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CLINICAL RESEARCH
Heart failure/cardiomyopathy

Iron deficiency in worsening heart failure is associated with reduced estimated protein intake, fluid retention, inflammation, and antiplatelet use

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Aims Iron deficiency (ID) is common in heart failure (HF) patients and negatively impacts symptoms and prognosis. The aetiology of ID in HF is largely unknown. We studied determinants and the biomarker profile of ID in a large international HF cohort.

Methods and results We studied 2357 worsening HF patients from the BIOSTAT-CHF cohort. ID was defined as transferrin saturation <20%. Univariable and multivariable logistic regression models were constructed to identify determinants for ID. We measured 92 cardiovascular markers (Olink Cardiovascular III) to establish a biomarker profile of ID. The primary endpoint was the composite of all-cause mortality and first HF rehospitalization. Mean age (±standard deviation) of all patients was 69 ± 12.0 years, 26.1% were female and median N-terminal pro B-type natriuretic peptide levels (+interquartile range) were 4305 (2360–8329) ng/L. Iron deficiency was present in 1453 patients (61.6%), with highest prevalence in females (71.1% vs. 58.3%; $P < 0.001$). Independent determinants of ID were female sex, lower estimated protein intake, higher heart rate, presence of peripheral oedema and orthopnoea, chronic kidney disease, lower haemoglobin, higher C-reactive protein levels, lower serum albumin levels, and $P2Y_{12}$ inhibitor use (all $P < 0.05$). None of these determinants were sex-specific. The biomarker profile of ID largely consisted of pro-inflammatory markers, including paraoxonase 3 (PON3) and tartrate-resistant acid phosphatase type 5. In multivariable Cox proportional hazard regression analyses, ID was associated to worse outcome, independently of predictors of ID (hazard ratio 1.25, 95% confidence interval 1.06–1.46; $P = 0.007$).

Conclusion Our data suggest that the aetiology of ID in worsening HF is complex, multifactorial and seems to consist of a combination of reduced iron uptake (malnutrition, fluid overload), impaired iron storage (inflammation, chronic kidney disease), and iron loss (antiplatelets).

Keywords Heart failure • Iron deficiency • Inflammation • Protein intake • Fluid retention • Antiplatelets

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Introduction

Numerous studies showed the adverse clinical and prognostic consequences of iron deficiency (ID) in patients with chronic heart failure (HF).[1–4] Despite the significant prevalence of ID in HF, its pathophysiology and aetiology are not well-understood. Suggested mechanisms for ID in HF are poor dietary iron intake, drug interactions, (occult) gastrointestinal blood loss due to antiplatelet drugs and anticoagulants, and hepcidin-induced iron entrapment due to chronic low-grade inflammation.[5] While ID is present in approximately half of all HF patients, its prevalence seems highest in females.[1,4] It is currently unclear which factors are driving this sex difference. In the present study, we identified determinants of ID in a large international cohort of worsening HF patients and sought to find sex-specific clinical and biochemical predictors of ID. Moreover, we established a cardiovascular biomarker profile of patients with ID.

Methods

Study population

We included HF patients from the BIOSTAT-CHF study (A systems BIOlogy Study to TAilored Treatment in Chronic Heart Failure). This cohort has been described in full detail elsewhere.[6–8] In short, the BIOSTAT-CHF study included patients either hospitalized for HF or presenting with worsening HF in the outpatient setting. Patients were eligible to participate with a left ventricular ejection fraction (LVEF) of ≤40% or, alternatively, brain natriuretic peptide or N-terminal pro B-type natriuretic peptide (NT-proBNP) levels of >400 ng/L or >2000 ng/L, respectively. Additionally, patients had to receive suboptimal evidence-based HF treatment (i.e. ≤50% of target dose of angiotensin-converting enzyme inhibitors, angiotensin receptor blockers, and/or beta-blockers). After study inclusion, treating physicians were encouraged to up-titrate these drugs during a 3-month treatment optimization phase. The BIOSTAT-CHF study was conducted in accordance with the Declaration of Helsinki. All patients provided written informed consent prior to any study-related activities.

Of all 2516 patients enrolled in the BIOSTAT-CHF cohort, serum for iron status analysis was available in 2357 (93.7%) patients.

Laboratory measurements

Iron parameters were assessed from venous blood. Blood samples were centrifuged at 2500 *g* for 15 min (4°C) and stored at -80°C afterwards. Samples were never thawed before laboratory analyses. The following blood markers reflecting iron metabolism were assessed on a Roche modular cobas 8000 using standard methods: serum iron, ferritin, and transferrin. Transferrin saturation (TSAT) was calculated as follows: [72.17 * iron (mg/dL)]/transferrin (mg/dL).[9]

Renal function was expressed as the estimated glomerular filtration rate (mL/min/1.73 m^2), calculated using the Chronic Kidney Disease Epidemiology collaboration equation (CKD-EPI). Serum NT-proBNP levels were determined using an immunoassay based on electrochemiluminescence (Elecsys, Roche Diagnostics, Mannheim, Germany). Serum hepcidin levels were measured using a competitive ELISA as described previously.[10] Serum soluble transferrin receptor (sTfR) levels were measured using immunonephelometry on a BNII Nephelometer (Siemens AG, Erlangen, Germany).

To establish a biomarker profile for patients with and without ID, 92 cardiovascular-related biomarkers from the Olink Cardiovascular III panel were measured, which were selected based on literature search on bioinformatics (e.g. Uniprot and DisGeNET) and by consulting experts in the cardiovascular field. All biomarkers were measured by Olink Proteomics (Uppsala, Sweden) using the Proximity Extension Assay technology, as previously described.[11] Results are reported as Normalized Protein eXpression (NPX) on a $\log_2$ scale.

