UNIVERSITAT DE BARCELONA

New pathophysiological pathways connecting heart failure and iron deficiency

Carles Diez Lopez

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Doctoral thesis dissertation presented by Carles Diez Lopez to apply for the degree of doctor at the University of Barcelona

Directed and Tutorized by Josep Comin Collet Professor of Medicine, Department of Clinical Sciences, School of Medicine, University of Barcelona, Cardiology Department, Bellvitge University Hospital, Bio-Heart Cardiovascular Diseases Research Group, Bellvitge Biomedical Research Institute and Marta Tajes Orduña, Bio-Heart Cardiovascular Diseases Research Group, Bellvitge Biomedical Research Institute.

Doctoral Program in Medicine and Translational Research

School of Medicine and Health Sciences. University of Barcelona

June 2024.

Acknowledgements

The present work would not have been possible without the help of many. Someone once told me that acknowledging is risky because it always carries the potential to hurt someone that might not feel represented. To my family, which has grown and shrunken during the working process, for being always the lowest common denominator in my life. For the ones who left. To José, which was the first to leave us: a visionary of his time who would always look for reflection and the understanding between each other; a life advice so necessary in these times. "*Avi, al final tenías razón: "este si sale bien, hará lo que quiera*". To Paulina, for her infinite love and for being the beacon of each all of us, even after her depart: she coined the expression "Work hard, play hard" even before any Yankee did; "*Yaya, tu "medicucho" se ha hecho mayor*". To Orestes, who would never miss a football game of his grandson; because he knew how to appreciate the little things that life gives us like no one else; "*Yayo, el "Capitán" te saluda, allá donde estés*". To Rosa, the last one to depart, whose life lesson was humility and silent work: Few people were as central and unassuming as she was, a fact that became clear only after she left more than a year ago. "*Yaya, descansa en paz, te recordamos cada día*".

For those with whom we share the physical world. To my mum, Anna, for her infinite and unconditional love and support, for always being an extra source of energy, and for knowing what I was thinking or how I was feeling before anyone else. To my dad, Lorenzo, who extended José and Paulina's mindset to his offspring. For his infinite capacity for work, only to provide us all the possible chances to succeed and for always pushing us forward to embrace life challenges. To Eva, my sister, because although she still doesn't know how to chew breakfast cereals, she is a life coach. Eva, it would not have been possible without you, always giving me confidence when the ground felt unsteady, and life was spinning out of control. To Pati, my love, my *"sherpa"*, who always showed me the other side of the moon. Thank you for all the time we've spent together; I know it hasn't always been easy, yet you chose to stay. To Paula, the littlest one, I am sorry your *"tiet"* is not always there but thank you for loving me so much despite that. To Cristina, Antonio, Andrea and David, for their warm welcome, for their credit and their love. For all their spontaneous messages, and again, to understand that I might not always be there

physically, but still always present. To Anna my aunt (Pati's favorite), and Adrià, who is no longer with us, but told me how to play chess and, so much more. To Pepe, Isa, and Elvira, who have adopted me as their son, and for being a harbor amid the worst storms. Pepe, thank you for taking me to Camp Nou when I was a kid to see the best club in the world, for your ham sandwiches. I will never be grateful enough.

To all my friends, who are our chosen family. To Ramon, my other (complementary) half: I wish "Labo" could see all this, he would not believe it, I am reading my thesis, and you became a father. Thank you, Labo, for showing us how to enjoy life. Your time was certainly shorter than you deserved, but we feel your presence beside us always. We will keep taking care of your mum, don't worry, she's in good hands. To Ali, Ángel, Belen, Laura and Nacho, for being a family, our family. To the *"Fridens"* who welcome me over and over again to any plan, despite my absence for months, years, ages. Incredible. To Maria (a Catalan from Madrid), Adrià (a life mentor), Marina (our writer), Anna C (my defender), Nuria L and Ingrid (our narrators) and to any of those who joined us on this long path, whom I unfairly did not mention.

To my work colleagues at Hospital Universitari de Bellvitge, probably the best possible. To Joe and Manito, to give me credit when I literally knew nothing about advanced heart failure. To Antoni Bayes, for seeing in me what nobody else could see, and to Josep Lupon, for giving me the opportunity to embrace heart failure and research as a passion. To Elena, for listening to me any time, for any reason, no matter what. To Eva Olivera, for guiding me through all the infinite grant-related bureaucracy and helping me go to the US. A life changing experience. To all the residents and fellows for asking challenging questions that make me rethink and grow.

Thanks to my directors Josep and Marta, for understanding me through all my ups and downs, for pushing me forward, and for giving me the chance to do research and become a doctor. Finally, to the patients, who are the main source of our inspiration and the reason behind everything we do.

Thank you all.

Funding

This study, titled “Blood differential gene expression in patients with chronic HF and systemic iron deficiency: pathways involved in pathophysiology and impact on clinical outcomes,” was supported by several grants: an unrestricted grant from Vifor Pharma and the Basic Research Competitive Grant in Cardiology from the Spanish Society of Cardiology (2015). Additionally, investigational support was provided by the Centro de Investigación Biomédica en Red Cardiovascular (CIBER CV 22/11/00027), Instituto Salud Carlos III, Madrid, Spain, and the CERCA Programme by the Generalitat de Catalunya and the Agència de Gestió d’Ajusts Universitaris i de Recerca (AGAUR) (2021 /SGR 00455). We extend our gratitude to the patients who contributed their samples and to the Biobank HUB-ICO-IDIBELL (PT17/0015/0024), which is part of the Spanish National Biobanks Network, for their collaboration.

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Abbreviations

ACO2: Aconitase 2

ADM: Adrenomedullin

AT2: Angiotensin II

ATP: Adenosine triphosphate

CI: Confidence interval

DMT1: Dimetal Transporter 1

ECE1: Endothelin converting enzyme

ET1: Endothelin 1

FTH1: Ferritin heavy chain

FTL1: Ferritin light chain

FTMT: Mitochondrial ferritin

GFR: Glomerular filtration rate

HAMP: Hepcidin antimicrobial peptide

HF: Heart failure

HFrEF: Heart failure with reduced ejection fraction.

