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RESEARCH ARTICLE

Cardiac iron concentration in relation to systemic iron status and disease severity in non-ischaemic heart failure with reduced ejection fraction

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Aims Low cardiac iron levels promote heart failure in experimental models. While cardiac iron concentration (CI) is decreased in patients with advanced heart failure with reduced ejection fraction (HFrEF), CI has never been measured in non-advanced HFrEF. We measured CI in left ventricular (LV) endomyocardial biopsies (EMB) from patients with non-advanced HFrEF and explored CI association with systemic iron status and disease severity.

Methods and results We enrolled 80 consecutive patients with non-ischaemic HFrEF with New York Heart Association class II or III symptoms and a median (interquartile range) LV ejection fraction of 25 (18–33)%. CI was 304 (262–373) µg/g dry tissue. CI was not related to immunohistological findings or the presence of cardiotropic viral genomes in EMBs and was not related to biomarkers of systemic iron status or anaemia. Patients with CI in the lowest quartile (CI_{Q1}) had lower body mass indices and more often presented with heart failure histories longer than 6 months than patients in the upper three quartiles (CI_{Q2-4}). CI_{Q1} patients had higher serum N-terminal pro-B-type natriuretic peptide levels than CI_{Q2-4} patients [3566 (1513–6412) vs. 1542 (526–2811) ng/L; $P = 0.005$]. CI_{Q1} patients also had greater LV end-diastolic ($P = 0.001$) and end-systolic diameter indices ($P = 0.003$) and higher LV end-diastolic pressures ($P = 0.046$) than CI_{Q2-4} patients.

Conclusion Low CI is associated with greater disease severity in patients with non-advanced non-ischaemic HFrEF. CI is unrelated to systemic iron homeostasis. The prognostic and therapeutic implications of CI measurements in EMBs should be further explored.

Keywords Iron deficiency • Non-ischaemic heart failure with reduced ejection fraction • Endomyocardial biopsy • Inductively-coupled plasma optical emission spectroscopy

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Introduction

Systemic iron deficiency (ID) is a frequent comorbidity in heart failure (HF).[1,2] Based on serum markers of depleted body iron stores, reduced systemic iron availability, and unmet cellular iron requirements, ~50% of patients with HF with reduced ejection fraction (HFrEF) are iron-deficient.[3–7] Systemic ID is associated with reduced exercise tolerance, increased symptom severity, and higher mortality rates independent of coexisting anaemia.[4–11] Iron supplementation using intravenous ferric carboxymaltose has been shown to improve exercise capacity and symptoms and to reduce the number of HF hospitalisations in iron-deficient patients with HFrEF.[12–14] Current European Society of Cardiology (ESC) guidelines therefore recommend assessing systemic iron status in patients with symptomatic HFrEF.[15]

Iron is an essential cofactor in haem and iron–sulphur cluster-containing proteins required for oxygen transport (haemoglobin) and storage (myoglobin) as well as cellular energy metabolism (e.g. components of the mitochondrial electron transport chain).[16] Using gene-targeted mice with cardiomyocyte-selective ID, we have recently observed that a ~30% decrease in cardiac iron content impairs cardiac contractile reserve and promotes adverse left ventricular (LV) remodelling after myocardial infarction.[17] Transgenic mice engineered to develop even more pronounced reductions in cardiac iron spontaneously develop HF and die prematurely.[18,19] Collectively, these studies indicate that a low cardiac iron content promotes HF independent of systemic iron status.

Cardiac iron concentration (CI) is 15–32% lower in patients with advanced HF undergoing heart transplantation compared to non-transplanted donor hearts.[17,20–22] Notably, CI has never been measured in non-advanced HF, and it is not known if CI is related to systemic iron status in these patients. We therefore measured CI in LV endomyocardial biopsies (EMBs) from patients with non-advanced non-ischaemic HFrEF and explored CI relationship to systemic iron status and disease severity.

Methods

Study population

We studied 80 consecutive patients older than 18 years with New York Heart Association (NYHA) class II or III symptoms and LV ejection fraction (LVEF) ≤40% referred to the Department of Cardiology and Angiology at Hannover Medical School between May 2017 and June 2018 for diagnostic work-up of HFrEF with unknown aetiology. According to the updated Heart Failure Association-ESC criteria for defining advanced HF,[23] all patients had non-advanced HF. All patients underwent transthoracic echocardiography and coronary angiography.

One day before coronary angiography, patients were informed about the study, which included performing an LV EMB in case coronary artery disease was excluded (no ≥50% diameter stenosis in a major coronary artery). Biopsies were not strictly indicated in some patients,[24,25] and we discussed this beforehand with the Ethics Committee of Hannover Medical School in light of previous studies indicating the safety of the procedure.[26–28] We obtained ethical approval (no. 7408–2017), and all patients provided written informed consent. LV EMBs were not associated with any complications in our patients.