Definitions and study endpoints

Anaemia was defined as a haemoglobin level <12 g/dL in women and <13 g/dL in men as per WHO standards.[12] Iron deficiency was defined as a TSAT <20%, as proposed by Grote Beverborg *et al.*[13] This definition has been validated against the gold standard test for ID (bone marrow iron staining) in HF patients and has previously been used.[4,13] Daily protein intake of patients was estimated using spot urinary nitrogen and body mass index.[14] Median follow-up of the study was 21 months. The primary endpoint of this study was the composite of all-cause mortality and first HF rehospitalization. Secondary endpoints included all-cause mortality and first HF rehospitalization.

Statistical analyses

Data are presented as mean ± standard deviation (SD) when normally distributed, as median and interquartile range (IQR) when non-normally distributed or as percentage when categorical. Baseline characteristics were compared using the Student's *t*-test (normally distributed variables), the Mann–Whitney *U* test (non-normally distributed variables), and the χ^2 test (categorical or binary variables). All baseline characteristics were stratified by iron status and sex. After baseline analyses, skewed variables were natural $\log_2$-transformed to obtain normal distributions. To identify independent predictors of ID, univariable and multivariable logistic regression models were constructed. Associates of ID with a univariable *P*-value of ≤0.1 were entered into the multivariable logistic regression models. Final multivariable models were established using backward elimination based on the significance of each variable. Bootstrap analyses with 1000 repeats (using the 'swboot' package in Stata) were performed to evaluate the robustness of the final models. Variables selected >700 times were considered robust predictors. As a sensitivity analysis, a regularization approach was performed using multivariable lasso regression. The final model was checked for multicollinearity by calculating the variance inflation factor. Restricted cubic splines (three knots for all variables) were constructed for better visualization of the predictive value of continuous parameters on ID. Multivariable interaction analyses for sex were performed with each predictor of ID.

The differential Olink biomarker expression pattern in iron-deficient patients was visualized using a volcano plot, displaying the magnitude in change of each biomarker ($\log_2$-fold change) against the significance of the difference in biomarker expression [negative $\log_{10}$ of the *P*-value (Mann–Whitney *U* test)]. Biomarkers in the top left or right of the plot are of interest (large magnitude fold change and high statistical significance). False discovery rate was controlled by correcting the *P*-values according to the Benjamini–Hochberg procedure (false discovery rate of 0.05). Biomarkers that were significantly up- or down-regulated in ID and had an absolute $\log_2$-fold change of >0.25 were entered in uni- and multivariable logistic regression analyses with ID as dependent variable.

Kaplan–Meier curves were constructed to determine the prognostic consequences of ID. Differences in survival rates were tested using the log-rank Mantel–Cox test. The influence of ID on outcome was further assessed with univariable and multivariable Cox proportional hazard regression models. In the multivariable models, adjustment was made for the BIOSTAT prediction models as described elsewhere.[8] Additionally, the prognostic consequences of continuous TSAT levels were analysed using univariable and multivariable fractional polynomial analyses. The proportional hazards assumption was evaluated using Schoenfeld

residuals and was applicable to all variables included in the outcome models. Missing predictor values were five times imputed as previously described.[8] Survival analyses were performed in all five imputation models and the results were averaged in agreement with Rubin's rules. The degree of missingness of all variables used in the model development is depicted in Supplementary material online, *Table S6*. A two-sided *P*-value <0.05 was considered statistically significant, while for interaction testing a *P*-value <0.1 was used. Data were analysed using Stata version 15.1 (StataCorp LLC, College Station, TX, USA) and R version 3.5.1 (R Foundation for Statistical Computing, Vienna, Austria).

Results

Baseline characteristics

Baseline characteristics of the present study cohort, stratified by iron status, are depicted in *Table 1*. Mean age (±SD) of all patients was 69 ± 12.0 years, median LVEF (+IQR) was 30% (25–36), and 37.2% of all patients were in New York Heart Association (NYHA) functional class III or IV. The overall prevalence of ID was 61.6% ($n = 1453$) with highest prevalence in females (71.1% vs. 58.3%, $P < 0.001$). According to the 'conventional' definition of ID in HF patients (i.e. ferritin <100 μg/L or ferritin 100–300 μg/L with a TSAT <20%), the prevalence of ID was even higher ($n = 1632$; 69.2%). Iron-deficient patients were older, had more comorbidities (including anaemia) and more signs of fluid overload compared to patients without ID (all $P < 0.05$). Furthermore, patients with ID had lowest estimated protein intake, more inflammation, and highest rate of proton-pump inhibitor and $P2Y_{12}$ inhibitor use (all $P < 0.001$). HF aetiology and type of HF event (i.e. new-onset HF or worsening HF) was comparable between patients with and without ID.

Similar in females and males, patients with ID had more severe HF at baseline (e.g. peripheral oedema, orthopnoea, and highest NT-proBNP levels), showed more signs of inflammation, had highest prevalence of anaemia and lowest estimated protein intake (all $P < 0.01$, see Supplementary material online, *Table S1*). Numerically, iron-deficient males had most comorbidities and highest rate of proton-pump inhibitor and $P2Y_{12}$ inhibitor use, although no significant interaction between these variables and sex was present. A higher prevalence of atrial fibrillation in iron-deficient patients was observed in females, but not in males (*P* for interaction, 0.028).

