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Relationship between iron deficiency and expression of genes involved in iron metabolism in human myocardium and skeletal muscle

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ABSTRACT

Background: Iron deficiency (ID) is associated with adverse prognosis in patients with heart failure. This study aims to investigate the relationship between ID and expression of genes involved in iron metabolism in human myocardium and skeletal muscle, focusing on Transferrin 1 receptor (TfR1), the main pathway of cellular iron uptake.

Methods: Patients undergoing elective CABG were assessed prior to surgery with echocardiography and serum iron parameters. Core needle biopsies were collected from the left and right ventricle (LV, RV), the right atrium and intercostal skeletal muscle (SM). Gene expression analyses were done by mRNA sequencing.

Results: Of 69 patients (median age 69 years, 91% men), 28% had ID. 26% had HFrEF, 25% had HFpEF physiology according to echocardiographic findings and NT-proBNP levels, and 49% had normal LV function. The expression of TfR1 was increased in patients with ID compared to patients without ID in ventricular tissue ($p = 0.04$) and in intercostal SM ($p = 0.01$). The increase in TfR1 expression in LV and RV was more pronounced when analysing patients with absolute ID (S-Ferritin<100 μg/L). Analysing the correlation between various iron parameters, S-Ferritin levels showed the strongest correlation with TfR1 expression. There was no correlation with NT-proBNP levels and no difference in TfR1 expression between different HF phenotypes.

Conclusions: In patients undergoing elective CABG we found an association between ID and increased TfR1 expression in myocardium regardless of LV function, indicating physiologically upregulated TfR1 expression in the presence of ID to restore intracellular iron needs.

Clinical Trial Registration: Clinicaltrials.gov NCT03671122

1. Introduction

The heart is the organ with the highest energy demand in the human body and is dependent on iron for normal mitochondrial function [1,2]. Muscle cells are especially vulnerable to iron deficiency (ID) since they need large amounts of iron to produce myoglobin [3]. Iron deficiency (ID) is associated with poor prognosis in heart failure (HF) patients with various degrees of left ventricular dysfunction, but underlying mechanisms are not fully understood [4,5].

Studies have shown contradicting results in HF regarding the myocardial response to ID. Transferrin receptor 1 (TfR1) is the primary pathway for iron uptake in cardiomyocytes and should normally be upregulated if intracellular iron levels are low [6,7]. Some studies comparing myocardial samples from end-stage HF patients undergoing transplantation with samples from donor hearts, showed reduced myocardial iron content as well as significant reduction of myocardial expression of TfR1 in HF [8–10]. In-vitro experiments of isolated cardiomyocytes from rats and mice show reduced levels of iron regulatory

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proteins including TfR1, after neuroendocrine stimulation, which suggests that the dysregulation of the myocardial iron metabolism is secondary to the neurohormonal activation seen in end stage HF [8,11]. In contrast, a study of myocardial samples from patients with HF with preserved EF (HFpEF) showed increased expression of TfR1 in patients with concomitant ID [12].

In summary, there are contradicting results regarding regulation of TfR1 gene expression in cardiomyocytes in HF patients with different phenotypes and disease severity. Is TfR1 gene expression inadequately reduced in both the myocardium and skeletal muscle of patients with HF and ID, or is gene expression increased as a physiological response to low intracellular iron levels? Therefore, the aim of this study was to investigate the relationship between iron deficiency and expression of genes involved in iron metabolism in human myocardium and skeletal muscle.

2. Methods

2.1. Subjects

All consecutive patients on the waiting list for elective coronary bypass surgery (CABG) at Karolinska University Hospital, Stockholm, Sweden, during 2014–2018 were considered for inclusion in this study which is a sub-study of the CABG-PREFERS study. Inclusion and exclusion criteria in the CABG-PREFERS study have been described previously [13].

2.2. Ethical conduct

The study was conducted according to principles outlined in the *Declaration of Helsinki* and was approved by the regional Ethics Committee Stockholm. After an amendment to the existing study protocol biopsies were also taken from the intercostal muscle next to the sternotomy in 20 patients. All subjects gave informed written consent prior to any study specific action was made.

