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Anemia, erythropoietin and iron in heart failure

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Summary and general discussion

Heart failure is a complex, multifactorial syndrome with a high mortality rate and no cure. Treatment is currently focused on preventing disease progression and improving symptoms and quality of life. One major aspect impacting symptoms and quality of life is the presence of comorbidities.¹ In this thesis, I focused on two very prevalent comorbidities: anemia and iron deficiency. Although there is large overlap, they are increasingly considered two separate entities. Iron deficiency has a detrimental effect on morbidity and mortality in HF independent of the hemoglobin level, suggesting separate pathophysiological roles of iron and hemoglobin in HF. We contribute to this thought by showing for the first time the direct effects of iron deficiency on mitochondrial and contractile function of the human cardiomyocyte *in vitro*, excluding the effects of anemia by study design. This possible impairment in cardiomyocyte contractility can contribute to the decreased peripheral oxygen delivery in HF subjects, which is also affected by decreases in hemoglobin, see **Figure 1**. Moreover, we studied the definition of iron deficiency in HF patients, its effects on prognosis and treatment effect and examined two different pathophysiological mechanisms of iron deficiency. Finally, we assessed causality between iron levels and coronary artery disease, the major cause of HF. In the regard of anemia, we more specifically focused on the role of erythropoietin (EPO). We studied the endogenous form of EPO and its association with cardiovascular disease in subjects from the general population, and studied the response to the administration of recombinant EPO in patients with HF.

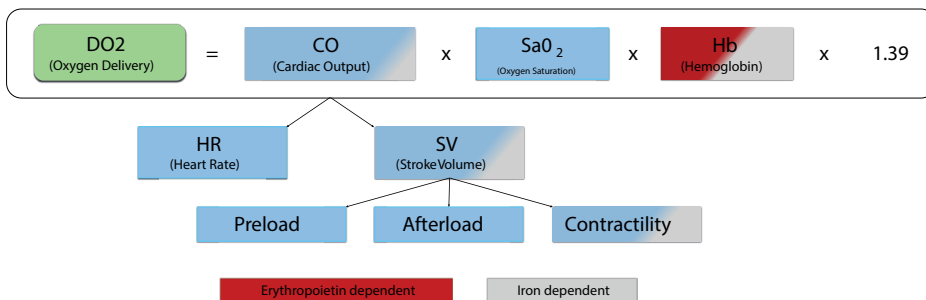


Figure 1 – Determinants of peripheral oxygen delivery with a focus on the role of iron and hemoglobin. An approximation of the formula to calculate peripheral oxygen delivery (DO₂, volume of oxygen delivered per minute). Variables depending on erythropoietin are colored red, variables depending on iron are colored gray. Peripheral oxygen delivery is dependent on the cardiac output (CO) and arterial O₂ content, both in turn dependent on other factors, of which the most important are depicted here. The amount of oxygen dissolved in plasma does not make a significant contribution to the total amount of oxygen delivered, except when a patient is inhaling air with a very high fraction of oxygen (FiO₂), in that case the arterial oxygen tension x 0.003 should be added to the total sum.

PART I

Anemia is highly prevalent in patients with HF (30-50%), higher than in age-matched control subjects.² HF patients with anemia experience more symptoms such as dyspnea and fatigue and have a twofold increased risk of death.^{3,4} Correcting anemia would therefore seem a logical approach to improve morbidity and mortality in these patients. This has been tried with blood transfusions and erythropoiesis-stimulating agents. Both are capable of rapidly increasing the hemoglobin level, however both have not shown a favorable effect on morbidity and mortality. So far, there is no proof that correcting anemia results in substantial clinical benefits, while both therapies bear a risk in terms of side-effects such as anaphylaxis and stroke. One could therefore question if anemia is not merely a sign of disease severity. We discuss this question, and others, in **chapter 2**, where we review the current literature on this topic. Much attention goes to the results obtained with recombinant EPO. Since EPO levels are already thought to be increased in HF patients, treatment with recombinant EPO might sound counterintuitive.⁵⁻⁸ We aimed to further study the role of endogenous EPO levels in HF and its possible association with the response to treatment with recombinant EPO. However, before doing so, we noticed that the data on endogenous EPO levels was limited. We therefore studied EPO levels in a large cohort of subjects from the general population in **chapter 3** and **chapter 4**.