Determinants of iron deficiency

Univariable and multivariable logistic regression prediction models for ID are shown in *Table 2*. Independent determinants of ID were lower estimated protein intake, higher heart rate, presence of peripheral oedema and orthopnoea, history of renal disease, lower haemoglobin, higher C-reactive protein (CRP), lower serum albumin, and use of $P2Y_{12}$ inhibitors (all $P < 0.005$). The c-statistic of this model was 0.76. None of the determinants had a significant interaction with sex. All determinants in the final model remained highly selected in additional bootstrap analyses. Additional Lasso regression analysis of the multivariable model confirmed our findings and selected haemoglobin and CRP as best predictors for ID. The variance inflation factors of variables in the multivariable model were not suggestive of multicollinearity (range of factors 1.02–1.27). Restricted cubic splines showing the association between ID and estimated daily protein intake, serum levels of CRP and albumin, and haemoglobin are displayed in Figure *1A–D*. Sex-specific restricted cubic splines for these determinants can be found in Supplementary material online, *Figure S1A–D*.

Biomarker profile of iron deficiency

Median $\log_2$ levels of the 92 cardiovascular biomarkers from the Olink Cardiovascular III panel are depicted in Supplementary material online, *Table S2*. In patients with ID, the following biomarkers were significantly up-regulated with largest magnitude of change: fatty acid binding protein 4 (FABP4), growth differentiation factor 15 (GDF15), NT-proBNP, osteopontin (OPN), ST2 protein (ST2), tumour necrosis factor receptor 1 (TNF-R1), and transferrin receptor protein 1 (TR). Only paraoxonase 3 (PON3) and tartrate-resistant acid phosphatase type 5 (TR-AP) were strongly and significantly down-regulated in ID (*Figure 2*). After correcting for the determinants for ID originating from *Table 2*, only PON3, TR-AP, ST2, NT-proBNP, and TR remained significantly associated with ID (all $P < 0.05$; Supplementary material online, *Table S3*).

Prognostic consequences of iron deficiency

During a median follow-up of 21 months, overall rates of mortality and first HF hospitalization were 26.9% ($n = 615$) and 24.5% ($n = 578$), respectively. Event rates were comparable between males and females (see Supplementary material online, *Figure S3A and B*). Kaplan–Meier estimator curves (for all-cause mortality and the composite endpoint of all-cause mortality and first HF hospitalization) and cumulative incident curves (for first HF hospitalization), stratified by iron status, are shown in Supplementary material online, *Figure S2A–C*. Iron deficiency was a significant predictor for all endpoints (all $P < 0.05$). No interaction was observed between iron status and sex on all endpoints. Univariable and multivariable Cox proportional hazard regression analyses for all endpoints are depicted in Supplementary material online, *Tables S4 and S7*. Iron deficiency remained independently associated with the primary composite endpoint of all-cause mortality and first HF rehospitalization after correcting for the BIOSTAT prediction model [hazard ratio (HR) 1.30, 95% confidence interval (CI) 1.12–1.50; $P = 0.0005$] and the logistic regression prediction model for ID (HR 1.25, 95% CI 1.06–1.46; $P = 0.007$). In a multivariable fractional polynomial analysis, lower TSAT levels were associated to an increased risk of all-cause mortality (see Supplementary material online, *Figure S4*). Finally, in the prognostic models including haemoglobin (all-cause mortality and the composite endpoint), we compared the prognostic power of haemoglobin and ID (defined as TSAT < 20% and using TSAT as a continuous variable). Exchanging haemoglobin for either ID or TSAT did not alter the prognostic power of both models (see Supplementary material online, *Table S8*).

Discussion

In a large cohort of patients with worsening HF, we identified the following independent determinants of ID: female sex, lower estimated protein intake, higher heart rate, presence of peripheral oedema and orthopnoea, history of renal disease, lower haemoglobin, higher CRP levels, lower serum albumin levels, and antiplatelet use. None of