2.3. Data collection

Included patients were assessed at a baseline visit 1–2 months prior to the CABG surgery with clinical evaluation including assessment of HF signs and symptoms, 12 lead electrocardiogram, blood samples including iron levels, and standardized echocardiography. Clinical variables were entered in a structured electronic health record system. Venous blood samples were collected at the baseline visit, with the study patient fasting and not having ingested caffeine or nicotine during the last 12 h.

The transthoracic echocardiography was performed according to a standardized protocol by two experienced sonographers. The Vivid 9 ultrasound system (Vingmed-General Electric, Horten, Norway) was used. Images were stored on a dedicated server and were analyzed offline on the EchoPAC workstation (GE EchoPAC sw only, Norway). The mean value of 3 cardiac cycles was reported for each variable.

The CABG operations were performed according to standard clinical routines. After midline sternotomy core needle biopsies (diameter one millimetre) were taken from the left and right ventricle (LV, RV) and the right atrium appendix (RAA) prior to initiation of cardiac arrest and start of extracorporeal circulation to minimize the risk of induced tissue inflammation or hypoxia. Biopsies were also taken from the intercostal muscle next to the sternotomy in a smaller group of patients. The biopsies were immediately stored in −70 °C.

2.4. Definitions

ID was defined as serum (*S*-)-Ferritin <100 μg/L, or S-Ferritin 100–299 μg/L and plasma transferrin saturation (P-TSAT) <0.2, in accordance with current guidelines for HF [14]. Absolute ID was defined as S-Ferritin <100 μg/L and functional ID as P-TSAT <0.2 and S-Ferritin 100–299 μg/L [15].

Patients were categorized into three prospective groups according to LV function variables. We followed the classification previously described in the PREFERS design paper [13]. HFpEF physiology was defined as EF ≥45%, NT-proBNP >125 ng/L and a minimum of three of five of the following variables indicating structural impairment and/or diastolic dysfunction: 1. left atrial volume indexed for body surface area (LAVI) > 34 milliliter/m^2, 2. LV mass index ≥115 g/m^2 for males or ≥ 95 g/m^2 for females, 3. ratio of early mitral inflow wave velocity (E) to myocardial tissue early diastolic wave velocity (e') defined as E/e' ≥ 8, 4. e' septal <0.07 m/s or e' mean septal/lateral <0.09 m/s, 5. tricuspid regurgitation velocity > 2.8 m/s. HFrEF was defined as EF <45%. Patients not fulfilling HFpEF physiology or HFrEF criteria were considered to have normal LV function.

2.5. Gene expression analyses

The methods have previously been reported in detail [16]. Total RNA was extracted from myocardial and skeletal muscle biopsies using the RNeasy Fibrous Tissue Mini Kit (#74704, Qiagen, Hilden Germany). RNA libraries for sequencing were prepared using poly-A selection and the Illumina RNA strand-specific TruSeq Stranded mRNA Sample prep kit with 96 dual indexes (Illumina, CA, USA) according to the manufacturer's instructions. Clustering was done by 'cBot' and samples were sequenced on HiSeq. 2500 (HiSeq Control Software 2.2.58/RTA 1.18.64). Whole transcriptome sequencing was performed for each biopsy and normalization and batch correction was done as previously reported. We analyzed gene expression data for genes involved in the regulation of the intracellular iron metabolism including Transferrin receptor 1 (TfR1), Iron Responsive Element Binding Protein 2 (IREB2), Ferroportin, Divalent Metal Transporter 1 (DMT1), Transferrin (TF), Melanotrasferrin (MTF), Lactotransferrin (LTF), Hemokromatos protein (HFE) and Metaloreductase (MR-STEAP2),

2.6. Statistical analysis

Descriptive data are presented as median with quartiles [Q1;Q3] or number and and percentages. The *t*-test of independent samples was used for between-group comparisons of normally distributed continuous variables, and the Mann-Whitney *U* test was used for non-parametric variables. Normality was assessed with histograms, Q-Q-plots and Shapiro-Wilks test. The Kruskal-Wallis test was used to compare continuous variables between multiple groups. Spearman's rank correlation coefficient (rho) was used for bivariate correlations. Linear regression was used to adjust for age and sex. A two-sided *p*-value <0.05 was considered to be statistically significant. Statistical analyses were performed using IBM SPSS Statistics (version 28.0).