We excluded patients with advanced HF, moderate to severe aortic stenosis, uncontrolled arterial hypertension, acute or chronic infections, advanced liver disease, end-stage chronic kidney disease, or malignant disease. We also excluded patients who had received oral iron (>100 mg/day), intravenous iron, or erythropoiesis-stimulating agents during the previous 6 weeks. Using these exclusion criteria, we ended up including one patient who had received 40 mg oral iron daily within the last 6 weeks. No patient recollected ever having received intravenous iron or erythropoiesis-stimulating agents. Further, patients with non-HF-related anaemia (e.g. due to haemoglobinopathies), patients with active bleeding or in need of blood transfusions, and patients with known iron overload were also excluded. A flowchart is presented in online supplementary *Figure S1*.

Echocardiography

We performed two-dimensional (2D) transthoracic echocardiography in the left lateral decubitus position with a Philips EPIQ 7 Cardiology Ultrasound Machine equipped with X5-1 xMATRIX array transducer. LV internal cavity diameters were directly measured in 2D from the parasternal long-axis view perpendicular to the LV long axis at the level of the mitral valve leaflet tips at end-systole and end-diastole.[29] Volume measurements were based on tracings of the blood tissue interface in the apical four- and two-chamber views. LV mass was calculated based on measurements of LV internal cavity diameter and wall thicknesses of the interventricular septum and the inferolateral wall at end-diastole, using the cube formula.[29] All parameters were indexed for body surface area. LVEF was calculated by the biplane method of disks (modified Simpson's rule). All images were digitally stored for subsequent analysis.

Endomyocardial biopsy and blood sampling

Patients received a 5000 IU bolus of unfractionated heparin. LV end-diastolic pressure and maximum rate of pressure change in the left ventricle (LV dP/dt_{max}) were measured with a 5 Fr pigtail catheter. No patient was decompensated at the time of LV EMB. After LV angiography, a 7 Fr long sheath (Cordis) was introduced into the LV cavity. The sheath tip was positioned towards the LV lateral wall. A 5.5 Fr biopsy forceps (Cordis) was introduced under fluoroscopic guidance. The bioptome jaws were opened within the LV cavity before wall contact. We took at least 6 (range 6–11) biopsies. Five biopsies were sent to the Institute for Cardiac Diagnostics and Therapy (IKDT, Berlin) for immunohistological and virological analyses. A median of four biopsies (range 1–6) were rinsed in ice-cold isotonic saline, transferred to plastic tubes (CryoPure Tube, Sarstedt), and stored in liquid nitrogen for later CI measurement. To avoid iron contamination, the tubes had previously been cleansed with 2.5% nitric acid and ultrapure water in an ultrasonic bath. Venous serum and EDTA-treated plasma samples were collected in the morning and either immediately analysed or stored at −80°C for later hepcidin and growth differentiation factor 15 (GDF-15) measurements.

Laboratory measurements

Assays for measuring haemoglobin concentration, serum ferritin, serum iron, transferrin saturation (TSAT), soluble transferrin receptor (sTFR), hepcidin, creatinine, C-reactive protein (CRP), interleukin-6

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(IL-6), cardiac troponin T, GDF-15, and N-terminal pro-B-type natriuretic peptide (NT-proBNP) are described in online supplementary *Table S1*. Following current ESC guidelines,[15] diagnosis of systemic ID required a serum ferritin concentration <100 μg/L or a serum ferritin concentration between 100 and 299 μg/L in combination with a TSAT <20%. Anaemia was diagnosed using World Health Organisation haemoglobin thresholds (<13 g/dL in men, <12 g/dL in women). Estimated glomerular filtration rate (GFR) was calculated by the Chronic Kidney Disease-Epidemiology Collaboration equation.[30] CI in EMBs was measured by inductively-coupled plasma optical emission spectroscopy (ICP-OES; a detailed description is provided in online supplementary *Methods S1*). To enable a comparison of our results with previous studies that have measured CI in advanced HF using inductively-coupled plasma mass spectrometry (ICP-MS), we established the ICP-MS method as previously described[22] and measured iron concentration in five EMBs with ICP-OES and ICP-MS. Iron concentrations determined with both methods were almost identical [289 (242–406) vs. 291 (246–416) μg/g dry tissue].

