hematologic variables, and DASI (Table 2). Furthermore, patients with low MCHC levels at baseline had significantly increased HF severity assessed by BNP levels (neurohormonal upregulation) and DASI (functional capacity) but not with LVEF (Table 3).

A total of 552 (35%) subjects had follow-up Hb levels that met our inclusion criteria. Mean time between baseline and follow-up samples was 169 ± 42 days. No significant differences were found between selected patients with follow-up level and those without, in regard to HF severity (LVEF, DASI, and BNP), adverse outcomes (5-year mortality), and hematologic indexes (Hb, MCV, and MCHC), suggesting this subgroup is a good representative of the total study population (Supplemental Table 1). In this longitudinal cohort, mean baseline Hb and MCHC were 12.6 ± 1.6 and 33.9 ± 1.2 g/dl, respectively. Mean follow-up Hb and MCHC were 12.8 ± 2.0 and 33.0 ± 1.3 g/dl, respectively. About half of the patients were nonanemic (n = 271, 49.1%) at baseline, and most were nonanemic on follow-up (n = 311, 56.3%). In the nonanemic group at baseline,

Table 1
Baseline characteristics of total population and non anemic population based on MCHC levels

MCHC levels, g/dL	Whole cohort	Non Anemic population					p value*
		All Non anemic	Quartile1 <33.6	Quartile2 33.6 34.3	Quartile3 34.3 35	Quartile4 ≥35	
Number	1579	785	196	195	197	197	
Age (years)	66±11	64±11	65±11	66±11	64±11	63±10	<0.001
Male sex	65%	65%	50%	57%	71%	82%	<0.001
Body mass index (kg/m$^{2)}$	30±7	31±7	32±9	31±8	31±7	30±6	<0.001
Systolic blood pressure (mmHg)	131±22	131±21	134±23	131±21	131±21	128±19	<0.001
Diabetes mellitus	39%	34%	38%	35%	29%	34%	0.311
Hyperlipdemia	83%	82%	81%	77%	83%	86%	0.182
Smoker	72%	73%	70%	74%	76%	72%	0.542
Creatinine clearance (mL/min)	83±4	94±43	87±45	91±40	98±43	98±44	<0.001
Coronary artery disease	76%	70%	62%	70%	72%	77%	0.007
Myocardial infarction	58%	56%	50%	54%	56%	64%	0.058
Ejection fraction (%)	40 (25 − 55)	35 (25 55)	35 (20 55)	40 (30 55)	40 (30 55)	35 (25 50)	0.124
Brain natriuretic peptide (pg/mL)	310 (114 695)	204 (91 499)	398 (125 951)	266 (107 686)	173 (78 376)	140 (73 371)	<0.001
Heart failure with preserved EF	37%	36%	35%	42%	38%	28%	0.048
Angiotensin converting enzyme inhibitors/ angiotensin receptor blockers	68%	67%	66%	70%	61%	70%	0.226
Beta blockers	67%	66%	64%	63%	68%	68%	0.644
Aspirin	66%	66%	59%	66%	61%	76%	0.003
Hemoglobin (g/dL)	12.6±1.6	13.8±1.1	13.7±1.1	13.6±1.1	13.8±1	14.1±1.1	<0.001
MCHC (g/dL)	33.9±1.2	34.2±1.1	32.8±0.8	34±0.2	34.6±0.2	35.5±0.4	<0.001
MCV (fl)	88.8±5.7	89±5	89.3±5.8	89.5±5.2	89±4.4	88.3±4.3	<0.001

MCHC mean corpuscular hemoglobin concentration; MCV mean corpuscular volume.
* p Value for the difference between MCHC quartiles in non anemic population.