3. Results

3.1. Clinical characteristics

Myocardial biopsies were obtained from 69 CABG patients (Fig. 1). In 20 of these patients, biopsies were also taken from the intercostal skeletal muscle next to the sternotomy. The clinical and echocardiographic characteristics are presented in Table 1 and Appendix Table 3, respectively. Median age was 69 [Q1;Q3 = 63;75] years and 91% were men. The patients were divided into three groups according to LV function; 26% (*n* = 18) HFrEF, 25% (*n* = 17) HFpEF physiology and 49% (*n* = 34) normal LV function. Patients with HFrEF and HFpEF physiology were older than patients with normal LV function. ID was present in 38% of HFrEF, 21% of HFpEF patients and 33% of those with normal LV function.
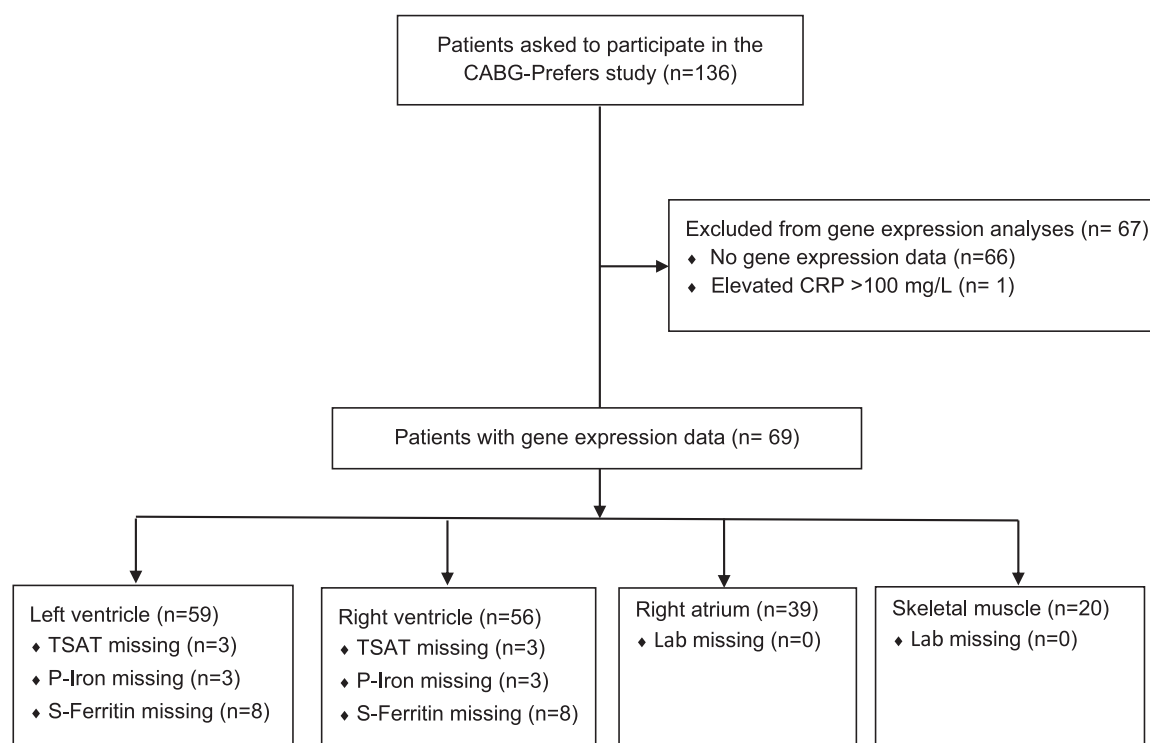
Fig. 1. Flow chart of patients included in the currrent study.
CABG denotes Coronary artery bypass graft, *hsCRP* High-sensitivity C-reactive protein, *TSAT* Transferrin saturation.