Table 1 Baseline characteristics for the total cohort, stratified by iron status

Variables	Total cohort	No ID	ID	*P*-value
N	2357	904	1453	
Clinical parameters				
Age (years)	68.9 ± 12.0	68.1 ± 12.1	69.3 ± 11.9	0.016
Females (%)	616 (26.1)	178 (19.7)	438 (30.1)	<0.001
BMI (kg/m^2)	27.9 ± 5.5	27.8 ± 5.3	27.9 ± 5.6	0.65
Estimated protein intake (g/day)	55.0 ± 11.2	56.8 ± 12.2	53.9 ± 10.5	<0.001
Ischaemic aetiology	1069 (46.2)	395 (44.6)	674 (47.1)	0.24
LVEF (%)	30 (25–36)	30 (25–35)	30 (25–37)	0.93
HFrEF	1707 (80.9)	687 (82.6)	1020 (79.8)	0.034
HFmrEF	271 (12.8)	107 (12.9)	164 (12.8)	
HFpEF	132 (6.3)	38 (4.6)	94 (7.4)	
Previous hospitalization for HF	736 (31.2)	278 (30.8)	458 (31.5)	0.70
NYHA functional class III/IV	1417 (61.8)	455 (51.6)	962 (68.3)	<0.001
Systolic blood pressure (mmHg)	125 ± 22	124 ± 20	125 ± 23	0.36
Heart rate (b.p.m.)	80 ± 19	77 ± 18	82 ± 20	<0.001
Peripheral oedema	1165 (59.5)	354 (48.8)	811 (65.7)	<0.001
Elevated JVP	518 (33.5)	156 (26.4)	362 (37.8)	<0.001
Hepatomegaly	333 (14.2)	118 (13.1)	215 (14.8)	0.24
Orthopnoea	818 (34.8)	231 (25.6)	587 (40.5)	<0.001
6MWT (m)	316 (225–393)	345 (250–416)	300 (210–374)	<0.001
KCCQ (overall score)	49 ± 22	56 ± 22	45 ± 22	<0.001
Comorbidities				
Atrial fibrillation	1063 (45.1)	393 (43.5)	670 (46.1)	0.21
Diabetes mellitus	759 (32.2)	238 (26.3)	521 (35.9)	<0.001
COPD	406 (17.2)	133 (14.7)	273 (18.8)	0.011
Renal disease	649 (27.5)	177 (19.6)	472 (32.5)	<0.001
Device therapy	582 (24.7)	215 (23.8)	367 (25.3)	0.42
Laboratory				
Haemoglobin (g/dL)	13.2 ± 1.9	13.9 ± 1.8	12.8 ± 1.8	<0.001
Anaemia[a]	778 (36.2)	181 (23.0)	597 (43.8)	<0.001
Haematocrit (%)	40.0 ± 5.4	41.6 ± 5.2	39.1 ± 5.2	<0.001
Mean corpuscular volume (fL)	90 ± 9	92 ± 9	89 ± 8	<0.001
Iron (mg/dL)	45 (28–73)	78 (62–101)	34 (22–45)	<0.001
Ferritin (µg/L)	103 (50–193)	142 (80–240)	78 (39–162)	<0.001
Transferrin (mg/dL)	200 (160–250)	200 (170–240)	210 (160–250)	0.17
Transferrin saturation (%)	17 (11–25)	27 (23–33)	12 (9–16)	NA
sTfR (mg/L)	1.5 (1.2–2.1)	1.3 (1.0–1.7)	1.7 (1.3–2.3)	<0.001
Hepcidin (nmol/L)	6.3 (2.2–16.5)	8.4 (4.4–20.0)	4.6 (1.4–13.1)	<0.001
CRP (mg/L)	13.0 (5.8–26.4)	8.0 (3.5–17.2)	16.9 (8.4–32.1)	<0.001
Leucocytes (10^9/L)	7.8 (6.4–9.6)	7.5 (6.3–9.1)	8.0 (6.6–9.8)	<0.001
AST (U/L)	25 (19–35)	26 (20–35)	25 (19–35)	0.28
ALT (U/L)	25 (17–38)	26 (18–40)	24 (16–37)	0.005
γ-GT (U/L)	55 (28–109)	53 (29–107)	56 (27–110)	0.89
Alkaline phosphatase (µg/L)	85 (65–118)	83 (63–117)	86 (66–120)	0.26
Total bilirubin (µmol/L)	14 (10–21)	14 (10–20)	14 (10–22)	0.14
Sodium (mmol/L)	140 (137–142)	140 (137–142)	139 (137–142)	<0.001
Potassium (mmol/L)	4.2 (3.9–4.6)	4.3 (4.0–4.6)	4.2 (3.9–4.6)	0.012
NT-proBNP (ng/L)	4305 (2360–8329)	3300 (1833–6767)	4812 (2688–8991)	<0.001
Creatinin (µmol/L)	101 (82–128)	97 (80–122)	104 (84–133)	<0.001
eGFR (mL/min/1.73 m^2)	60 (44–79)	64 (48–83)	57 (43–76)	<0.001
Albumin (g/L)	32 ± 9	34 ± 8	31 ± 9	<0.001
Urea (mmol/L)	11.4 (7.6–18.2)	10.3 (7.1–16.4)	12.0 (7.8–19.0)	<0.001

Continued

Table 1 *Continued*

Variables	Total cohort	No ID	ID	*P*-value
Medication				
Loop diuretics	2346 (99.5)	902 (99.8)	1444 (99.4)	0.17
Beta-blockers on target dose	128 (5.4)	54 (6.0)	74 (5.1)	0.36
ACEi/ARB on target dose	314 (13.3)	128 (14.2)	186 (12.8)	0.35
Aldosterone antagonist	1259 (53.4)	514 (56.9)	745 (51.3)	0.008
Proton-pump inhibitors	825 (35.0)	260 (28.8)	565 (38.9)	<0.001
Antiplatelets				
$P2Y_{12}$ inhibitors	363 (15.4)	108 (11.9)	255 (17.5)	<0.001
Acetylsalicylic acid	1168 (49.6)	443 (49.0)	725 (49.9)	0.67
Anticoagulants	915 (38.8)	349 (38.6)	566 (39.0)	0.87
Vitamin K antagonists	898 (38.1)	341 (37.7)	557 (38.3)	0.77
DOACs	17 (0.7)	8 (0.9)	9 (0.6)	0.46

6MWT, 6-min walk test; ACEi, angiotensin-converting enzyme inhibitor; ALT, alanine aminotransferase; ARB, angiotensin receptor blocker; AST, aspartate aminotransferase; BMI, body mass index; COPD, chronic obstructive pulmonary disease; CRP, C-reactive protein; DOAC, direct-acting oral anticoagulant; eGFR, estimated glomerular filtration rate; γ-GT, gamma-glutamyltransferase; HFmrEF, heart failure with mid-range ejection fraction; HFpEF, heart failure with preserved ejection fraction; HFrEF, heart failure with reduced ejection fraction; ID, iron deficiency; JVP, jugular venous pressure; KCCQ, Kansas City Cardiomyopathy Questionnaire; LVEF, left ventricular ejection fraction; NT-proBNP, N-terminal pro B-type natriuretic peptide; sTfR, soluble transferrin receptor.
[a]Anaemia was defined as a haemoglobin level <12 g/dL in women and <13 g/dL in men.

these factors had a significant interaction with sex. The adverse prognostic consequences of ID are independent of these identified predictors. Finally, we provided a biomarker profile of patients with ID, in which predominantly pro-inflammatory markers seem up-regulated.