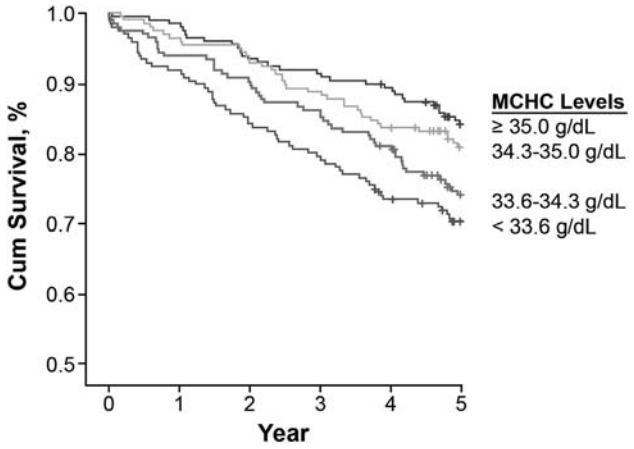

Figure 2. Kaplan Meier plot for 5 year mortality stratified by baseline MCHC levels in nonanemic patients with heart failure (n 785). Log rank <0.001.

patients with low MCHC did not have increased risk of developing anemia (less than vs more than quartile 1, odds ratio 1.8, 95% CI 0.96 to 3.2, p = 0.067).

Effect of MCHC change over time on long-term survival was studied in patients with normal Hb both at baseline and at follow-up (n = 206). Patients with low MCHC ("relative hypochromia," less than quartile 1) were compared with those with high MCHC ("normochromia," quartile 1 or more). Most of the patients with low MCHC on baseline have also low MCHC on follow-up "persistent hypochromia," whereas about half of the patients with high MCHC at baseline continued to have high MCHC levels "no hypochromia" (Figure 1). In comparison with patients with no hypochromia, patients with persistent hypochromia had significant increased mortality risk (log rank <0.001, Figure 3).

We also examined the relation between MCHC levels in nonanemic patients with heart failure and markers of inflammation and systemic oxidative stress (Table 3). Of the 790 patients with available biomarkers levels, 398 patients were nonanemic. In nonanemic patients with HF with low MCHC levels, we observed graded and significantly high-level hsCRP and ceruloplasmin, consistent with heightened systemic inflammation. Serum levels of arylesterase activity, a cardioprotective antioxidant activity catalyzed by high-density lipoprotein-associated paraoxonase-1,[20] were significantly lower in patients with low MCHC an indication of heightened oxidative stress (Table 3).

Discussion

The primary finding of our study is the association between the presence and persistence of relative hypochromia (as defined by low MCHC levels, a readily available parameter in complete blood count analysis) and increased risk of 5-year all-cause mortality. This was especially notable in nonanemic patients with HF, a subgroup for whom no clinical consensus yet exists regarding approach, monitoring, and treatment. Furthermore, we observed significant impairment in functional activity in patients with relative hypochromia and heightened inflammatory and reduced antioxidative processes. Interestingly, low MCHC

Table 2
Association between MCHC levels and 5 year all cause mortality in non anemic HF patients

MCHC levels (g/dL)	Quartile 1 <33.6	Quartile 2 33.6 34.3	Quartile 3 34.3 35	Quartile 4 ≥35
5 year Death	58/196 29.6%	50/195 25.6%	37/197 18.8%	32/197 16.2%
Unadjusted Hazard ratio	2.1 (1.4 3.3)**	1.7 (1.1 2.7)*	1.2 (0.8 2.0)	1
Adjusted Hazard ratio (Model 1)	1.9 (1.2 3.1)*	1.7(1.0 2.7)*	1.3 (0.8 2.1)	1
Adjusted Hazard ratio (Model 2)	1.9 (1.1 3.0)*	1.7 (1.0 2.7)*	1.3 (0.8 2.1)	1
Adjusted Hazard ratio (Model 3)	1.7 (1.0 2.7)*	1.5 (0.9 2.4)	1.2 (0.7 1.9)	1

Model 1: Age, sex, diabetes, systolic blood pressure, smoking, hyperlipidemia, coronary artery disease, creatinine clearance, angiotensin converting enzyme inhibitor/angiotensin receptor blocker, beta blockers, aspirin use, body mass index and LVEF; Model 2: Model 1 + baseline hemoglobin and MCV; Model 3: Model 2 + DASI.