In **chapter 3** we set out by measuring serum EPO levels in 6,777 patients from the **P**revention of **R**enal and **V**ascular **E**nd-stage **D**isease (PREVEND) study.^{9,10} We report age and sex specific reference ranges, which can be used in future studies and clinical care to provide context for measurements in diseased patients. Using a genome wide association approach, we detected a novel SNP near the *HBS1L-MYB* locus to be related to EPO levels. This locus is known to have pleiotropic effects on other hematological parameters, all weaker than its association with EPO, suggesting EPO as the driver of the effects. We showed that higher levels of EPO are associated with cardiovascular risk factors, more specifically the components of the metabolic syndrome including a larger waist circumference, higher plasma glucose level and blood pressure. The association of EPO levels with abdominal obesity is reported by others as well and suggested to be caused by adipose tissue hypoxemia, which in turn could lead to insulin resistance.^{11,12} Interestingly, cholesterol levels are lower in subjects with higher EPO levels, consistent with the observation that cholesterol levels decrease after administration of recombinant EPO.¹³ Finally, we show that a decent renal function, here assessed by glomerular filtration rate ($\text{eGFR} \geq 60 \text{ mL/min/1.73m}^2$), is necessary for an appropriate EPO level in response to a low hemoglobin concentration. However, functional studies are required to further untangle cellular EPO regulation in health and disease.

High serum EPO levels are a sign of hypoxia and inflammation and associated with poor outcome in the elderly (age >85 years), and patients with HF or chronic kidney disease.^{7,14–16} No data on the prognostic value in the general population is available. We studied the association of serum EPO levels with cardiovascular events, and specifically new-onset HF in the PREVEND study in **chapter 4**. We show that a doubling of serum EPO levels is associated with a 32% increased risk of new-onset HF, independent of other known risk modifiers, including the components of the metabolic syndrome and renal function. Importantly, this association interacts with the presence of albuminuria (a urinary albumin concentration $\geq 10\text{mg/L}$). PREVEND was originally set-up to investigate the association of urinary albumin with renal and cardiovascular diseases. For this reason, the cohort was enriched with subjects having an increased urinary albumin concentration.¹⁰ Only these subjects have an increased risk of new-onset HF with higher EPO levels, the reason of which we can only speculate upon. An increased urinary albumin concentration is an very early cardiovascular risk identifier and it's increase might precede any increase in serum EPO levels.^{9,10,17} Alternatively there might be an interplay between stimulation of hemostatic factors by both EPO and albuminuria, worsened by the endothelial damage associated with albuminuria.^{18,19} Finally, we showed that EPO levels are not associated with increased risks of cardiovascular events, except stroke. This is also seen in patients treated with recombinant EPO, where treatment led to increased stroke and thrombo-embolic events in HF and chronic kidney disease patients in respectively the TREAT study and **Reduction of Events with Darbepoetin alfa in Heart Failure trial (RED-HF)**.^{20,21} That EPO does not seem to lead to increased cardiac ischemic events might be explained by the protective ischemic pre-conditioning effects of EPO.^{22,23} This regulation is not effective in the brain, as that organ has its own local regulation in response to hypoxia and is not affected by systemic EPO levels.²⁴ The vasculature of the brain is still exposed to the detrimental effects of increased systemic EPO levels. This imbalance might cause the increased rates of stroke observed. We do not have data supporting this, which makes this only theory, but it is worth further investigation since new treatments also target the same EPO pathway.

The findings of the previous chapters reinforced our hypothesis that the administration of recombinant EPO might be detrimental in HF patients with already high endogenous EPO levels. An interesting study by Solomon et al. showed that approximately 25% of patients with chronic kidney disease do not respond to recombinant EPO, and that these patients had the worst prognosis.²⁵ As chronic kidney disease and HF share many disease characteristics, we hypothesized that this would be similar in the HF patients. Additionally, we hypothesized that a high serum EPO level would identify patients who would not respond to recombinant EPO, as in these patients the production of endogenous EPO would not be the cause of the anemia. In chronic kidney disease it was proven