Table 1
Baseline characteristics of participating patients.

Variable	All patients $n = 69$
Age, years	69 [63;75]
Male gender, n (%)	63 (91)
BMI	26 [24;29]
LV function, n (%)	
Normal	34 (49)
HFpEF physiology	17 (25)
HFrEF, EF <45%	18 (26)
Comorbidities, n (%)	
Diabetes mellitus	23 (33)
Atrial fibrillation	8 (12)
Anaemia	16 (23)
Iron deficiency	19 (28)
Absolute iron deficiency	14 (20)
Functional iron deficiency	5 (7)
TSAT<0.2	13 (19)
B-Haemoglobin (g/L)	139 [128;146]
P-Iron (μmol/L)	16 [11;18]
P-Transferrin (g/L)	2.25 [2.01;2.42]
P-TSAT (%)	0.27 [0.21;0.31]
S-Ferritin (μg/L)	159 [103;239]
eGFR MDRD (ml/min/1.73m^2)	83 [65;93]
P-Creatinine (μmol/L)	84 [76;103]
P-NT-proBNP (ng/L)	256 (127;636)
P-hsCRP (mg/L)	1.2 [0.4;2.0]
P-Troponin T (ng/L)	13 [8;19]

Data are presented as displayed as median and quartiles [Q1;Q3] or as number (n) and percentage (%).

EF denotes Ejection Fraction; *eGFR MDRD* estimated Glomerular Filtration Rate Modification of Diet in Renal Disease; *HFpEF* heart failure with preserved ejection fraction; *HFrEF* heart failure with reduced ejection fraction; *hsCRP* High-sensitivity C-reactive protein; *NT-proBNP* N-terminal pro-B-type natriuretic peptide; *P-* plasma; *S-* serum; *TSAT* Transferrin saturation.

3.2. Associations of gene expression and iron parameters

We found an increased expression of TfR1 in patients with ID compared to patients without ID in ventricular myocardium (pooling mean expression in LV and RV, $n = 57$) (mean difference − 0.32, 95% CI -0.63 to −0.01) and in intercostal skeletal muscle (SM, $n = 20$), (mean difference − 0.95, 95% CI -1.60 to −0.30) (Fig. 2). The increase in TfR1 expression in biopsies from myocard was more pronounced when analysing patients with absolute ID (Fig. 3), but did not significantly differ in patients with functional ID nor in patients with P-TSAT<0.2 ($p = 0.41$ and $p = 0.11$, respectively) Preoperative S-Ferritin concentrations were significantly correlated with TfR1 expression in LV, RV and RAA, but we found no correlations between TfR1 expression and P-Iron, P-Transferrin, or P-TSAT, except for P-TSAT in RV (Table 2). There was no difference in TfR1 expression between different phenotypes of LV function (Fig. 4 appendix) and no association with NT-proBNP (in myocard β ≤0.00, $R^2 = 0.014$, $p = 0.41$, in SM β = 0.001, $R^2 = 0.07$, $p = 0.28$) or LVEF (in myocard β = −0.001, $R^2 = 0.000$, $p = 0.95$, in SM β = −0.03, $R^2 = 0.14$, $p = 0.11$).

We found no correlation between age and expression of TfR1 in myocard (β = −0.002, $R^2 = 0.001$, $p = 0.85$), nor in SM (β = 0.01, $R^2 = 0.016$, $p = 0.59$). The association between TfR1 expression and S-ferritin, ID and absolute ID respectively, was unchanged after adjusting for age group (tertials) and sex (Table 5 Appendix).