**p <0.01; *p <0.05.

Table 3
Distribution of cardiac severity, inflammatory and oxidative markers across MCHC quartiles in non anemic HF patients

MCHC levels	All	Quartile 1 <33.6	Quartile 2 33.6 34.3	Quartile 3 34.3−35	Quartile 4 ≥35	P value
LVEF (%)	35 (25 55)	35 (20 55)	40 (30 55)	40 (30 55)	35 (25 50)	0.124
BNP (pg/ml)	204 (91 499)	398 (125 951)	266 (107 686)	173 (78 376)	140 (73 371)	<0.001
DASI Score	30 (18 43)	24 (13 38)	26 (17 43)	35 (19 50)	38 (23 50)	<0.001
hsCRP (mg/L)	3 (1 6)	4 (3 8)	3 (1 7)	3 (1.3 5.8)	2 (1 5)	<0.001
Ceruloplasmin (mg/dL)	25 (21 29)	26.5 (23.6 30.9)	25.8 (21.8 30.7)	24.5 (20.5 28.3)	23 (20 26)	<0.001
Arylesterase activity (μmoles/min/mL)	99 (81 117)	89 (75 113)	99 (86 114)	103 (83 120)	100 (81 117)	0.118

BNP B type natriuretic peptide; DASI Duke Activity Status Index score; hsCRP high sensitive C reactive protein; LVEF Left ventricular ejection fraction.

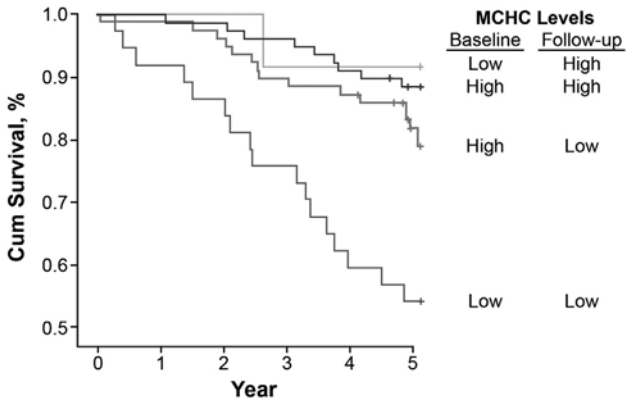

Figure 3. Kaplan Meier plot for 5 year mortality by change in MCHC levels at follow up in nonanemic patients with heart failure. Log rank <0.001.

levels on baseline were not associated with increased risk of developing anemia on follow-up. These findings highlight the importance of recognizing and exploring the mechanistic underpinnings of relative hypochromia in the setting of chronic HF, above and beyond its contribution to anemia.

Because MCHC is a direct assessment of the amount of Hb incorporated into the erythrocytes, MCHC may serve as a reliable and readily accessible indicator of erythrocyte iron load,[11 13,21] which has a high specificity (up to 96%) in detecting iron deficiency.[21] Iron is involved in multiple physiological functions including oxygen transport (Hb), oxygen storage (myoglobin), oxidative metabolism (mitochondrial oxidative enzymes),[7,22] and antioxidant activity (ferritin, myoglobin) and also is involved in the synthesis and degradation of lipids, carbohydrates, and nucleotides.[4,7,22] Traditionally, iron deficiency is only considered important in the presence of anemia. Our current findings argue that strict cut-points for assessing iron deficiency may miss out on identifying patients with relative impairment in iron metabolism/utilization.