difficult to predict which patients would respond, but the level of endogenous EPO was not taken into account in these analyses.²⁵ We studied the response to darbepoetin-alfa in **chapter 5** and indeed report comparable results to those obtained in chronic kidney disease patients. We studied patients based on their response in hemoglobin to the first 2 weight-based doses of recombinant EPO. The lowest quartile of responders actually shows a reduction in hemoglobin levels of 0.2 g/dL (interquartile range: -0.6 to 0.0 g/dL) (in mmol/L: 0.1 mmol/L and an interquartile range: -0.4 to 0.0 mmol/L). These patients are considered poor responders. A poor response is, independently of other characteristics, associated with the worst prognosis. Subsequently, we tried to predict who would and would not be a responder. Using a large set of baseline variables we could only achieve a Harrell C statistic of 0.68, were 0.5 is equal to flipping a coin and 1.0 to a perfect prediction. The level of serum EPO does marginally improve the classification of subjects into their risk categories, but does not increase the predictive value of the model. Additionally, the baseline serum EPO level does not identify patients who might benefit from recombinant EPO in terms of prognosis. Currently, the use of erythropoiesis stimulating agents is not recommended in patients with HF, as the side-effects outweigh the limited beneficial effects on symptoms and quality of life.²⁶ Our data do not provide any rationale to reconsider this recommendation. Importantly, erythropoiesis stimulating agents are still recommended and often used in patients with chronic kidney disease to reduce blood transfusions and anemia-related symptoms.²⁷ Because of the large overlap in patient population (three quarters of patients in our study had concomitant chronic kidney disease), our data provide relevant guidance in identifying hypo-responsive patients, which could lead to the discontinuation of treatment in these patients. Since other therapies for anemia have not shown to result in an improvement of prognosis, the management of anemia should include assessing and correcting possible underlying causes. Of these, iron deficiency is the most prevalent and important.²⁸

PART II

Iron is an essential trace element which we acquire through our diet, mainly in the form of heme bound iron in high protein containing foods such as meat and fish. It is necessary for many cellular processes as cofactor in enzymes and oxygen transporting proteins. Because of this, its metabolism is highly controlled and iron is stored in different locations and different forms. Levels of free iron in the blood are kept to a minimum to prevent oxidative damage. Iron is bound to transferrin in the circulation and to ferritin, heme or iron-sulfur clusters intracellularly. As iron is so diffusely stored, assessing iron status is challenging and many circulating markers have been proposed as a potential marker of iron status. In patients with HF, a combination of two markers

has been used. Ferritin, which represent the levels of intracellular stored iron, and transferrin saturation (TSAT), the percentage of transferrin that has iron bound to it, which reflects the systemic iron availability. The definition of a deficiency in iron has been set as follows: a ferritin <100ng/mL or a ferritin 100 – 300ng/mL if TSAT <20%. Although this definition has been used in the majority of clinical trials, various definitions have been used in epidemiological research and none of these has ever been validated. We aimed to provide a definition of iron deficiency constructed using the gold standard: iron assessment in the bone marrow, the major iron storage site. Using iron staining of bone marrow aspirations, we aimed to construct a simple definition using practical circulating biomarkers in **chapter 6**. We show that 40% of HF patients have iron deficiency according to their bone marrow results (see **figure 2** for comparison with healthy control subjects) and that TSAT <20% (or serum iron $\leq 13 \mu\text{mol/L}$) is the optimal definition of iron deficiency using a single circulating biomarker. We studied this definition in a cohort of outpatient HF patients and show that it identifies the patients at highest risk of mortality. Interestingly, there is a group of patients who are conventionally considered to have iron deficiency but are not included in our new definition. These are patients with a TSAT >20%, but included in the old definition because they had a low ferritin (<100ng/mL). We studied this group with an “isolated low ferritin” in more detail and found that they have a similar prognosis as patients without iron deficiency and do not benefit from intravenous ferric carboxymaltose in terms of an improved prognosis. New trials investigating the effects of intravenous iron on prognosis are underway (FAIR-HF2 and IRONMAN), and do include patients with an “isolated low ferritin”. It will be interesting to see how these patients respond to treatment.