HFpEF: Heart failure with preserved ejection fraction.

HR: Hazard ratio

ID: Iron deficiency

IRPS: Iron responsive proteins

LVEF: Left ventricular ejection fraction

lncRNA: Long non-coding ribonucleic acid

mRNA: Messenger ribonucleic acid

MYH7: Myosin Heavy Chain

Nor: Norepinephrine

NTproBNP: N-terminal pro b-type natriuretic peptide

PCR: Polymerase chain reaction

RNA: Ribonucleic acid

ROS: Radical oxygen species

SIRT7: Sirtuin 7

SMIM20: Small integral membrane protein 20

TFR1: Transferrin receptor 1

TSAT: Transferrin saturation

Compendium of Articles Thesis

The present thesis is based on 2 articles covering 10 different objectives.

Article 1#

Objectives 1 to 4:

Authors: Tajes M, Díez-López C, Enjuanes C, Moliner P, Ferreiro JL, Garay A, Jiménez-Marrero S, Yun S, Sosa SG, Alcoberro L, González-Costello J, García-Romero E, Yañez-Bisbe L, Benito B, Comín-Colet J.

Title: Neurohormonal activation induces intracellular iron deficiency and mitochondrial dysfunction in cardiac cells.

Journal: Cell Bioscience. 2021 May 17;11(1):89.

Doi: 10.1186/s13578-021-00605-5. PMID: 34001233; PMCID: PMC8130332.

Area of Knowledge, Impact Factor and Quartile: Science Edition – BIOCHEMISTRY & MOLECULAR BIOLOG. IF 7.133. Q1 (2021).

Article 2#

Objectives 5-8:

Authors: Díez-López C, Tajes Orduña M, Enjuanes Grau C, Moliner Borja P, González-Costello J, García-Romero E, Francesch Manzano J, Yun Viladomat S, Jiménez-Marrero S, Ramos-Polo R, Ras Jiménez MDM, Comín-Colet J.

Title: Blood Differential Gene Expression in Patients with Chronic HF and Systemic Iron Deficiency: Pathways Involved in Pathophysiology and Impact on Clinical Outcomes

Journal: Journal of Clinical Medicine. 2021 Oct 26;10(21):4937.
Doi: 10.3390/jcm10214937. PMID: 34768457; PMCID: PMC8585093.

Area of Knowledge, Impact Factor and Quartile: Science Edition – MEDICINE, GENERAL & INTERNAL. IF 4.242. Q1 (2021).

Thesis Summary

1. Title

New pathophysiological Pathways Connecting Heart Failure and Iron Deficiency

2. Introduction

Heart failure (HF) remains a global challenge, affecting millions of individuals and it is characterized by myocardial dysfunction, congestion, and multi-organ failure. It typically starts with an either episodic or persistent insult, affecting the heart's structure or function. Patients with HF experience a declining clinical trajectory, marked by diminished quality of life and adverse prognosis. Current research underscores the relevance of metabolism and myocardial energetics to understand HF syndrome. Iron deficiency (ID) affects 40-50% of patients with HF and has emerged as an important pathophysiological factor in disease onset and progression, independent from anemia. Iron is intimately related with metabolism and ID is associated with decreased contractility and myocardial remodeling, but the mechanisms that lead to ID and the specific consequences into the cardiovascular function and disease progression are broadly unknown.

We sought to investigate novel regulatory pathways influencing iron homeostasis and define the relationship between iron regulation, neurohormonal activation, metabolism, and oxidative stress both at a myocardial and systemic levels.

3. Hypothesis and Objectives

1. The high prevalence of ID in HF patients suggests that it might be an intrinsic component of the HF syndrome rather than a consequence of it.
2. HF may induce changes in the expression of genes involved in myocardial iron regulation, leading to ID and promoting disease progression.
3. Neurohormonal activation in HF may be a key factor responsible for the disruption of intramyocardial iron regulation.
4. The effect of ID on myocardial function might be mediated through mitochondrial dysfunction and increased oxidative stress.

5. ID might induce changes in the expression of specific genes involved in systemic metabolism, iron regulation, and cardiovascular function, independent of anemia.
6. Alterations in myocardial and systemic metabolism due to ID might play a significant role in the progression of HF.

To test our hypothesis, we developed the following research plan and objectives:

1. Analyze the impact of HF on iron status and regulation in the myocardium.
2. Investigate the impact of neurohormonal stimuli in HF on cardiac cell iron levels and regulatory pathways.
3. Evaluate the influence of HF neurohormonal stimuli on cardiac cell mitochondrial function and iron regulation.
4. Study alterations in mitochondrial iron regulation under neurohormonal stimuli intracellular ID.
5. Examine the systemic transcriptomic profile in HF patients with systemic ID but without anemia and identify systemic biological pathways linking HF and ID, in the absence of anemia
6. Discover candidate genes associated with iron regulation, cardiovascular function, and metabolism within these shared pathways and analyze the gene expression patterns of the identified genes related to cardiovascular disease and metabolism in the context of ID without anemia.
7. Correlate biological pathways observed in mice and cellular models with gene expression patterns in transcriptomic analyses of patient blood samples.
8. Evaluate the clinical implications of variations in genetic expression profiles of selected biological pathways and candidate genes in blood samples from HF patients.