Myocardial expression of other genes involved in the regulation of the intracellular iron metabolism such as IREB2, Ferroportin, DMT1, Transferrin, Melanotrasferrin, Lactotransferrin HFE and Metaloreductase -STEAP2 were not significantly associated with the iron parameters measured in serum except for a weak correlation between P-Transferrin and DMT1 in LV biopsies and HFE in RV biopsies (Table 4 Appendix). DMT1 was significantly higher in patients with absolute ID compared to those with S-Ferritin ≥100 μg/L both in LV (mean difference − 0.16, 95% CI -0.28 to −0.05, $p = 0.008$) and SM (mean difference − 0.48, 95% CI -0.96 to −0.003, $p = 0.049$), but none of the other genes differed in expression according to ID status, functional ID or P-
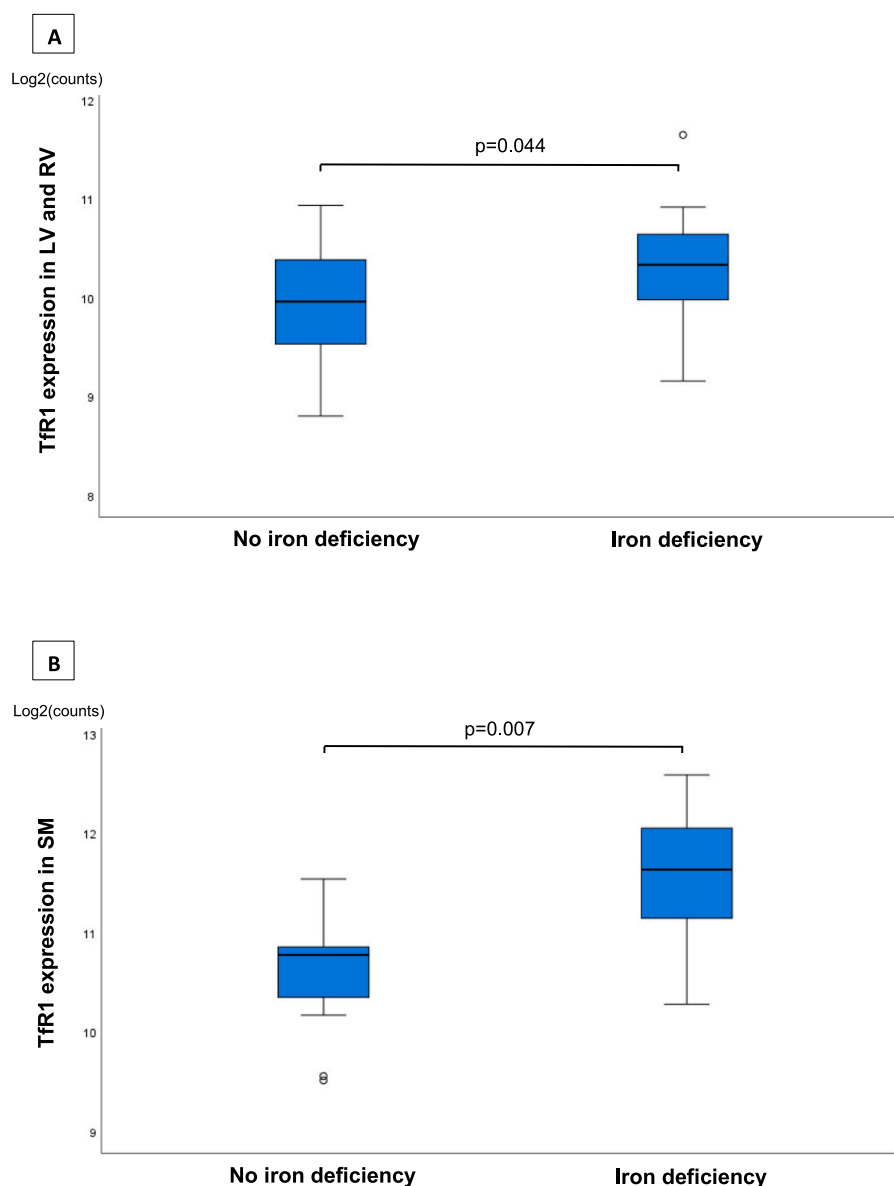
Fig. 2. Transferrin receptor 1 expression in relation to iron deficiency.
A myocardial biopsies from myocard ($n = 57$), B skeletal muscle next to the sternotomy ($n = 20$).
ID denotes iron deficiency, *LV* left ventricle, *RV* right ventricle, *SM* skeletal muscle, *TfR1* Transferrin receptor 1.