Immunohistological and virological analyses

Cardiomyocyte diameters were determined in haematoxylin- and eosin-stained sections. For immunohistological evaluation, specimens were embedded in Tissue-Tek O.C.T. compound (Sakura), snap-frozen in methylbutane that had been cooled in liquid nitrogen, and stored at −80°C until processing. Serial 5 μm cryosections were placed on 10% poly-L-lysine-precoated slides. Type I and type III collagens were detected with antibodies from Biotrend and Calbiochem, respectively. Sections were also stained with CD3 (Dako), CD45R0 (Dako), LFA-1 (ImmunoTools), MAC-1 (ImmunoTools), and perforin (BD Biosciences) antibodies and EnVision peroxidase-conjugated secondary antibodies (Dako). 3-Amino-9-ethylcarbazole (Merck) was used as chromogenic substrate. Slides were then counterstained with haematoxylin and mounted in Aquatex (Merck). Inflammatory cells were quantified at 200-fold magnification by digital image analysis applying colour-coded thresholds.[31] Active myocarditis was defined by the presence of ≥14 leucocytes/mm^2, including up to 4 monocytes/mm^2 with the presence of ≥7 CD3$^+$ T-lymphocytes/mm^2, which are focally associated with cardiomyocyte degeneration and necrosis.[25] Non-ischaemic cardiomyopathy with high-grade inflammation was defined by the presence of ≥14 leucocytes/mm^2, including up to 4 monocytes/mm^2 with the presence of ≥7 CD3$^+$ T-lymphocytes/mm^2, without cardiomyocyte degeneration and necrosis. Cardiotropic viral genomes were detected by nested polymerase chain reaction on RNA (enterovirus); DNA (adenovirus, Epstein–Barr virus, human herpesvirus 6); or RNA and DNA (parvovirus B19).[32]

Statistical analysis

Categorical variables are reported as numbers and percentages, continuous variables as median with interquartile range (IQR). Proportions were compared by the chi-square test, continuous variables by the Mann–Whitney test. We used ANOVA for comparisons among more than two groups followed by Dunn's post hoc test for comparisons between two groups. Binary logistic regression analyses were applied to identify factors that were independently associated with low CI or NT-proBNP. Spearman's correlation analysis was applied to assess the relationship between two variables. Linear regression analysis was used to identify factors associated with systemic iron parameters and CI. A two-tailed *P*-value of <0.05 was considered to indicate statistical significance. Analyses were performed with GraphPad Prism 7.04 (GraphPad Software) and SPSS Statistics 25 (IBM).

Results

Patients

The patient population included 53 men and 27 women. Patients had a median (IQR) age of 61 (51–71) years, a LVEF of 25 (18–33)%, and NT-proBNP concentrations of 1859 (698–3740) ng/L. Patients presented with NYHA class II (64%) or class III symptoms (36%). Median HF duration was 2 (1–6) months.

Systemic iron status

Overall, 46% of the patients (37 out of 80) had systemic ID. Patient characteristics according to systemic iron status are shown in online supplementary *Table S2*. Systemic ID was more frequent in women and patients with HF histories longer than 6 months. Patients with systemic ID had lower haemoglobin concentrations and were more often anaemic. Additionally, patients with systemic ID had lower serum concentrations of ferritin and iron, lower TSATs, lower plasma hepcidin concentrations, and higher serum concentrations of sTFR and IL-6. Estimated GFR and serum concentrations of CRP, GDF-15, and NT-proBNP were not significantly different between patients with or without systemic ID (online supplementary *Table S2*).

Lower serum iron concentration and a higher ferritin concentration were related to higher serum concentrations of CRP and IL-6. TSAT was also inversely related to the IL-6 concentration. Higher sTFR level and a lower haemoglobin concentration were related to a higher GDF-15 concentration (online supplementary *Figure S2*).

Cardiac iron concentration

Median CI was 304 (IQR 262–373; range 132–1339) μg/g dry tissue. CI was similar in patients with or without systemic ID [301 (228–382) vs. 312 (266–367) μg/g; $P = 0.44$]. A total of 60% of patients (12 out of 20) in the lowest CI quartile (CI_{Q1}) and 42% of patients (25 out of 60) in the top three CI quartiles (CI_{Q2-4}) had systemic ID ($P = 0.15$). Systemic iron homeostasis parameters (serum ferritin, serum iron, TSAT, sTFR, hepcidin) and haemoglobin concentrations were not significantly different between CI_{Q1} and CI_{Q2-4} patients (*Table 1*) and not associated with CI in linear regression analyses (online supplementary *Figure S3*).