4. Methodology

We designed a stepwise experimental approach, including an *in vivo* isoproterenol-induced mouse HF model, an *in vitro* model using H9c2 cells, and a cohort of chronic HF patients. The cells were exposed to angiotensin 2 (AT2) and noradrenaline (Nor) as main components of neurohormonal activation and for the analysis of the genetic expression signatures, we designed a cross-sectional study with patients with reduced ejection fraction from the DAMOCLES study with and without ID and without anemia. We

performed comprehensive whole blood transcriptomic analyses on a subset of patients with the GeneChip Human Gene 2.0 ST Array. Subsequently, we assessed the gene expression of selected candidate genes representatives of iron regulation, metabolism, and microvascular function in an independent cohort, using TaqMan Low-Density Array (TLDA) analysis. We conducted statistical analyses, to evaluate gene expression's association with outcomes.

5. Main results

1. We observed disrupted intramyocardial iron regulation mechanisms, including impaired iron uptake and decreased iron levels in mice treated with isoproterenol.
2. Neurohormonal activation in cardiomyocytes was shown to disrupt intracellular iron regulation, primarily affecting iron uptake and causing decreased intracellular iron levels.
3. We observed a decreased expression and protein levels of mitochondrial ferritin in our cells challenged with the neurohormonal stimuli and in the myocardium of mice stimulated with isoproterenol.
4. We observed structural alterations in mitochondrial membranes, reduced adenosine triphosphate production and increased levels of reactive oxygen species the cardiac cells.
5. Patients with HF and reduced ejection fraction, who have systemic ID without anemia, exhibit a differential transcriptomic footprint in blood samples that suggests changes in biological pathways related to systemic metabolism, oxidative stress, cellular proliferation, autophagy, and senescence.
6. Patients with HF and reduced ejection fraction, systemic ID without anemia, exhibit differential genetic expression in specific genes involved in iron regulation, metabolism, and cardiovascular function that can be measured in blood samples.
7. Patients with HF, reduced ejection fraction, and ID without anemia also show altered genetic expression profiles of mitochondrial ferritin and transferrin receptor 1, as previously underscored in animal and cellular models.

8. Genetic expression patterns of Sirtuin7, Small integral membrane protein 20, and mitochondrial ferritin associated with ID correlate with adverse clinical outcomes in HF patients with reduced ejection fraction.

6. Conclusions

1. Our findings highlight that ID might not merely a comorbidity but an integral component of the HF syndrome and takes a central role in the interaction between HF and myocardial metabolism.
2. HF induces changes in the expression of iron-related genes within the heart that provoke myocardial iron depletion in experimental models.
3. Neurohormonal activation is crucial to understand intracellular iron depletion and making cardiac cells more metabolically inefficient and vulnerable to oxidative stress.
4. ID also plays a critical role in the interplay between the heart and the broader cardiovascular system, resulting in changes in biological pathways related to systemic metabolism, oxidative stress, inflammation, cellular proliferation, autophagy, and senescence.
5. These metabolic changes happen irrespective of anemia and are represented by distinct genetic expression patterns in Sirtuin7, Small integral membrane protein 20, and Mitochondrial ferritin that were associated with adverse clinical outcomes.
6. Our findings underscore the importance of metabolism and antioxidant capacity in understanding HF pathophysiology and the central role of iron in this intricate regulation.

Resum

1. Títol

Noves vies fisiopatològiques que correlacionen el dèficit de ferro amb la insuficiència cardíaca

2. Introducció

La insuficiència cardíaca (IC) continua sent un repte global, que afecta milions de persones i es caracteritza per disfunció miocàrdica, congestió i insuficiència multiorgànica. El procés fisiopatològic de IC s'inicia amb un insult episòdic o persistent, que afecta l'estructura o la funció del cor. Els pacients amb IC experimenten una trajectòria clínica en declivi, marcada per una disminució de la qualitat de vida i un pronòstic advers. Les investigacions actuals subratllen la rellevància del metabolisme i l'energia del miocardi per entendre la síndrome d'IC. La deficiència de ferro (DF) afecta el 40-50% dels pacients amb IC i s'ha convertit en un factor fisiopatològic important en l'inici i la progressió de la malaltia, independentment de l'anèmia. El ferro està íntimament relacionat amb el metabolisme i la DF s'associa amb la disminució de la contractilitat i la remodelació del miocardi, però els mecanismes que condueixen al DF en IC i les conseqüències específiques en la funció cardiovascular i la progressió de la malaltia són àmpliament desconeguts.

En la present tesi, hem tractat de descobrir noves vies reguladores que influeixen en l'homeòstasi del ferro i definir la relació entre la regulació del ferro, l'activació neurohormonal, el metabolisme i l'estrès oxidatiu tant a nivell miocàrdic com sistèmic.

3. Hipòtesi i Objectius

1. L'elevada prevalença de la DF en pacients amb IC suggereix que podria ser un component intrínsec de la síndrome d'insuficiència cardíaca més que una conseqüència d'aquesta.

2. La IC pot induir canvis en l'expressió dels gens implicats en la regulació del ferro del miocardi, donant lloc a una deficiència de ferro i afavorint la progressió de la malaltia.
3. L'activació neurohormonal en la insuficiència cardíaca pot ser un factor clau responsable de la interrupció de la regulació intramiocàrdica del ferro.
4. L'efecte de la deficiència de ferro en la funció del miocardi podria estar mediat per la disfunció mitocondrial i l'augment de l'estrès oxidatiu.
5. La DF pot induir canvis en l'expressió de gens específics implicats en el metabolisme sistèmic, la regulació del ferro i la funció cardiovascular, independentment de l'anèmia.
6. Les alteracions en el metabolisme miocàrdic i sistèmic a causa de la DF podrien tenir un paper important en la progressió de la IC.