Determinants of iron deficiency

Our observational data suggest factors which may be involved in the aetiology of ID in HF. These determinants are depicted in *Take home figure* and are discussed below.

Sex difference

In the final prediction model for ID, we could not identify significant interactions with sex. Instead, female sex was an independent predictor for ID, which has been reported in other studies as well.[1,4,15] Several mechanisms might be underlying this association. First, to exclude menstrual blood loss as a confounding factor, we performed a sensitivity analysis in which we excluded premenopausal women (i.e. age <52 years; Supplementary material online, *Table S5*). This yielded a nearly identical prediction model for ID. Second, female patients in our cohort had a higher prevalence of HFpEF, a HF subtype which has been linked to highest prevalence of ID.[16]

Reduced estimated protein intake

As depicted in *Figure 1A*, the prevalence of ID rapidly increases in patients with lower estimated protein intake. Additionally, we identified lower serum albumin levels as an independent predictor of ID (*Figure 1C*). These findings might suggest a poor nutritional status as an aetiological pathway of ID. Several risk scores for estimating malnutrition have included serum albumin levels as predictor, also in HF patients.[17–20] Although we did not study dietary iron intake per se, daily protein intake should provide a fair estimation of daily dietary iron intake, as a significant amount of dietary iron intake is provided by protein-rich food, such as meat (haem iron), nuts and legumes (non-haem iron).[21] It should be acknowledged that we only estimated total daily protein intake using surrogate markers; we did not have data on the exact daily protein intake, nor exact daily iron intake.

Venous congestion

Heart failure is associated with right-sided venous congestion which leads to increased gastrointestinal wall thickness and malabsorption.[22–25] Intestinal wall oedema due to right-sided congestion might also negatively influence nutrient absorption, including dietary iron. In the multivariable prediction model for ID, we showed that peripheral oedema, as an indicator of right-sided congestion, was an independent predictor of ID. Consequently, malabsorption due to venous congestion may also play a role in the aetiology of ID in HF.

Antiplatelet drugs

Iron-deficient patients had higher prevalence of antiplatelet drug use compared to patients without ID. These patients might be more prone to (sub)clinical gastrointestinal blood loss, for example due to gastrointestinal malignancies or angiodysplasia, which might eventually lead to ID due to iron loss. A recent study by Meijers *et al.*[26] revealed that HF patients may be at risk for incident cancer, including colorectal cancer, possibly due to circulating cardiac and inflammatory markers.

We did not find differences in vitamin K antagonist use in patients with and without ID. Due to the very low use of direct-acting oral anticoagulants (DOACs) in the present cohort ($n = 17$, 0.7%), it was not possible to study this drug group in relation to ID. Given the conflicting gastrointestinal bleeding risk of DOACs compared to vitamin K antagonists, the prevalence of anticoagulant-related bleeding as a cause of ID might change in the future as DOAC prescriptions become more common in general practice.

Table 2 Univariable and multivariable logistic regression prediction models for iron deficiency