CI_{Q1} patients had lower body mass indices (BMIs) ($P < 0.001$) and more often presented with HF histories longer than 6 months ($P = 0.027$) than CI_{Q2-4} patients (*Table 1*). NYHA class, estimated GFR and serum concentrations of CRP, IL-6, and GDF-15 were not significantly different between CI_{Q1} and CI_{Q2-4} patients (*Table 1*). A multivariate analysis established low BMI and HF duration longer than 6 months as independent predictors of low

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Table 1 Patient characteristics according to cardiac iron concentration

	All patients (*n* = 80)	Cardiac iron Q_1 (*n* = 20)	Cardiac iron Q_{2-4} (*n* = 60)	*P*-value
Age (years)	61 (51–71)	64 (49–70)	61 (51–72)	0.88
Female sex	27 (34)	8 (40)	19 (32)	0.49
BMI (kg/m^2)	28.4 (24.1–31.8)	23.3 (21.4–27.6)	30.1 (25.0–33.5)	<0.001
HF history >6 months	21 (26)	9 (45)	12 (20)	0.027
Prior HF hospitalisation*	30 (38)	9 (45)	21 (35)	0.42
NYHA class II/III	51 (64)/29 (36)	15 (75)/5 (25)	36 (60)/24 (40)	0.23
Systolic BP (mmHg)	105 (95–125)	101 (87–117)	105 (97–133)	0.13
Hypertension	47 (59)	9 (45)	38 (63)	0.15
Diabetes mellitus	22 (28)	3 (15)	19 (32)	0.15
Treatment				
ACEI/ARB/ARNI	65 (81)	15 (75)	50 (83)	0.41
Beta-blocker	58 (73)	13 (65)	45 (75)	0.39
MRA	35 (44)	11 (55)	24 (40)	0.24
Diuretic	52 (65)	16 (80)	36 (60)	0.10
Oral anticoagulation	23 (29)	7 (35)	16 (27)	0.48
Aspirin	22 (28)	4 (20)	18 (30)	0.39
Calcium antagonist	8 (10)	1 (5)	7 (12)	0.39
Device therapy	9 (11)	3 (15)	6 (10)	0.54
Laboratory parameters				
Ferritin (µg/L)	154 (79–293)	170 (35–283)	141 (840–328)	0.34
Iron (mg/L)	13.0 (10.0–17.8)	12.0 (8.5–15.8)	13.0 (10.0–18.0)	0.45
TSAT (%)	23.0 (17.0–32.0)	23.0 (16.3–30.8)	23.0 (17.0–32.8)	0.71
sTFR (µg/dL)	3.0 (2.4–4.2)	2.8 (2.5–6.1)	3.1 (2.4–4.2)	0.79
Haemoglobin (g/dL)	13.4 (12.6–14.7)	13.0 (11.6–14.3)	13.6 (12.7–14.7)	0.15
Anaemia	22 (28)	7 (35)	15 (25)	0.39
Hepcidin (ng/mL)	26.9 (8.3–45.3)	17.3 (2.3–40.5)	28.7 (9.2–45.9)	0.09
eGFR (mL/min)	70.5 (56.3–88.0)	69.0 (50.3–87.5)	70.5 (57.3–88.0)	0.62
CRP (mg/L)	3.9 (2.0–11.0)	3.4 (1.8–11.2)	4.0 (2.1–11.0)	0.64
IL-6 (ng/L)	10.0 (4.3–18.8)	9.5 (3.5–18.0)	10.0 (4.3–19.0)	0.54
hs-cTnT (ng/L)	27 (16–43)	32 (16–43)	26 (16–42)	0.73
GDF-15 (ng/L)	1786 (1035–2859)	2303 (1426–3228)	1635 (951–2750)	0.32
NT-proBNP (ng/L)	1859 (698–3740)	3566 (1513–6412)	1542 (526–2811)	0.005

Data are *n* (%), or median (interquartile range).
ACEI, angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker; ARNI, angiotensin receptor–neprilysin inhibitor; BMI, body mass index; BP, blood pressure; CRP, C-reactive protein; eGFR, estimated glomerular filtration rate; GDF-15, growth differentiation factor 15; HF, heart failure; hs-cTnT, high-sensitivity cardiac troponin T; IL-6, interleukin 6; MRA, mineralocorticoid receptor antagonist; NT-proBNP, N-terminal pro-B-type natriuretic peptide; NYHA, New York Heart Association; Q_1, lowest quartile; Q_{2-4}, upper three quartiles; sTFR, soluble transferrin receptor; TSAT, transferrin saturation.
*No patient was previously hospitalised more than once.

CI (online supplementary *Table S3*). CI was not significantly different between patients who had or had not previously been hospitalised with decompensated HF and was not related to the time interval from decompensation (online supplementary *Table S4*).

CI_{Q1} patients had significantly higher NT-proBNP levels than CI_{Q2-4} patients [3566 (1513–6412) vs. 1542 (526–2811) ng/L; $P = 0.005$] (*Figure 1*). In a multivariate analysis that considered age, sex, BMI, HF symptom duration, and estimated GFR, CI_{Q1} patients had a 6.0-fold (95% confidence interval 1.5–24.1) higher risk of having an NT-proBNP concentration above the median than CI_{Q2-4} patients ($P = 0.011$).