Per comprovar la nostra hipòtesi, hem desenvolupat el següent pla de recerca i objectius:

1. Analitzar l'impacte de la insuficiència cardíaca en l'estat i la regulació del ferro en el miocardi.
2. Investigar l'impacte dels estímuls neurohormonals en la IC sobre els nivells de ferro de les cèl·lules cardíaques i les seves vies reguladores.
3. Avaluar la influència dels estímuls neurohormonals de la IC en la funció del ferro mitocondrial de les cèl·lules cardíaques.
4. Estudiar les alteracions en la regulació del ferro mitocondrial sota estímuls neurohormonals de deficiència intracel·lular de ferro.
5. Examinar el perfil transcriptòmic sistèmic en pacients amb insuficiència cardíaca amb deficiència de ferro sistèmica però sense anèmia i identificar les vies biològiques sistèmiques que vinculen la insuficiència cardíaca i la deficiència de ferro, en absència d'anèmia.
6. Revelar gens candidats associats amb la regulació del ferro, la funció cardiovascular i el metabolisme dins d'aquestes vies compartides i analitzar els patrons d'expressió gènica dels gens identificats relacionats amb la malaltia cardiovascular i el metabolisme en el context de la DF sense anèmia.

7. Avaluar les implicacions clíniques de les variacions en els perfils d'expressió genètica de les vies biològiques seleccionades i els gens candidats en mostres de sang de pacients amb insuficiència cardíaca.
8. Correlacionar vies biològiques observades en ratolins i models cel·lulars amb patrons d'expressió gènica en anàlisis transcriptòmiques de mostres de sang de pacients.

4. Metodologia

Hem dissenyat un enfocament experimental pas a pas, que inclou un model de ratolí induït per isoproterenol *in vivo*, un model *in vitro* amb cèl·lules H9c2 estimulades amb noradrenalina i angiotensina i una cohort de pacients amb IC crònica. Per a l'anàlisi de les signatures d'expressió genètica, vam dissenyar un estudi transversal amb pacients amb fracció d'ejecció reduïda de l'estudi DAMOCLES amb i sense dèficit de ferro, però sense anèmia. Hem realitzat anàlisis transcriptòmiques completes de sang sencera en un subconjunt de pacients amb GeneChip Human Gene 2.0 ST Array. Posteriorment, es va avaluar l'expressió gènica de gens candidats seleccionats representants de la regulació del ferro, el metabolisme i la funció microvascular en una cohort independent, mitjançant l'anàlisi TaqMan Low-Density Array (TLDA). Hem realitzat anàlisis estadístiques per avaluar l'associació de l'expressió gènica amb els resultats.

5. Resultats principals

1. Hem observat alteracions en els mecanismes de regulació del ferro intramiocàrdic, inclosa la captació deteriorada del ferro i la disminució dels nivells de ferro en ratolins tractats amb isoproterenol.
2. Hem evidenciat que l'activació neurohormonal en cèl·lules cardíaques altera la regulació intracel·lular del ferro, afectant principalment la captació de ferro i provocant una disminució dels nivells intracel·lulars de ferro.
3. Hem observat una disminució de l'expressió i dels nivells de proteïnes de ferritina mitocondrial a les nostres cèl·lules tractades amb estímul neurohormonal i en el miocardi de ratolins estimulats amb isoproterenol.

4. Hem observat alteracions estructurals a les membranes mitocondrials, una reducció de la producció de trifosfat d'adenosina i un augment dels nivells d'espècies reactives d'oxigen a les cèl·lules cardíaques.
5. Els pacients amb IC i fracció d'ejecció reduïda, que tenen ID sistèmica sense anèmia, presenten un perfil transcriptòmic diferencial a les mostres de sang que suggereix canvis en les vies biològiques relacionades amb el metabolisme sistèmic, l'estrès oxidatiu, la proliferació cel·lular, l'autofàgia i la senescència.
6. Els pacients amb IC i fracció d'ejecció reduïda, ID sistèmica sense anèmia, presenten expressió genètica diferencial en gens específics implicats en la regulació del ferro, el metabolisme i la funció cardiovascular que es poden mesurar en mostres de sang.
7. Els pacients amb IC, fracció d'ejecció reduïda i DI sense anèmia també mostren perfils d'expressió genètica alterats de la ferritina mitocondrial i del receptor 1 de transferrina, tal com s'ha subratllat anteriorment en models animals i cel·lulars.
8. Els patrons d'expressió genètica de Sirtuin7, la proteïna integral de membrana petita 20 i la ferritina mitocondrial associada a la ID es correlacionen amb resultats clínics adversos en pacients amb IC amb fracció d'ejecció reduïda.

6. Conclusions

1. Les nostres troballes posen de manifest que la DF pot no ser només una comorbiditat, sinó un component integral de la síndrome d'IC, i podria tenir un paper central en la interacció entre l'IC i el metabolisme del miocardi.
2. L'IC indueix canvis en l'expressió de gens relacionats amb el ferro miocàrdic que provoquen l'esgotament del ferro del intramiocàrdic en models experimentals.
3. L'activació neurohormonal és crucial per entendre l'esgotament intracel·lular de ferro i fer que les cèl·lules cardíaques siguin més metabòlicament ineficients i vulnerables a l'estrès oxidatiu.
4. La DF també té un paper crític en la interacció entre el cor i el sistema cardiovascular més ampli, donant lloc a canvis en les vies biològiques relacionades amb el metabolisme sistèmic, l'estrès oxidatiu, inflamació, la proliferació cel·lular, l'autofàgia i la senescència.

5. Aquests canvis metabòlics es produeixen independentment de l'anèmia i estan representats per diferents patrons d'expressió genètica amb implicacions pronòstiques en el receptor de la Sirtuina 7, la Proteïna integral de membrana petita 20 i la ferritina mitocondrial.
6. Les nostres troballes subratllen la importància del metabolisme i la capacitat antioxidant per entendre la fisiopatologia de la IC així com el paper central del ferro en aquesta regulació complexa.

Introduction

Heart failure (HF) is a multifaceted clinical syndrome characterized by congestion that develops from underlying structural and/or functional abnormalities in the heart (1,2). The inability to meet the systemic demands of circulation triggers an ongoing, silent increase of intracardiac pressures and/or insufficient cardiac output during rest and/or physical exertion. From a clinical perspective, HF results in diminished quality of life for patients, marked by recurrent hospitalizations, reduced physical capacity, and emotional distress (3,4).