Variables	Univariable				Multivariable		
	Odds ratio (95% CI)	*Z*-value	*P*-value	*P* for interaction with sex	Odds ratio (95% CI)	*Z*-value	*P*-value
Clinical parameters							
Age (per 5 years)	1.04 (1.01–1.08)	2.40	0.016	0.464			
Sex (female vs. male)	1.76 (1.44–2.15)	5.58	<0.001	—	1.42 (1.13–1.79)	2.99	0.003
BMI (per 5 kg/m^2)	1.02 (0.95–1.10)	0.60	0.551	0.368			
Estimated protein intake (per 10 g/day)	0.80 (0.74–0.86)	-5.74	<0.001	0.405	0.87 (0.79–0.94)	-3.32	0.001
Ischaemic aetiology (yes vs. no)	1.11 (0.93–1.31)	1.17	0.241	0.687			
LVEF (per 5%)	1.03 (1.00–1.08)	1.71	0.087	0.008			
Male	0.98 (0.93–1.02)	-0.95	0.340				
Female	1.11 (1.02–1.19)	2.54	0.011				
Previous HF hospitalization (yes vs. no)	1.04 (0.87–1.24)	0.39	0.695	0.261			
NYHA functional class III/IV (vs. I/II)	2.02 (1.70–2.40)	7.95	<0.001	0.899			
Systolic blood pressure (per 5 mmHg)	1.01 (0.99–1.03)	0.85	0.395	0.726			
Heart rate (per 5 b.p.m.)	1.08 (1.05–1.10)	6.10	<0.001	0.157	1.06 (1.04–1.09)	3.83	<0.001
Peripheral oedema (yes vs. no)	1.97 (1.67–2.34)	7.90	<0.001	0.649	1.36 (1.12–1.66)	3.03	0.002
Elevated JVP (yes vs. no)	1.69 (1.35–2.12)	4.59	<0.001	0.735			
Hepatomegaly (yes vs. no)	1.16 (0.91–1.48)	1.23	0.220	0.116			
Orthopnoea (yes vs. no)	1.96 (1.64–2.36)	7.28	<0.001	0.927	1.33 (1.07–1.66)	2.59	0.010
Comorbidities							
Atrial fibrillation (yes vs. no)	1.11 (0.94–1.31)	1.25	0.211	0.016			
Male	1.02 (0.84–1.23)	0.19	0.850				
Female	1.69 (1.17–2.44)	2.82	0.005				
Diabetes mellitus (yes vs. no)	1.56 (1.30–1.88)	4.80	<0.001	0.539			
COPD (yes vs. no)	1.34 (1.07–1.68)	2.54	0.011	0.165			
Renal disease (yes vs. no)	1.98 (1.62–2.41)	6.76	<0.001	0.474	1.62 (1.28–2.04)	4.07	<0.001
Device therapy (yes vs. no)	1.08 (0.89–1.31)	0.81	0.419	0.133			
Laboratory							
Anaemia[a] (yes vs. no)	2.49 (2.07–3.00)	9.64	<0.001	0.201			
Haemoglobin (per g/dL)	0.75 (0.71–0.78)	-11.77	<0.001	0.216	0.79 (0.74–0.83)	-7.87	<0.001
CRP (per doubling)	1.48 (1.40–1.57)	13.66	<0.001	0.805	1.39 (1.30–1.48)	10.14	<0.001
AST (per doubling)	0.97 (0.87–1.09)	-0.45	0.653	0.740			
ALT (per doubling)	0.94 (0.86–1.02)	-1.44	0.150	0.467			
γ-GT (per doubling)	0.99 (0.84–1.24)	-0.18	0.859	0.967			
Alkaline phosphatase (per doubling)	1.06 (0.89–1.26)	0.65	0.516	0.186			
Total bilirubin (per doubling)	1.08 (0.98–1.18)	1.57	0.116	0.942			
Sodium (per mmol/L)	0.97 (0.94–0.99)	-3.19	0.001	0.162			
Potassium (per mmol/L)	0.81 (0.70–0.94)	-2.73	0.006	0.182			
NT-proBNP (per doubling)	1.23 (1.16–1.30)	6.95	<0.001	0.028			
Male	1.26 (1.18–1.35)	6.91	<0.001				
Female	1.08 (0.96–1.22)	1.25	0.211				
Creatinin (per doubling)	1.35 (1.15–1.58)	3.74	<0.001	0.027			
Male	1.69 (1.40–2.05)	5.40	<0.001				
Female	1.09 (0.78–1.53)	0.52	0.604				
eGFR (per doubling)	0.79 (0.69–0.91)	-3.39	0.001	0.162			
Albumin (per 5 g/L)	0.81 (0.77–0.86)	-8.09	<0.001	0.776	0.93 (0.87–0.98)	-2.46	0.014
Urea (per doubling)	1.26 (1.15–1.38)	4.90	<0.001	0.387			
Medication							
Loop diuretics (yes vs. no)	NA						
Beta-blockers on target dose (yes vs. no)	0.84 (0.59–1.21)	-0.92	0.359	0.716			
ACEi/ARB on target dose (yes vs. no)	0.89 (0.70–1.13)	-0.94	0.346	0.472			

Continued

Table 2 *Continued*

Variables	Univariable				Multivariable		
	Odds ratio (95% CI)	*Z*-value	*P*-value	*P* for interaction with sex	Odds ratio (95% CI)	*Z*-value	*P*-value
Aldosterone antagonist (yes vs. no)	0.80 (0.68–0.94)	-2.64	0.008	0.745			
Proton-pump inhibitors (yes vs. no)	1.58 (1.32–1.88)	4.99	<0.001	0.197			
$P2Y_{12}$ inhibitors (yes vs. no)	1.57 (1.23–2.00)	3.64	<0.001	0.693	1.64 (1.24–2.16)	3.47	0.001
Acetylsalicylic acid (yes vs. no)	1.04 (0.88–1.22)	0.42	0.674	0.225			
Anticoagulants (yes vs. no)	1.01 (0.86–1.20)	0.17	0.866	0.156			

ACEi, angiotensin-converting enzyme inhibitor; ALT, alanine aminotransferase; ARB, angiotensin receptor blocker; AST, aspartate aminotransferase; BMI, body mass index; CI, confidence interval; COPD, chronic obstructive pulmonary disease; CRP, C-reactive protein; eGFR, estimated glomerular filtration rate; γ-GT, gamma-glutamyltransferase; JVP, jugular venous pressure; LVEF, left ventricular ejection fraction; NT-proBNP, N-terminal pro B-type natriuretic peptide.
[a]Anaemia was defined as a haemoglobin level <12 g/dL in women and <13 g/dL in men.