When CI quartiles were analysed individually, CI_{Q1} patients had a significantly lower BMI than CI_{Q2-4} patients and significantly higher NT-proBNP levels than CI_{Q2} and CI_{Q3} patients (online supplementary *Table S5*).

Cardiac iron and endomyocardial biopsy analysis

Clinically, all patients presented with non-ischaemic HFrEF. EMB evaluation revealed non-ischaemic cardiomyopathy with no or low-grade inflammation in 34 patients, non-ischaemic cardiomyopathy with high-grade inflammation in 42 patients, active myocarditis in 2 patients, and amyloidosis in another 2 patients. CI was not related to EMB-based diagnostic subgroups (online supplementary *Table S6*). Average cardiomyocyte diameters, collagen type I and type III area fractions, and accumulation of

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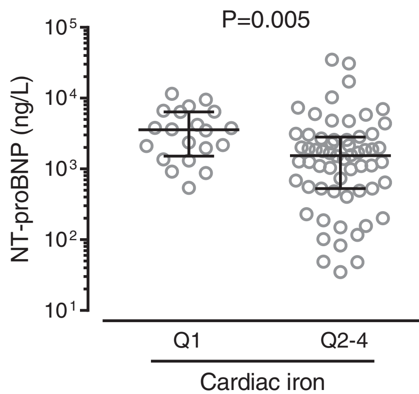

Figure 1 Cardiac iron and N-terminal pro-B-type natriuretic peptide (NT-proBNP). NT-proBNP in patients with cardiac iron concentrations in the lowest quartile (Q_1) or upper three quartiles (Q_{2-4}). Individual data points, median, and the upper and lower quartile boundaries are shown.

$CD3^+$, $CD45R0^+$, LFA-1^+, or MAC-1^+ inflammatory cells were not significantly different in CI_{Q1} or CI_{Q2-4} patients (*Table 2*). By linear regression analysis, CI was not associated with $CD3^+$, $CD45R0^+$, LFA-1^+, or MAC-1^+ inflammatory cell numbers in the myocardium, whereas CI was weakly associated with collagen type III area fraction (online supplementary *Figure S4*). Genomes of adenovirus, Epstein–Barr virus, enterovirus, human herpesvirus 6, and parvovirus B19 were detected at similar frequencies in CI_{Q1} and CI_{Q2-4} patients (*Table 2*).

Iron status and left ventricular remodelling

Left ventricular end-diastolic and end-systolic diameter indices, LV volume indices, LVEF, LV end-diastolic pressure, LV dP/dt_{max}, and interventricular septum thickness and LV mass indices were not significantly different in patients with or without systemic ID (online supplementary *Table S7*). Some of these echocardiographic parameters, however, aligned with CI. Specifically, CI_{Q1} patients had greater LV end-diastolic [31 (29–36) vs. 27 (25–31) mm/m^2; $P = 0.001$] and end-systolic diameter indices [27 (24–31) vs. 23 (20–27) mm/m^2; $P = 0.003$] than CI_{Q2-4} patients (*Figure 2*). LV end-diastolic and end-systolic volume indices were not significantly greater in CI_{Q1} than in CI_{Q2-4} patients; likewise, LVEF was not significantly different between the two groups [23 (17–28) vs. 27 (18–34)%; $P = 0.15$] (online supplementary *Table S7*). LV end-diastolic pressure was higher in CI_{Q1} than in CI_{Q2-4} patients [16 (11–21) vs. 11 (6–19) mmHg; $P = 0.046$] (*Figure 2*). When CI quartiles were analysed individually, CI_{Q1} patients had significantly greater LV end-diastolic and end-systolic diameter indices than CI_{Q2-4} patients (online supplementary *Table S8*).

Discussion

This study is the first to measure CI in patients with non-advanced HF and to use LV EMBs as source material. We present two main findings: first, patients with low CI have greater disease severity and, second, CI is not related to systemic iron status.

Although we studied a relatively homogeneous population of patients presenting with non-ischaemic HFrEF, CI varied widely in individual patients (range 132–1339 µg/g dry tissue). Lacking established normal values, we arbitrarily chose the lower quartile boundary to define patients with low CI. Patients with lower BMIs (still within the normal weight range in our population) and those with longer HF histories were more likely to have low CI. CI in patients with non-advanced HFrEF was approximately twofold higher than CI previously measured in patients with end-stage HF.[21,22] This difference does not appear to be related to the analytical methods (ICP-OES or ICP-MS) employed in these studies. While we cannot fully explain this difference, varied HF duration may have played