HF remains a worldwide leading cause of morbidity and mortality among the cardiovascular disease spectrum (5). Almost 1 million new cases are reported each year in the United States (5), and the latest ESC survey calculated a median annual incidence of 1 to 4 cases per 1000 person-years and a prevalence of 10 to 30 cases per 1000 persons (6). The prevalence of HF has been on the rise due to several factors, including an aging population, increased survival from acute cardiovascular events, and the global spread of lifestyle-related diseases like obesity and type 2 diabetes (7). Ischemic heart disease, hypertension, and degenerative valve disease remain the leading causes of HF. Notably, these conditions are significantly associated with traditional cardiometabolic risk factors such as diabetes, obesity, and smoking (8). Healthcare systems are challenged by the socioeconomic implications of HF, including the direct costs from hospitalizations, medical treatments, and outpatient care, and indirect costs due to lost productivity and the burden on caregivers(9,10). Moreover, the variations in care, access to treatments, and health infrastructure across countries further complicate the HF scenario. Prevention strategies aimed at promoting heart-healthy lifestyles, early intervention in at-risk populations, and improving access to guideline-directed medical therapies are crucial to a reduce the incidence and impact of the HF syndrome(1).

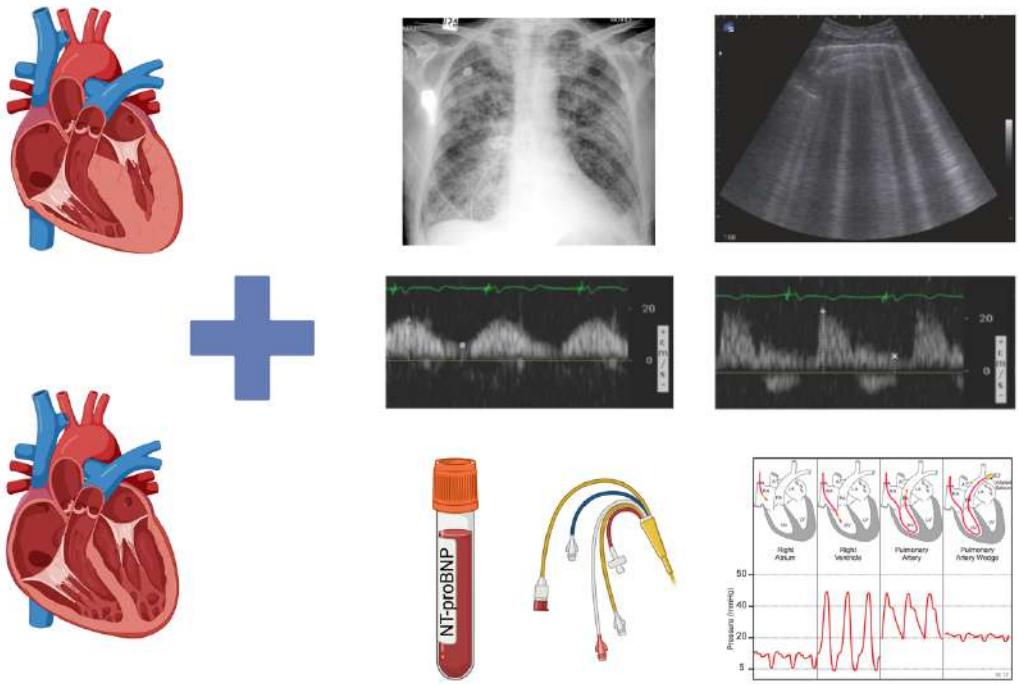
HF is a product of a complex interplay of multiple mechanisms involving altered heart coupling with systemic and pulmonary circulations, and different degrees of multiorgan dysfunction. These components intertwine into the disease’s pathophysiology and influence its management from diagnosis to treatment (11). The advent of new pharmacological and device treatments for HF over the past three decades, have largely

contributed to substantial reductions in morbidity and mortality (12,13). However, there is a significant subset of patients who either do not tolerate or respond to these interventions, progressing to advanced disease stages marked by severe HF symptoms, recurring decompensations, and ultimately, death (13).

1. Diagnosis and Classification of Heart Failure

HF is defined as a progressive disease, diagnosed in the presence of clinical signs or symptoms that result from cardiac functional or structural abnormalities that provoke congestion (14). This detail is crucial, as it underscores the relevance of identifying the presence of congestion as a marker of high cardiac filling pressures, for the diagnosis of HF (Figure 1). In this regard, the identification of natriuretic peptides has played a pivotal role in the understanding of HF. These peptides comprise a group of bioactive hormones released by cardiac cells as a response to high filling pressures inside the heart that ultimately result in diuretic and natriuretic effects, metabolic and immune response regulation within the cardiovascular system (15–17). The importance of natriuretic peptides is such that today, the detection and monitoring of natriuretic peptides has become an essential component for the diagnosis and management of HF, enabling healthcare providers to better assess disease severity and provide insights into more tailored treatment strategies(18).

Figure 1. Contemporary management of HF: HF is diagnosed in the presence of clinical signs and symptoms of congestion with the evidence of functional or structural heart abnormalities. The measurement of B natriuretic peptides, venous and lung ultrasound have become crucial for the diagnosis, avoiding the classical use of invasive hemodynamic data.