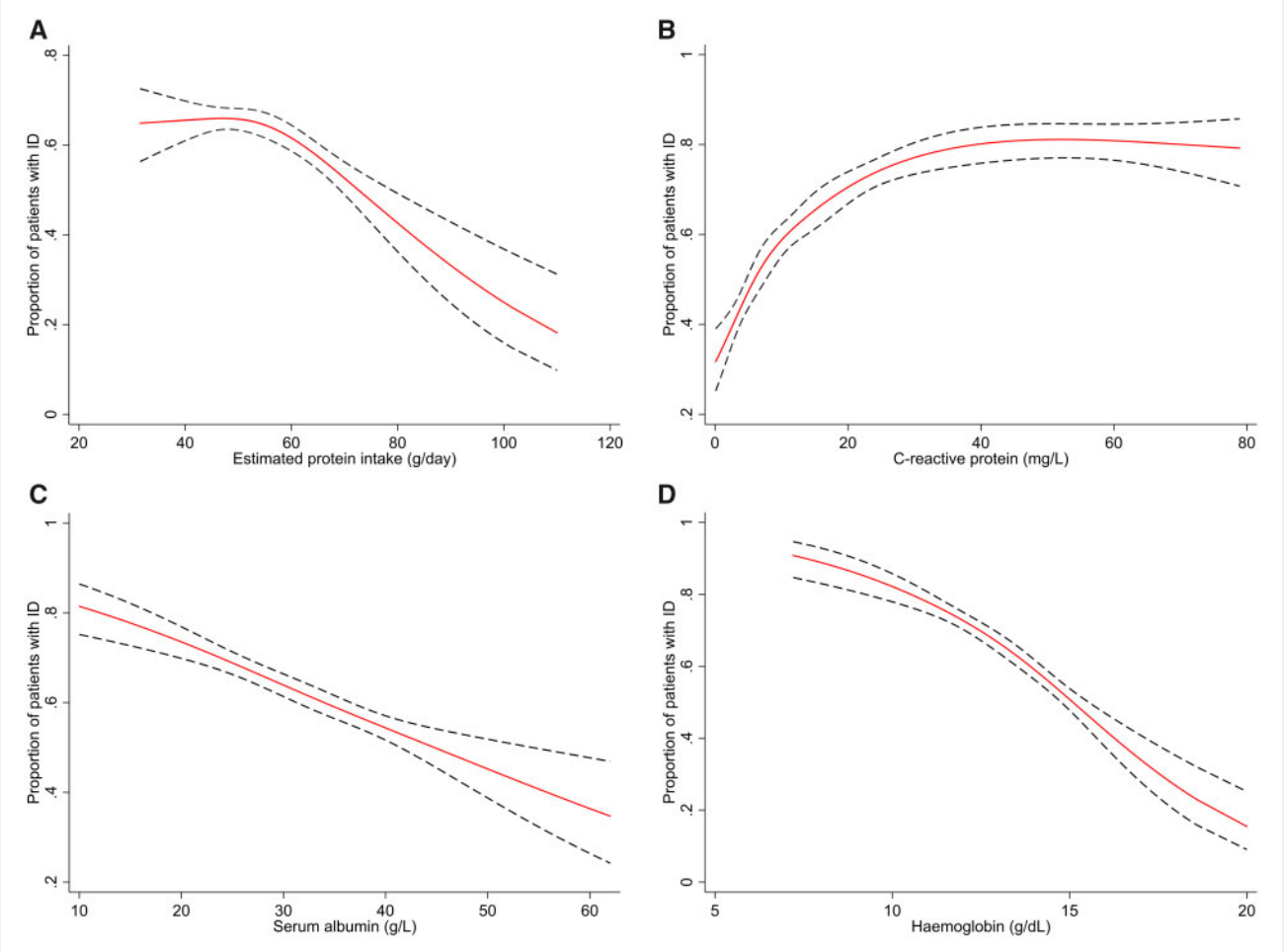


Figure 1 (*A*) Restricted cubic spline of the association between estimated protein intake and the prevalence of iron deficiency. (*B*) Restricted cubic spline of the association between C-reactive protein and the prevalence of iron deficiency. (*C*) Restricted cubic spline of the association between serum albumin and the prevalence of iron deficiency. (*D*) Restricted cubic spline of the association between haemoglobin and the prevalence of iron deficiency. The solid lines indicate estimates of the prevalence of iron deficiency across continuous levels of estimated protein intake, C-reactive protein, serum albumin, and haemoglobin, fitted using logistic regression analysis. The dashed lines indicate 95% confidence intervals.

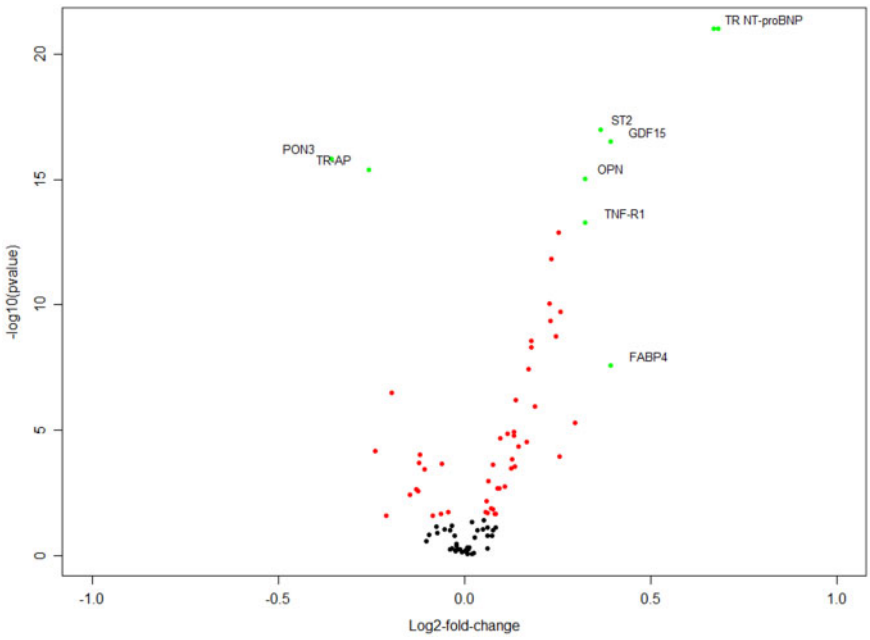
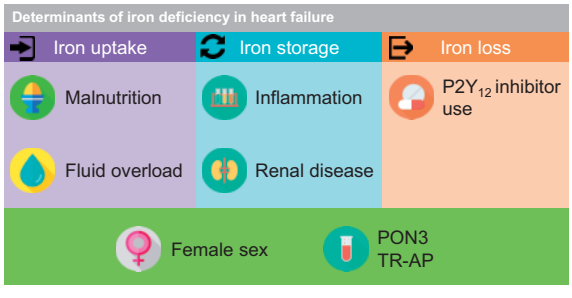