Iron deficiency has been studied in patients with heart failure.[4,22,23] Even in patients with normal Hb levels, patients with iron deficiency showed decreased functional capacity, LVEF, and adverse outcomes.[4,22,23] Nonanemic iron-deficient patients had a twofold greater risk for death than anemic iron-replete subjects.[13] Furthermore, treatment with iron compounds in nonanemic patients with low iron load improved symptoms, functional capacity, and quality of life but not survival.[23] The last could be related to short follow-up period in this study (only 24 weeks) and low total event rates (only 2%). Gaber et al[24] found that correction of iron deficiency improves functional class and walking distance in nonanemic iron-deficient patients with systolic HF. They also found significant improvement of diastolic and systolic functions using echocardiogram after therapy despite lack of improvement of LVEF.[24] Ongoing clinical trials are addressing the role of iron supplementation in both anemic and nonanemic patients with HF.

Patients with HF are more susceptible to development of iron deficiency.[25] This could be related to gradual depletion of iron stores (absolute iron deficiency) because of low iron intake, gastrointestinal blood loss, or iron malabsorption.[25]

In HF, there is also an activation of proinflammatory cytokines that block intestinal absorption of iron and divert iron from the circulation into the reticuloendothelial system (functional iron deficiency).[2,26] In both scenarios, there is decreased iron availability to targeted tissues and organs.[4] Iron level in the blood or stores once it reaches a minimum threshold is not as critical as how much iron that is getting into functioning erythrocytes.[27] Routine monitoring of blood iron levels in all patients with heart failure is costly. Using erythrocyte indexes (such as MCHC in our study) as a surrogate marker of iron deficiency (both absolute and functional),[10,14,21,27] high-risk patients who might benefit from further iron studies/replacement therapy could be identified.

Interestingly, the association among MCHC and HF outcomes, severity, and oxidative and inflammatory markers, was observed in a graded pattern. Thus, even patients with low normal MCHC showed adverse outcomes and increased oxidative stress. Both HF and iron deficiency are associated with increased oxidative stress.[28,29] Because iron plays a central role in the formation and scavenging of reactive oxygen species,[29] low MCHC could be either a contributing factor to increased oxidative in HF or a result of increased oxidative state because of HF, which impairs iron utilization. Taken together, the gradual decrease in MCHC may be more reflective of HF severity and iron metabolic disarrangement, more than arbitrary cutoffs of MCHC.

Limitations include lack of traditional measure of iron profiles (iron, transferrin, and ferritin) to establish clinical evidence of iron deficiency; hence, we can only establish the presence of relative hypochromia and not specifically iron deficiency. We do not have complete data about cause of death or other HF end points like hospitalization or 6-minute walking test. Our study population is also a selected group of high-risk patients who all had elective coronary angiogram for clinical purposes. However, with the relatively large sample size, these findings corroborated the existing literature in HF and anemia and iron deficiency, whereas providing new evidence to support the prognostic role of relative hypochromia as an independent predictor of increase mortality in patients with heart failure with normal Hb levels.

Disclosures

Dr. Hazen and Dr. Brennan are named as co-inventors on active and pending patents held by the Cleveland Clinic relating to cardiovascular diagnostics. Dr. Hazen reports having been paid as a consultant or speaker for the following companies: Cleveland Heart Lab (Cleveland, OH), Esperion (Ann Arbor, MI), Lilly (Indianapolis, IN), Merck & Co., Inc. (Kenilworth, NJ), and Pfizer (Cambridge, MA), Inc. Dr. Hazen reports receiving research funds from Abbott (Chicago, IL), Cleveland Heart Lab, and Pfizer Inc. Dr. Hazen reports having the right to receive royalty payments for inventions or discoveries related to cardiovascular diagnostics or therapeutics from the following companies: Abbott Laboratories, Cleveland Heart Lab., Esperion, Frantz Biomarkers, and Siemens (Malvern, PA). Dr. Brennan is currently an employee of Epinomics. Dr. Hazen is also

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.amjcard.2016.01.023.

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