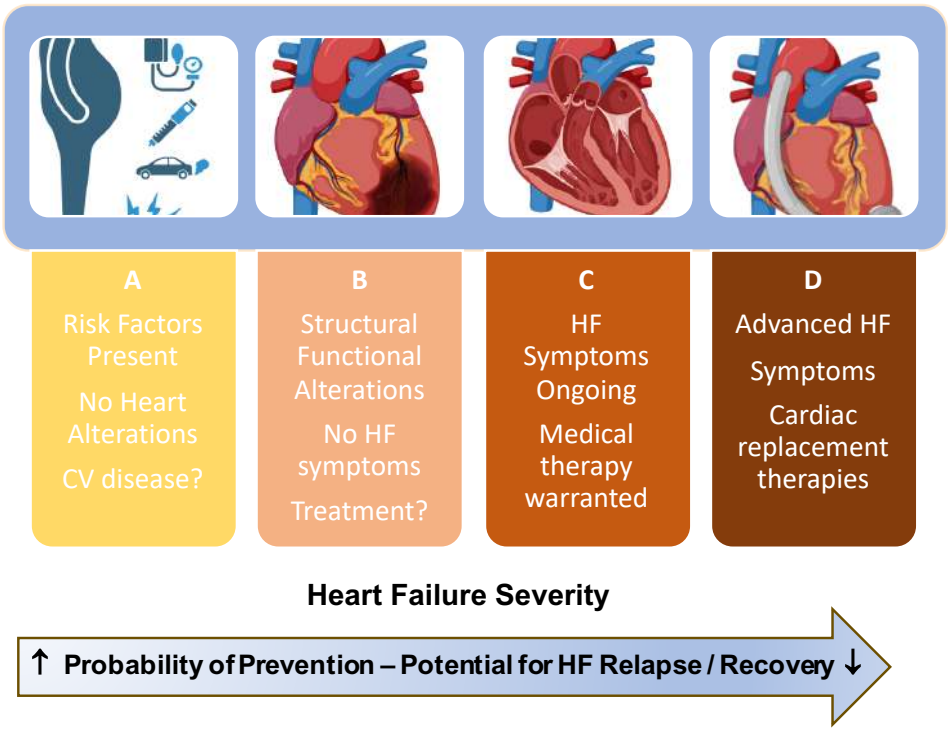
Images adapted from Chatterjee et al.(17), Swan HJC, et al.(19), Beaubien-Souligny, W, et al.(20), and modified from (21–23) and generated with BioRender ®.

Similarly, the stratification of HF has traditionally been based on evaluating factors such as symptom severity, frequency of hospital admissions, and foremost, the left ventricular ejection fraction (LVEF): HF with reduced LVEF <40%, mildly reduced LVEF: 50-40%, and preserved LVEF: >50%. (1,2). However, this classification has proven to be inaccurate, as there is a weak correlation with patients' symptoms, and outcomes and patients may exhibit varying ejection fraction levels throughout the disease course. Moreover, HF pharmacological treatment strategies have shown potential effectiveness across diverse patient phenotypes defined by ejection fraction (13,24,25). Indeed, HF is a more complex syndrome that involves the whole cardiac structure and function and includes the crosstalk between the different organs and systems within the cardiovascular system.

As a result, the current understanding of HF involves the recognition of the disease across different stages of cardiovascular health, leading to a classification of the syndrome into four distinct phases some of which precede the onset of clinical manifestations (Figure 2). This conceptualization explains the complex nature of HF and underscores its role and interactions within the continuum of the cardiovascular system and incorporates the potential for implementing preventive measures across all disease stages, aiming to increase the diagnosis and treatment for symptomatic patients and identifying high-risk individuals to prevent the development of overt clinical manifestations (2).

Figure 2. New classification within the comprehensive spectrum of HF disease continuum. Despite the diagnosis of HF is made in the presence of signs of congestion (Stages C and D), the current understanding of the HF syndrome includes the acknowledgment of prior stages of subclinical HF in which patients are either at risk of developing structural changes which might lead to the development of overt HF (Stage A) or even compensated stages of cardiac abnormalities that precede the development of over HF (Stage B).(2)

This novel concept is essential as it underscores HF as a dynamic condition, influenced by a myriad of interrelated factors that collectively determine patient outcomes. Finally, it further enables both clinicians and patients to determine and administer the most appropriate and tailored interventions, acknowledging that varied fundamental factors can be addressed to prevent, alleviate, or even reverse disease progression for each patient at a determined stage of the disease. This is particularly relevant for patients with subclinical valvular heart disease, systemic diseases and treatments with potential adverse effects within the cardiovascular system and to complex conditions like genetic



cardiomyopathies, some of them characterized by their tendency for progression and an elevated risk of sudden cardiac death. Remarkably, all these conditions are usually characterized by a high morbidity and mortality when the HF are already present. Therefore, the ability to further understand the fundamentals of heart disease and the

determinants of progression will enable to initiate preventive strategies into high-risk patients and situation, in order to avoid the development of cardiac dysfunction and congestion.

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2. State of the art in heart failure pathophysiology

Beyond structural and functional abnormalities in the heart, the current perspective on HF pathophysiology is based on biological interactions between the heart and the rest of the cardiovascular system that culminate in fluid retention and inadequate oxygen and nutrient delivery. HF is initiated as an initial either episodic or persistent insult tackling the myocardial structure and/or function. From an hemodynamical standpoint, the persistent elevation in cardiac preload and/or afterload initiates a cardiovascular uncoupling that result in clinical HF. However, this model remains incomplete, as a substantial proportion of HF cases commence in the absence of primary myocardial alterations, pointing to alternative triggers from additional cardiovascular or external sources.