Figure 2 Biomarker expression profile (volcano plot) in iron-deficient heart failure patients compared to patients without iron deficiency. The volcano plot shows the difference in cardiovascular biomarker expression in patients with and without iron deficiency. Each dot represents one of the 92 biomarkers of the Olink Cardiovascular III panel. On the *x*-axis, the $\log_2$-fold change in biomarker expression is depicted (positive $\log_2$-fold change is higher biomarker expression in patients with iron deficiency; negative $\log_2$-fold change is lower biomarker expression in patients with iron deficiency), while the *y*-axis shows the magnitude of the biomarker expression difference as $-\log_{10}$ of the *P*-value. Red dots are biomarkers with a significant up- or down-regulation in patients with iron deficiency (corrected for a false discovery rate of 5%); green dots indicate biomarkers with an absolute $\log_2$-fold change of >0.25. Most biomarkers were significantly up-regulated in patients with ID (n = 39, 42.4%), while 15 biomarkers had significantly lower expression (16.3%). FABP4, fatty acid binding protein 4; GDF15, growth differentiation factor 15; NT-proBNP, N-terminal prohormone brain natriuretic peptide; OPN, osteopontin; PON3, paraoxonase 3; ST2, ST2 protein; TNF-R1, tumour necrosis factor receptor 1; TR, transferrin receptor protein 1; TR-AP, tartrate-resistant acid phosphatase type 5.



Take home figure Determinants of iron deficiency in heart failure. Several graphical elements in this figure are provided by Freepik and DinosoftLabs from www.flaticon.com.

Chronic inflammation

Iron-deficient patients in the present study had higher levels of inflammatory markers compared to patients without ID, whereas hepcidin levels were lowest in iron-deficient patients. This is an interesting finding, as hepcidin levels are expected to be elevated in inflammatory states. Despite the pro-inflammatory state in iron-deficient patients, there must be mechanisms lowering hepcidin levels in these patients. It seems conceivable that these patients have ID due to iron unavailability, malabsorption or loss rather than inflammation, which might explain lower hepcidin levels. In this hypothesis, the influence of chronic inflammation on iron status via the inflammation-hepcidin-iron axis seems limited: systemic iron status itself seems to dictate hepcidin release over inflammatory status. This hypothesis has been postulated by Weber *et al.*[27] in their study comprising 60 stable chronic HF patients with anaemia. They showed that iron-deficient patients have lower hepcidin levels despite increased levels of the pro-inflammatory cytokine tumour necrosis factor-α (TNF-α). Our study yields comparable results using CRP and pro-inflammatory biomarkers from the Olink Cardiovascular III panel. Furthermore, some pro-inflammatory cytokines directly influence iron status independently of hepcidin, for example TNF-α.[28]

Biomarker profile of iron deficiency

As shown in *Figure 2*, many biomarkers from the Olink Cardiovascular III panel were significantly up- or down-regulated in ID. After correcting for HF severity, renal function and predictors for ID, several biomarkers remained independently associated with iron status. Of these, paraoxonase 3 (PON3) and TR-AP are both down-regulated in ID. PON3 is a liver-derived, HDL-bound protein, which

has several antiatherogenic and antioxidative properties. In animal studies, overexpression of PON3 seemed protective against atherosclerosis and cardiac hypertrophy, while PON3-deficient mice show mitochondrial and fatty acid oxidation dysfunction.[29–32] Second, TR-AP is predominantly an osteoclast-derived, iron-containing protein reflecting bone turnover rate and is expressed by activated macrophages. *In vitro* studies show that the expression of TR-AP is regulated by iron status.[33,34] TR-AP knockout mice display altered osteoclastic function leading to mild osteopetrosis and an increased proinflammatory response.[35,36] Unfortunately, both PON3 and TR-AP are poorly studied in human (patho)physiology. Although our study shows an independent link between PON3, TR-AP, and iron status, the clinical significance of the biomarker expression pattern in ID need to be elucidated.

Clinical implications

Our data confirm the adverse prognostic consequences of ID, which are independent of established predictors of outcome. While ID is frequently observed in HF patients, the aetiology is often unknown. However, it is essential to explore the underlying cause(s), since some of them are treatable and reversible.[37] For example, if ID is caused by gastrointestinal blood loss, the underlying cause of this blood loss (e.g. malignancy, angiodysplasia or antiplatelet use) needs to be detected and treated. When ID is caused by the use of antiplatelets or anticoagulants, their use should be reconsidered, especially in patients without a direct treatment indication. Finally, when poor nutritional status is causing ID, this should be treated as well.

Strengths and limitations

To our knowledge, this is the largest cohort with clinical and biochemical parameters available, providing more knowledge on the drivers of ID in HF patients. However, an important limitation of this study is its observational character, making it challenging to directly study aetiological and pathophysiological mechanisms. Nevertheless, we hope our data encourage more studies on the determinants identified in our study. As iron indices were only measured at a single time point, differences in iron status over time could not be studied. Second, there were no data available on the presence or absence of recent blood loss (e.g. blood donations, surgery), which could possibly affect iron status. Third, protein intake was estimated using a formula based on urinary urea and body mass index, and not directly measured. Finally, the majority of patients in BIOSTAT-CHF is male. Therefore, we cannot rule out that the prediction models for ID in males had more statistical power to identify independent determinants of ID compared to the models in female patients.

Supplementary material

Supplementary material is available at *European Heart Journal* online.

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