Table 2 Immunohistology and viral polymerase chain reaction

	All patients (n = 80)	Cardiac iron Q_1 (n = 20)	Cardiac iron Q_{2-4} (n = 60)	*P*-value
Cardiomyocyte diameter (µm)	23 (20–25)	23 (20–25)	23 (20–25)	0.87
Collagen type I area fraction (%)	9.8 (2.7–21.3)	8.8 (4.0–19.8)	10.5 (2.4–21.4)	0.94
Collagen type III area fraction (%)	9.8 (6.2–16.9)	8.4 (4.4–18.1)	10.9 (6.3–16.6)	0.34
$CD3^+$ cells (mm^{-2})	9.8 (2.0–22.5)	4.3 (0.8–21.2)	10.7 (2.6–22.5)	0.18
$CD45R0^+$ cells (mm^{-2})	52.5 (33.0–72.5)	38.0 (27.0–67.0)	53.3 (34.7–75.8)	0.10
LFA-1^+ cells (mm^{-2})	16.7 (6.7–33.0)	11.1 (5.3–28.2)	18.2 (8.2–37.1)	0.16
MAC-1^+ cells (mm^{-2})	36.3 (20.4–61.7)	28.3 (17.6–49.6)	40.9 (21.4–68.9)	0.16
Adenovirus detectable	0	0	0	1.0
Epstein–Barr virus detectable	0	0	0	1.0
Enterovirus detectable	0	0	0	1.0
Human herpesvirus 6 detectable	6 (8)	2 (10)	4 (7)	0.64
Parvovirus B19 detectable	51 (64)	14 (70)	37 (62)	0.60

Data are n (%), or median (interquartile range). Collagen type I area fraction (n = 56), collagen type III area fraction (n = 55).
CD, cluster of differentiation; LFA-1, lymphocyte function-associated antigen 1; MAC-1, macrophage-1 antigen; Q_1, lowest quartile; Q_{2-4}, upper three quartiles.

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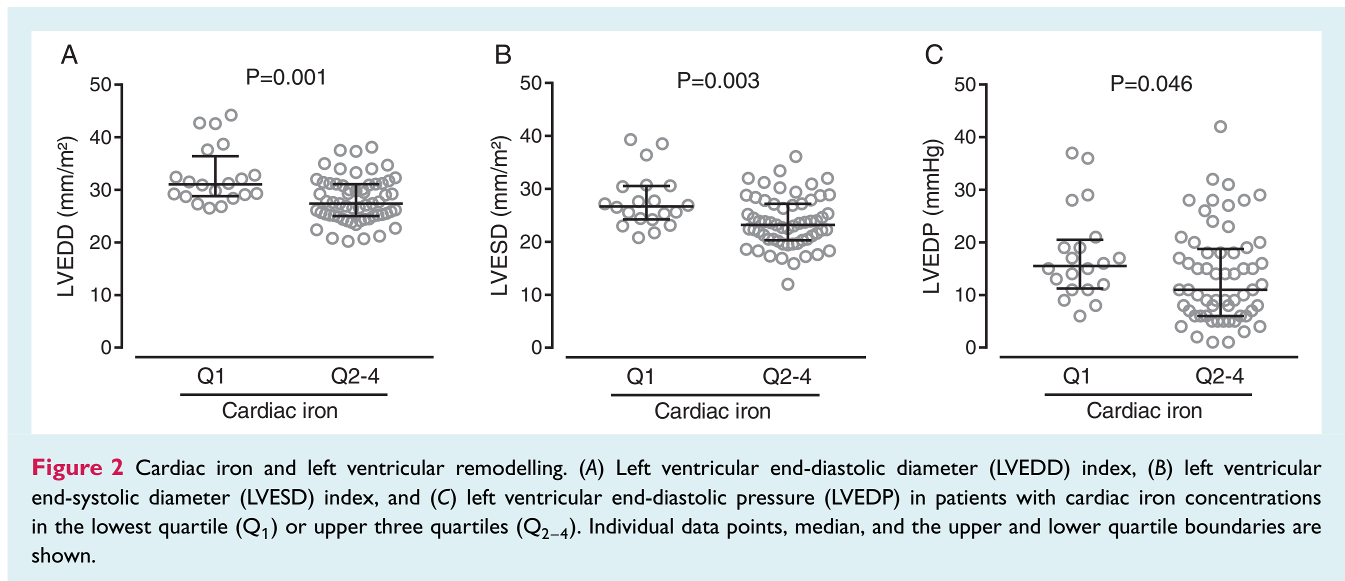

Figure 2 Cardiac iron and left ventricular remodelling. (*A*) Left ventricular end-diastolic diameter (LVEDD) index, (*B*) left ventricular end-systolic diameter (LVESD) index, and (*C*) left ventricular end-diastolic pressure (LVEDP) in patients with cardiac iron concentrations in the lowest quartile (Q_1) or upper three quartiles (Q_{2-4}). Individual data points, median, and the upper and lower quartile boundaries are shown.

a role. Indeed, we found HF duration to be independently associated with low CI, suggesting that cardiac ID may develop or worsen during the course of the disease.