This concept is emphasized through the secretion of various peptides within distinct segments of the cardiovascular system, including the myocardium and the endothelium. These peptides exhibit pleiotropic regulatory properties, influencing different organs and systems that collectively form the neurohormonal axis. The neurohormonal hypothesis in HF states that the effects of long-term and sustained maladaptive secretion of neurohormones such as renin, angiotensin, aldosterone and catecholamines throughout the cardiovascular system is the main driver of fluid retention, myocardial and vascular malfunction, and adverse remodeling (26,27). The sympathetic nervous system (SNS) and the renin angiotensin and aldosterone system (RAAS) are the main representatives of this hypothesis yet the complexity of the neurohormonal axis transcend these elements, encompassing a broader and bidirectional interaction between different components and biological processes within the human organism, as we will discuss further on (28–30)

2.1 The sympathetic nervous system activation

Catecholamines are a group of biochemical elements that act as both neurotransmitters and hormones that participate in regulating various physiological processes such as heart rate, blood pressure and stress response. These compounds include adrenaline (also known as epinephrine), noradrenaline (or norepinephrine), and dopamine, which are mainly synthesized in the adrenal glands and in specific neurons. Their physiological effects are mediated through binding to adrenergic receptors distributed widely

throughout the body but with nuanced and organ-specific actions mediated by distinct receptor subtypes. In this regard, sympathetic overactivation is one of the main drivers of HF As a matter of fact, individuals with chronic HF exhibit significantly increased adrenaline and noradrenaline (NOR) plasma levels, and this catecholamine spillover correlate with worse HF symptoms and adverse outcomes (31–35).

In HF, the overactivation of the sympathetic nervous system stem from different mechanisms such as the reduction in baroreflex sensitivity and the intensified activity of sympathetic afferents. As a result, in the acute phase, catecholamines augment ventricular contractility, heart rate, and induce systemic vasoconstriction to maintain blood pressure and perfusion. However, this compensatory physiology increases the myocardial afterload and the myocardial oxygen consumption inducing progressive cardiac decline. In addition, it induces renal vasoconstriction at the efferent arteriole to preserve the glomerular filtration at the expense of more glomerular damage and reduced renal blood flow. This in turn, activates the RAAS, amplifying this abnormal signaling pathways which culminate in increased fluid and sodium retention and myocardial adverse remodeling.

At the cardiomyocytes, the activation of β1 adrenergic receptors, predominantly Nor, increases the production of cyclic adenosine monophosphate (cAMP) and the activation of protein kinase A, which induces the phosphorylation of phospholamban and the ryanodine receptor and results in enhanced Ca^{2+} uptake into the sarcoplasmic reticulum stimulating the process of contraction and relaxation (36). Per contra, chronic sympathetic stimulation triggers myocardial hypertrophy and increases oxygen consumption, exceeding the contra regulatory mechanisms and leading to intracellular Ca2+ uncoupling and impairing the myocyte contraction-relaxation cycle (37). Subsequently, different oxidation products derived from catecholamines increase the oxidative stress, impairing metabolism and stimulating proapoptotic pathways that lead to progressive myocardial dysfunction and remodeling (38,39).

Beta blockers play a crucial role in the treatment of HF. They contribute to the management of HF by reducing heart rate and left ventricular wall stress, resulting in decreased myocardial oxygen consumption. Additionally, beta-blockers exhibit benefits

such as enhancing the intrinsic contractile function of cardiomyocytes and improving myocardial energetics, possibly through a favorable shift in substrate utilization and reduced apoptosis(40). In fact, the use of beta-blockers is associated with beneficial myocardial remodeling, leading to reduced decompensations and improved survival rates(41) and recently, autonomic nervous system modulatory based device therapies have shown to be beneficial in a subset of patients with chronic HF(42).

2.2 The renin angiotensin aldosterone system activation

The renin–angiotensin–aldosterone system (RAAS) plays a central role in HF pathophysiology. The dysfunctional activation of this system is initiated by the release of renin from the juxtaglomerular apparatus, which is triggered by stimuli such as decrease in renal blood flow, affecting the glomerular filtration, reducing the levels of sodium reaching the macula densa and activating the juxtaglomerular apparatus. Renin then converts angiotensinogen into angiotensin 1, which is further converted to angiotensin 2 (AT2) by the angiotensin-converting enzyme, stimulate aldosterone secretion and is cleaved into other peptides with vasoconstrictive properties. In addition, other downstream effects including microvascular vasoconstriction and generation of radical oxygen species (ROS), activate a series of secondary signaling pathways that regulate inflammation, intracellular metabolism and substrate use, generating myocyte hypertrophy and apoptosis leading to myocardial remodeling and HF progression (43–45,37). Indeed, the degree of this atypical activation of the RAAS system correlates with an increased HF hospitalizations and mortality (46).

However, beyond all these individual functions, both: SNS and RAAS neurohormonal activation, are deeply and intricately interconnected through numerous secondary effectors, spanning across multiple sites within the cardiovascular system. For instance, AT2 enhances adrenergic pre and postsynaptic transmission and the production of vasopressin at the hypothalamus (47), causing an increase in water and salt intake and vasoconstriction of the arterioles (48,49). On the other hand, the sympathetic nervous system reinforces RAAS by increasing local renin and AT2 release at the juxtaglomerular apparatus enhancing the reabsorption of sodium and the use of β-adrenergic blockers inhibits renin secretion (32,50). All these maladaptive processes, initiated by either

singular or combined stimuli involving myocardial stretch and neuroendocrine activation, result in myocyte hypertrophy, an imbalance in the collagen synthesis at the extracellular matrix and contractile dysfunction (51,52). This leads to gradual myocyte loss and fibrosis that leads to progressive ventricular enlargement and impairment of both contractile and relaxation processes, a phenomenon known as ventricular remodeling (51–53), As cardiac filling pressures increase and cardiac output gradually decreases, this remodeling becomes clinically evident when all counterregulatory mechanisms are overwhelmed, culminating in the manifestation of clinical HF.

Arguably, the most significant proof highlighting the significance of neurohormonal activation is the effectiveness of pharmacologic interventions tackling the neurohormonal modulation, especially in patients with systolic HF (reduced ejection fraction)(54). Moreover, a substantial proportion of patients (20-25%) exhibit HF recovery, a phenomenon characterized by myocardial reverse remodeling and symptom resolution, consequent to the initiation and sustained administration of targeted medical therapy (55–58). This is further reinforced by the fact that some individuals present recurrence of HF symptoms and myocardial dilatation and dysfunction upon discontinuation of the medications(59). However, despite the growing range of therapeutic interventions, approximately 10-15% of these patients show no clinical improvement after treatment initiation and continue to progress to advanced disease stages, where the therapeutic options are scarce and mostly relegated to replacement therapies like ventricular assist devices or cardiac transplantation (1,2,60).