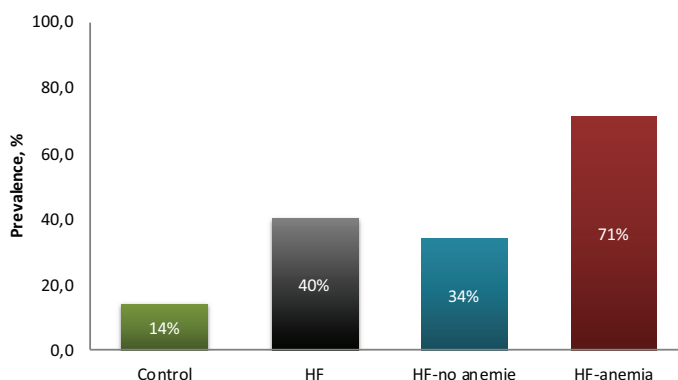


Figure 2 – Prevalence of iron deficiency according to bone marrow iron staining. The prevalence of iron deficiency in healthy control subjects (n=7) and HF patients (n=42) undergoing coronary artery bypass graft surgery, additionally stratified for the presence of anemia according to the WHO definition (men: Hb < 8.1 mmol/L [13.0 g/dL], women: Hb < 7.45 mmol/L [12.0 g/dL]). Iron deficiency is defined as less than 10% of erythroblasts containing iron (i.e. sideroblasts). HF – heart failure.

The pathophysiology of iron deficiency can be divided into two pathways: 1) an insufficient uptake and/or increased loss of iron resulting in a shortage of iron molecules and 2) sufficient iron present but trapped inside the mononuclear phagocyte system.^{29,30} Both are treated as one condition, as we lacked practical tools to distinguish both from each other. The bone marrow aspirations from the study in the previous chapter allowed us to discriminate between the two pathophysiology's and assess possible differences in **chapter 7**. We show that in HF patients with iron deficiency (i.e. a TSAT <20%), ferritin is a good indicator of iron storage. A low ferritin (<128ng/mL) can identify patients with an insufficient amount of iron stored in the bone marrow, a condition we named "low iron storage". The group with a TSAT <20% and a ferritin ≥128ng/mL is termed "defective iron utilization", as these patients have appropriate iron stores but insufficient iron incorporation into the erythroblasts. A low iron storage is associated with the clinical characteristics we generally contribute to iron deficiency: anemia, poor quality of life and a poor prognosis. On the other hand, defective iron utilization is mainly associated with increased inflammation and hepcidin levels, consistent with the hypothesis that hepcidin traps iron intracellularly in chronic inflammation. Importantly, while defective iron utilization is associated with increased inflammation, it did not result in worse prognosis compared to patients with a normal iron status. Similar to the patient group we described in chapter 5 as "isolated low ferritin", it would be interesting to see if the patients with defective iron utilization do benefit from treatment with intravenous iron in the currently conducted trials. This might not be the case, since the low iron availability in these patients does not result in an impaired prognosis.

In all analyses we performed in the previous two chapters, we corrected for the confounding effect hemoglobin could have had on our analyses. Therefore, we can conclude that the effects of iron deficiency reported, are independent of its effect on the hemoglobin concentration. This is in line with the observation that HF symptoms improve after treatment with intravenous iron, even in non-anemic patients.^{31,32} Iron deficiency could therefore have a direct effect on cardiac function. Indeed, several *in vivo* mouse studies with cardiac specific deletions of the transferrin receptor (*TfRC*), hepcidin (*HAMP*), or iron-regulatory proteins showed that low cardiac iron levels result in an impaired function and increased mortality.³³⁻³⁶ We assessed the effect of low iron levels on human cardiomyocytes in **chapter 8**. We differentiated embryonic stem cells into cardiomyocytes, and depleted them from iron using deferoxamine, an iron chelator also used to treat patients with an iron overload. After 4 days of deferoxamine, this resulted in an 84% reduction of intracellular ferritin levels and the upregulation of several genes involved in iron uptake (*TfRC* and *SLC39A14* and *SLC11A2*, two divalent metal transporters). From animal experiments studying skeletal muscle, it is known that iron deficiency leads to an impaired oxidative metabolism, mitochondrial complex I and II activity and

iron-sulfur cluster synthesis.³⁷ Heme and, heme based mitochondrial complexes IV and V are relatively spared.³⁷ In patients with advanced HF, even increased levels of heme are found in cardiac tissues.³⁸ In line with this, we observed an impaired mitochondrial respiration with decreased activity of the iron-sulfur containing mitochondrial complexes I-III, resulting in lower levels of ATP. The contraction-relaxation cycle of cardiomyocytes is highly ATP demanding, and we observed impaired contraction and relaxation velocities of iron deficient cardiomyocytes. This might partly explain why HF subjects with iron deficiency have a lower exercise capacity and poorer prognosis.^{3,4} However, on a critical note, the phenotype we provoked here is very severe. The cardiomyocytes died when we treated them for one more day with deferoxamine. This is therefore probably not completely representative of the phenotype observed in patients, but it does provide us with insights in the underlying pathophysiology. Direct comparison of ferritin levels is unfortunately not easily performed. Ferritin levels in patients are measured in the circulation, which is only a proxy for intracellular levels; we directly measured intracellular ferritin levels in this study. Importantly, the phenotype could be restored by providing the cardiomyocytes access to transferrin bound iron. After only 3 days of iron supplementation, the mitochondrial respiration returned towards baseline and contractility fully restored, thus providing a potential mechanism for the improved echocardiographic parameters seen after iron treatment in patients with HF.^{39,40}