TSAT<0.2 either in LV nor in SM.

4. Discussion

The primary finding in the present study was that ID was associated with an increased myocardial expression of TfR1, especially in absolute ID with S-Ferritin <100 μg/L, in patients undergoing elective CABG. The increased TfR1 levels were independent of LV function and did not correlate with NT-proBNP. Also, the increased TfR1 gene expression was independent of age and sex. Secondly, we found similar response to ID in skeletal muscle as in the myocardium, and no difference between LV and RV. This implies TfR1 is physiologically upregulated in a compensatory manner in the presence of ID to secure intracellular iron levels sufficient for oxidative metabolism.

Our findings are in contrast to previous studies of myocardial TfR1 where patients with end-stage HF undergoing heart transplant had a decreased TfR1 gene expression [8–10], but are in line with the findings by Kasner et al. [12] showing up-regulated TfR1 expression in patients with HFpEF and ID. One explanation of these differences in TfR1

expression could lie in the different degree of HF severity and LV dysfunction between these populations. Myocardium from end-stage HF patients undergoing transplantation might be so remodulated and fibrotic that it no longer can respond adequately to low iron levels. This might even be a part of a vicious circle where low intracellular iron contributes to worsening myocardial function. On the other hand, patients with less severe HF as in our study, seem to still be able to respond to ID and attempt to secure sufficient myocardial iron by upregulating the TfR1 receptors on the cell surface. Interestingly, we also saw an increased TfR1 expression in skeletal muscle from patients with ID. Further studies are needed to elucidate if skeletal muscle in heart transplant patients have a similar response, as it would contribute to the understanding if the fatigue seen in HF patients with ID is partly a result of low intracellular iron levels in the myocardium, in the skeletal muscle or low serum levels of iron.