We studied patients with non-ischaemic HFrEF some of who presented with myocardial inflammation as typically observed in patients clinically diagnosed with dilated cardiomyopathy undergoing EMB evaluation.[33,34] Low CI was not related to EMB-based diagnostic subgroups, immunohistological findings (fibrosis, cardiomyocyte diameters, inflammation), or the presence of cardiotropic viral genomes in the biopsies. It is possible that in pathophysiological conditions associated with more pronounced myocardial inflammation (e.g. active myocarditis; not studied here) or in more heterogeneous patient populations presenting with low, intermediate, or very high levels of cardiac inflammation, CI will be associated with cardiac inflammation. Notably, low CI was also not associated with systemic ID, anaemia, or systemic iron homeostasis biomarkers, indicating that CI cannot be inferred from these routine blood measurements. These data add to a growing body of evidence that systemic iron homeostasis and CI are differentially regulated. We have previously shown in mice with post-infarction HF that iron-regulatory proteins 1 and 2 cell-autonomously secure iron availability in cardiomyocytes independent of systemic iron status.[17] Rats with volume overload-induced HF have been shown to develop cardiac ID although iron absorption and systemic iron levels are preserved.[35] In a study of 33 patients with advanced HF, serum iron markers did not reflect LV myocardial iron levels, except for sTFR which tended to display a negative correlation.[21]

Based on higher serum NT-proBNP levels, LV end-diastolic and end-systolic diameters, and LV end-diastolic pressure, patients with low CI had more severe disease than patients with higher CI. Increased LV diameters did not translate into significantly greater LV volumes (although, numerically, LV volumes were larger and LVEF was smaller in patients with low CI). Compared with LV diameters, which are a highly reproducible measure of LV size, measurements of LV volumes may be less accurate in some patients, especially when endocardial borders are not well defined and/or if imaging quality is affected by body habitus.[29] A previous study in 33 patients found no association between CI and NT-proBNP.[36] Another study in 91 patients reported no association between CI and BNP, LV end-diastolic diameter, or LVEF.[22] Both studies, however, investigated heterogeneous patient populations with advanced HF, mostly related to ischaemic cardiomyopathy, and with much higher (NT-pro)BNP levels than in our population.[22,36]

Our study cannot establish a causal relationship between low CI and increased disease severity. In other words, it remains to be investigated if CI is a marker of disease severity and/or a factor contributing to disease progression in patients with HF. Experimental studies indicate that low CI can directly promote contractile dysfunction and HF. Indeed, mice with genetically-engineered cardiomyocyte-specific ID develop HF due to impaired mitochondrial respiration and disturbed cardiac energy reserve.[17–19] Notably, depleting intracellular iron also impairs mitochondrial function and contractility in human-induced pluripotent stem cell-derived cardiomyocytes.[37]

In the future, patients with low CI and a preserved systemic iron status may be candidates for iron supplementation therapy. In mice with genetically-engineered cardiomyocyte-specific ID, intravenous iron supplementation increases CI even when cellular iron regulation is defective, e.g. by deletion of iron regulatory proteins or the transferrin receptor in cardiomyocytes.[17,19] This, however, remains to be shown in patients. It will also be interesting to explore if improvements in symptoms and exercise tolerance by iron supplementation relate to cardiac and/or peripheral iron repletion.[38,39]

Our study has limitations that merit consideration. First, CI in LV EMBs reflects iron levels not only in cardiomyocytes but also in other cellular constituents. In mice genetically engineered to develop cardiomyocyte-selective ID, a ~30% reduction in cardiomyocyte iron resulted in a ~30% reduction of total LV myocardial iron, suggesting that cardiac iron is mainly localised within cardiomyocytes.[17] CI was not related to LV myocardial inflammation, interstitial fibrosis, or cardiomyocyte hypertrophy in our study, thereby showing that interindividual variations in