Our epidemiological data suggest a pathophysiological role for iron in the worsening of HF, and our *in vitro* data support this by providing the underlying mechanisms in human cardiomyocytes. A large meta-analysis also provided evidence that higher levels of iron parameters are protective for the development of coronary heart disease, the major cause of HF.⁴¹ These results are in contrast with the “iron hypothesis”.⁴² The iron hypothesis was introduced in 1981 by Sullivan and states that iron deficiency is the reason for the lower incidence of cardiovascular disease in premenopausal women compared to men and postmenopausal women.⁴² Additionally, improved markers of cardiovascular risk were observed in blood donors.^{43,44} In line with this, previous work from our group in the PREVENT cohort showed an increased risk of new onset HF in women with higher ferritin levels.⁴⁵ However, all these epidemiological data have the limitation of possible residual confounding and reverse causation. Especially ferritin levels could be influenced by these factors, as it is raised by inflammation, a major cardiovascular risk factor. To better address residual confounding and reverse causation, we applied the Mendelian Randomization approach in **chapter 9**. This approach uses the random allocation of genetic variants that influence iron status to assess potential causality. We can compare the incidence of coronary artery disease in subjects that have been exposed to a lifetime higher iron status because of their genes, to subjects with a lower iron status.⁴⁶ Using the UK Biobank, a large population based cohort of

the United Kingdom, we report a significant protective effect of higher genetic levels of both ferritin and iron on coronary artery disease. The effects of TSAT and transferrin are consistent with a protective effect of higher iron status but are not significant, mainly due to the inclusion of one SNP (rs9990333). This SNP is located near the *TFRC* gene and has a pronounced effect of coronary artery disease while it has a very marginal effect of the levels of TSAT and transferrin. In contrast, all other SNPs have generally larger effects on the iron parameters, which are directionally consistent with the overall effect. In an earlier report, rs9990333 was the only iron SNP without known pleiotropic effects that was associated with the presence of plaque in 1,819 subjects from the general population of Nijmegen, the Netherlands.⁴⁷ These data suggest a causal role for rs9990333 but possibly through mechanisms independent from iron status. Residual confounding can therefore not be completely excluded, even not by using the Mendelian Randomization approach applied here. A previous report showed similar results in the CARDIoGRAM database with 3 SNPs.⁴⁸ We meta-analyzed all 11 SNPs from the UK-Biobank with the CARDIoGRAM database and found the same results, see **Figure 3**.⁴⁹ Our results are clinically relevant. Since there is no cure for HF, prevention is essential. Iron can be easily supplemented using oral or intravenous preparations. If this could lead to a reduction in coronary artery disease, and thereby HF, is a question worth further study.

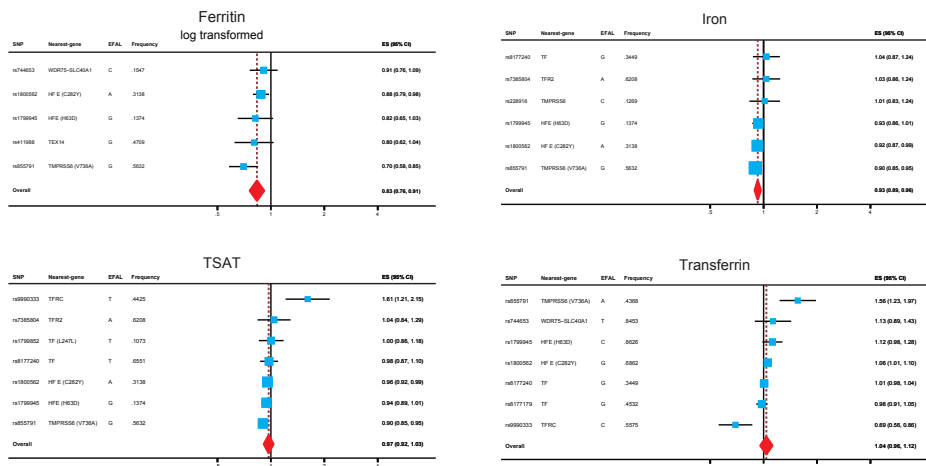


Figure 3 – Genetically determined iron marker levels and risk of coronary artery disease in a meta-analysis from UK-Biobank and CARDIoGRAM. The genetically determined levels of iron markers and risk of coronary artery disease. Overall effect is obtained using random effects meta-analysis. Odds ratios (OR) with 95% confidence intervals (CI) relate to a change per SD change in iron parameter.