When analysing the other genes related to iron metabolism, only DMT1 showed a significantly higher expression in patients with absolute ID. This was seen both in LV and SM biopsies and might reflect the synergistic role of TfR1 and DMT1 in the intracellular iron metabolism
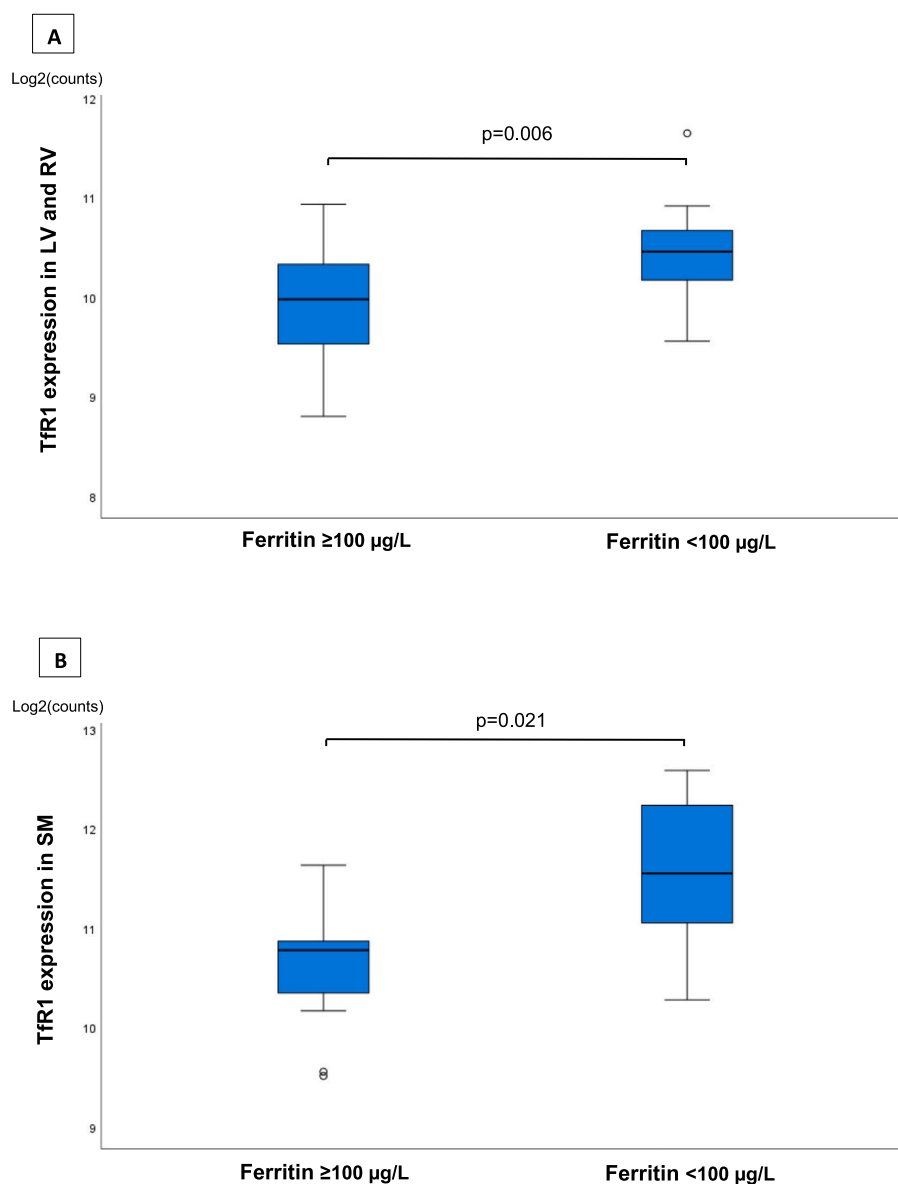
Fig. 3. Transferrin receptor 1 expression in relation to absolute ID (S-Ferritin <100 microg/L).
A myocardial biopsies from myocard (n = 57), B skeletal muscle next to the sternotomy (n = 20).
ID denotes iron deficiency, *LV* left ventricle, *RV* right ventricle, *SM* skeletal muscle, *TfR1* Transferrin receptor 1.

Table 2
Correlations between iron parameters and TfR1 expression in different tissues.

Tissue	S-Ferritin		P-Iron		P-Transferrin		P-TSAT	
	rho	p	rho	p	rho	p	rho	p
LV (n = 59)	−0.311	**0.026**	−0.222	0.100	0.043	0.747	−0.248	0.065
RV (n = 56)	−0.458	**0.001**	−0.256	0.064	0.033	0.812	−0.330	**0.016**
RAA(n = 39)	−0.384	**0.016**	−0.271	0.096	−0.183	0.265	−0.096	0.562
SM (n = 20)	−0.356	0.123	−0.032	0.894	0.227	0.337	−0.140	0.557

Spearman's rho correlation coefficient and significance.[*] Bold highlights that correlation is significant at the 0.05 level (2-tailed).
LV denotes left ventricle; *P-* plasma; *RV* right ventricle; *RAA* right atrium appendix; *S-* serum; *SM* skeletal muscle; *TfR1* Transferrin receptor 1; *TSAT* Transferrin saturation.

[17]. Both TfR1 and DMT1 are regulated by iron regulatory proteins (IRP) which are directly affected by the intracellular iron levels and under circumstances of low iron levels stabilises the mRNA transcripts of TfR1 and DMT1 leading to higher import of iron and at the same time destabilising ferritin and ferroportin leading to reduced iron export and iron storage [17,18]. This co-variation of Tfr1 and DMT1 might be reflected in our results, while the other genes possibly have more of a house keeping function and may not vary in expression levels to the same extent.