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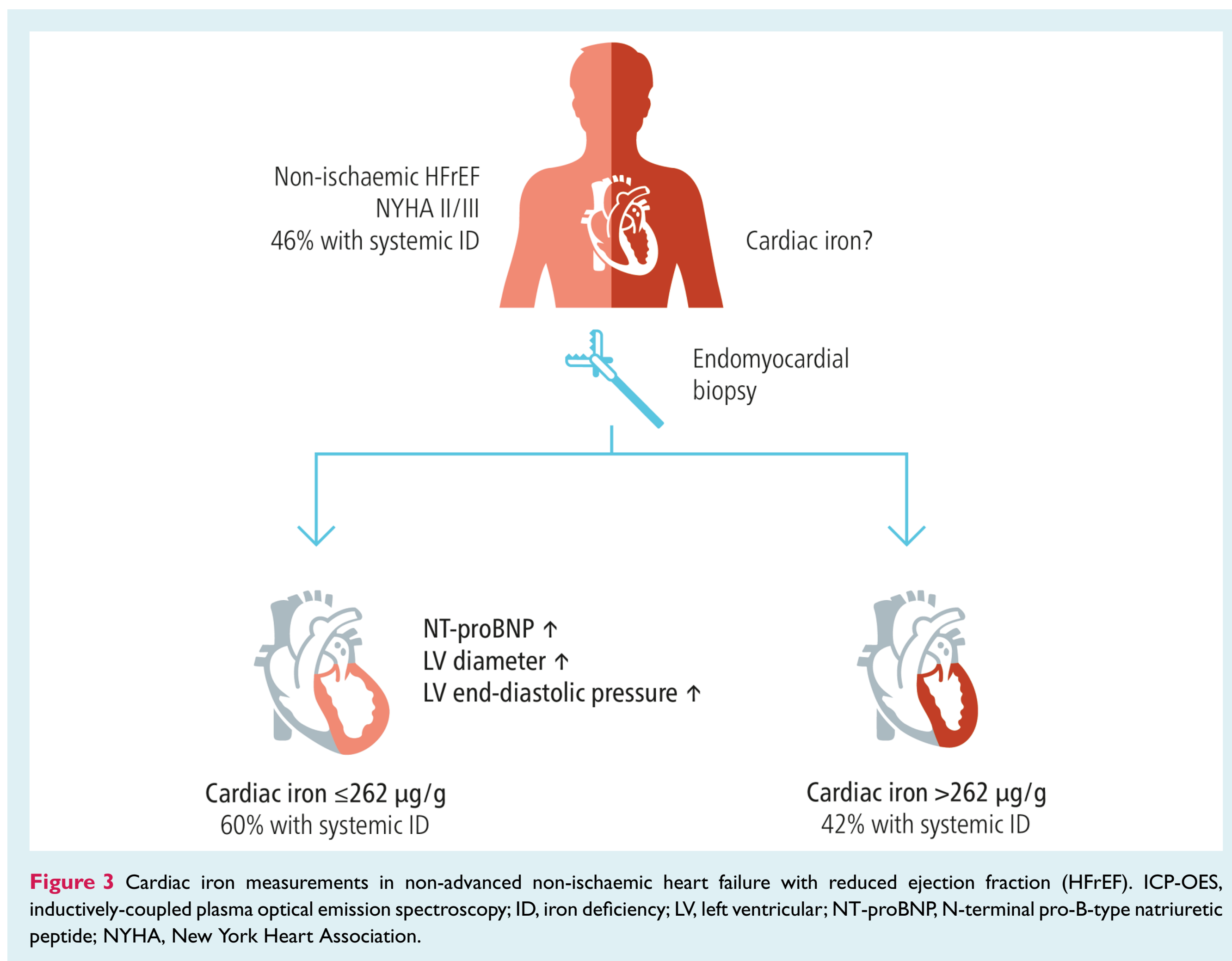

Figure 3 Cardiac iron measurements in non-advanced non-ischaemic heart failure with reduced ejection fraction (HFrEF). ICP-OES, inductively-coupled plasma optical emission spectroscopy; ID, iron deficiency; LV, left ventricular; NT-proBNP, N-terminal pro-B-type natriuretic peptide; NYHA, New York Heart Association.

CI were not related to differences in tissue composition in our patients (this may be different in other HF aetiologies, e.g. in ischaemic cardiomyopathy with more pronounced and variable intramyocardial scarring). We therefore believe that in our study, CI primarily reflects iron localised within cardiomyocytes. Second, we are unable to define a reference range for CI. For ethical reasons, we could not obtain EMBs from healthy individuals; non-transplanted donor hearts were not investigated as they differ from EMBs in tissue composition and pre-analytic handling. Third, we investigated younger patients with non-ischaemic HFrEF and a low number of comorbidities. Therefore, our findings need to be validated in other HF aetiologies and patient cohorts. Fourth, our cohort includes a few patients with active myocarditis ($n = 2$) and amyloidosis ($n = 2$). After excluding these patients, low CI remained significantly associated with higher NT-proBNP levels and larger LV diameter indices (online supplementary *Table S9*).

In conclusion, patients with non-advanced non-ischaemic HFrEF and low CI have more severe disease. CI needs to be directly measured and cannot be predicted based on systemic iron status (*Figure 3*). Future studies should explore whether low CI influences outcome in HFrEF and analyse the effects of iron supplementation therapy in patients with low CI.

Supplementary Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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