Future perspectives

Anemia

The future perspective concerning anemia in HF relates to the title of **chapter 2**, is anemia still relevant in HF? The answer is a clear yes. The current guidelines emphasize the importance of anemia as a comorbidity.^{26,50} However, the guidelines can only recommend to search and treat the underlying etiology. Blood transfusions are rarely used, as these evidence is almost non-existent and the potential complications such as infections and allergic reactions are severe. No treatment has yet shown to be successful in reducing morbidity and mortality. This could have several reasons which need to be assessed in future research: 1) anemia does not lead to morbidity/mortality in HF, but is an indicator of more advanced disease, 2) the wrong patients are treated, 3) the wrong treatment is given and 4) the wrong hemoglobin level is targeted. The hemoglobin target that should be achieved in patients with HF has not been studied but is a topic of debate. We know from studies in chronic kidney disease that adverse events, such as hypertension, stroke, thrombosis and death occur when aiming at hemoglobin levels >8.1 mmol/L (13g/dL).⁵¹⁻⁵⁴ Currently, it is advised to aim at 6.2mmol/L (10g/dL).²⁷ Importantly, this is true for treatment with recombinant EPO in chronic kidney disease patients and might be different for other treatment strategies and patient populations. Therapies under development focus on compounds that more stably and physiologically mimic the function of EPO to stimulate red blood cell production such as hypoxia-inducible factor stabilizers and EPO receptor antibodies. Other therapies aim at reducing the hepcidin level, a major factor in the anemia of chronic disease. Finally, treatment with intravenous iron is promising, not exclusively for anemia, but for all patients with iron deficiency.

Iron deficiency

Hepcidin is the main iron regulating hormone. As I have shown, defective iron utilization, caused by increased hepcidin levels are present in around a fifth of patients with HF. Targeting hepcidin might therefore be a potential future strategy for iron deficiency as well, as it directly addresses the pathophysiological mechanism involved in these patients. But first, the right patient should be identified and assigned to the therapy fitting the etiology of iron deficiency in that specific patient. Although it is clear that the etiology of iron deficiency is multifactorial and diverse, the specific causal factors have not been clearly identified in patients with HF. We provided the first insights by dividing patients into defective iron utilization and low iron stores. Future studies should focus on the role of specific factors such as gastro-intestinal iron uptake and the role of

gastro-intestinal edema or the microbiome herein, or on the role of chronic low-grade inflammation. After the identification of these possible contributors, future therapies could be aimed at the specific etiology.

Our work showed that human cardiomyocytes are dependent on iron for their mitochondrial respiration and contraction/relaxation. Skeletal myocytes have a similar dependence on oxygen metabolism.³⁷ The effects of iron deficiency on exercise capacity might therefore also be caused by impaired peripheral muscle oxidative metabolism.⁵⁵ It is our goal to study the oxidative skeletal muscle metabolism in HF patients with iron deficiency and to subsequently assess the effects of intravenous iron on the observed phenotype.

Intravenous iron has shown improve signs and symptoms of patients with chronic HF and a reduced ejection fraction with iron deficiency. In an individual patient data meta-analysis, beneficial effects of intravenous ferric carboxymaltose on cardiovascular mortality and hospitalizations were observed.⁵⁶ The next step consists of studies designed to assess the effects of intravenous iron on morbidity and mortality, and other HF populations such as acute HF and HF with a preserved ejection fraction. At this moment, these studies are running. Ongoing studies assess the effects of intravenous iron on morbidity and mortality in chronic (HEART-FID, FAIR-HF2, Ironman) and acute HF (AFFIRM-AHF) and the 6-minute walking test in HF patients with a preserved ejection fraction (FAIR-HFpEF). All these studies include patients based on the conventional criteria of iron deficiency, and thus include the patients with an "isolated low ferritin". It will be interesting to see the effects of intravenous iron in this patient group in pre-specified subgroup analyses.

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