The current definition of ID has been debated and it has been suggested that P-TSAT <0.20 or S-Iron have better accuracy in detecting ID when compared to the golden standard of bone marrow staining and

have bigger impact on the clinical prognosis than the current ID definition [19–23]. However, apart from serum soluble TfR1, no other serum iron parameter has previously been shown to reflect low myocardial iron levels [9,24,25].In our study we found correlations between low concentrations of S-Ferritin and increased TfR1 expression in various biopsy locations, while P-TSAT only showed weak non-significant correlations in all tissues except for RV. TfR1 expression was higher in patients with ID and those with Ferritin<100 μg/L, but not in patients with functional ID or P-TSAT<0.2. Our findings suggest that S-Ferritin reflects the intracellular iron demand to a higher extent than P-TSAT, P-Iron and P-Transferrin, and that low ferritin levels impacts the intracellular iron metabolism resulting in an upregulated expression of TfR1 on the myocardial cell surface to facilitate iron import. This indicates that the concept of absolute ID with low S-Ferritin levels, reflecting depleted intracellular iron stores, is still clinically relevant in addition to the concept of functional ID with low P-TSAT reflecting low bioavailability of iron. However, the effects of acute inflammation on S-Ferritin levels have to be considered in the clinical setting.

4.1. Limitations and strengths

To address the ethical dilemma of obtaining myocardial biopsies, these were taken during planned CABG by a specified surgeon with extensive experience in this procedure. As a result of this, all patients in the present study had underlying atherosclerotic chronic coronary artery disease, which can induce a limitation in the generalizability of the results to cohorts without coronary heart disease. This also made it more difficult to assess the presence of, and degree of, HF symptoms since all patients had concomitant ischemic symptoms. The definition of HFpEF physiology was therefore limited to mainly rely on echocardiographic signs of structural, systolic or diastolic abnormalities and NT-proBNP elevation. Another limitation in the study is that the classification of HF used at the time of the study design lately has been changed to include also the category of mildly reduced HF, HFmrEF [13,14]. We chose to keep the original study design since introducing another category of LV function would further reduce the sample size of the groups. The number of biopsies from skeletal muscle was low which may have introduced a type 2 error in the statistical analysis. However, the present study collected biopsies from both myocardial tissue (RAA, RV and LV) and biopsies from skeletal muscle, which is a strength in relation to previous studies.

Further limitations include that our biopsy material did not allow measurement of the myocardial iron content since this requires larger quantity of biopsy material. In some previous studies larger tissue samples have been taken from transplanted hearts and myocardial iron content has been determined with atomic adsorption spectroscopy (Instrumental Neutron Activation) [8,24,26]. However, in our study biopsies were taken from patents undergoing CABG which restricted the possibility to take larger samples. The biopsy material was largely used for RNA sequencing, not leaving the possibility of further tissue analysis. However, we do believe that our data is more representative of every day clinical practice than previous studies including end-stage cardiac failure.

Strengths of the study include expression analysis of several genes involved in iron metabolism in human myocardium as well as skeletal muscle, from well-characterised patients who underwent structured collection of echocardiographic data, analyzed and interpreted by experienced investigators in a blinded manner to ensure reliable results.

5. Conclusion

In conclusion, we found an association between low serum ferritin and increased TfR1 gene expression in myocardium regardless of LV function. The TfR1 gene expression was significantly higher in patients with ID, and the difference was even more evident in absolute ID. The response was similar in biopsies from LV and RV myocard and from

skeletal muscle. This indicates that TfR1 is physiologically upregulated in a compensatory manner in the presence of ID to secure intracellular iron levels sufficient for oxidative metabolism. Our findings might improve both the understanding of the pathophysiology and the clinical implications of iron deficiency in HF patients and emphasizes the need for screening and treatment of iron deficiency in these patients.

Declaration of Competing Interest

CC: Speaker honoraria from Astra Zeneca and Boeringer Ingelheim.
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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijcard.2023.03.032.

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