This suggests the presence of numerous pathophysiological pathways and interactions that are yet to be uncovered, that collectively shape the clinical presentation for each individual at a specific moment along the disease trajectory.

2.3 New insights on the interactions within the cardiovascular system

Current evidence advocates for a more integrated view highlighting that beyond hemodynamic stress, persistent inflammation and aberrant metabolism are crucial elements in perpetuating multiorgan damage by activating biological pathways that promote hypertrophy, fibrosis, and apoptosis within the cardiovascular network. In this

regard, microvascular dysfunction, as exemplified by endothelial dysfunction, is one of the principal mechanisms in this novel understanding of the HF physiology.

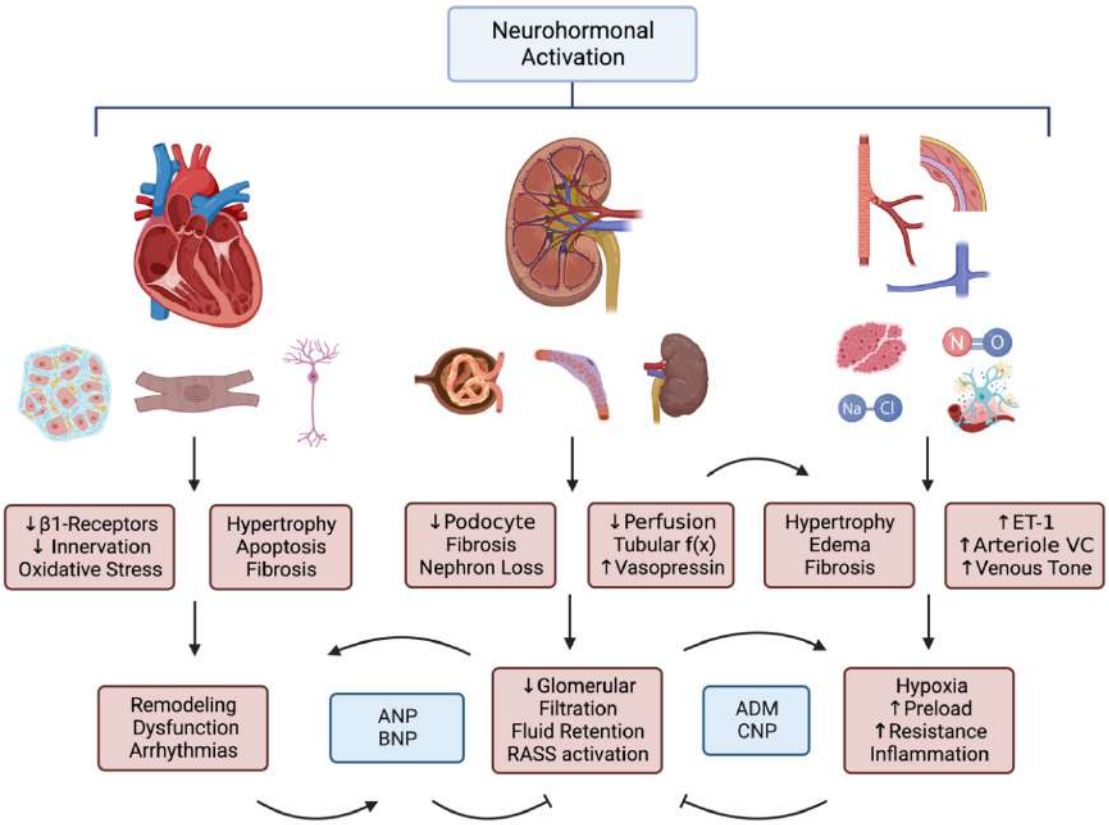
The endothelium is responsible for preserving vascular integrity, overseeing and regulating both the structural and functional aspects of the vessel wall. Endothelial dysfunction is characterized by a compromised vasodilatory efficacy and is thought to be related to various cellular, molecular, and hemodynamic factors and regulated by mediators such as endothelial nitric oxide synthase, vascular endothelial growth factor, neurohormonal activation and the presence of ROS (61). Among these, the natriuretic peptides are thought to be intimately related with endothelial integrity and function. Atrial natriuretic peptide (ANP) and b-type natriuretic peptide (BNP) are predominantly secreted by cardiomyocytes at the atria and the c-type natriuretic peptide (CNP) in endothelial cells. These peptides act as active hormones that rest stored in dedicated intracytoplasmic granules until their activation by stimulus such as muscle stretching, hormones such as AT2 or endothelin 1 (ET1) and inflammation. In the kidneys, natriuretic peptides lead to a decrease in afferent arteriole tone and an increase in efferent arteriole tone, leading to elevated glomerular filtration and blood flow, resulting in natriuresis and diuresis and reduced renin release, reduce aldosterone synthesis and antidiuretic hormone secretion. Interestingly, at the endothelium of the nephrons, these peptides influence the vessel tone resulting in lowered blood pressure and increased capillary permeability, and at the nervous system level, they reduce renal sympathetic activity and pulmonary chemoreceptor and baroreceptor activity (16,62). A significant stride in HF pharmaceutical treatment involves neprilysin inhibition. Neprilysin is an enzyme cleaving peptides like bradykinin, adrenomedullin (ADM), endothelin, and natriuretic peptides. Combining angiotensin receptor antagonism with neprilysin inhibition enhances natriuretic peptide vasodilatory effects and curbs angiotensin system counter regulation. The dual-action drug antagonizing the RAAS and inhibiting the degradation of natriuretic peptides, has markedly improved the prognosis of patients with HF and reduced ejection fraction.(63,64).

The natriuretic peptide-signaling pathway involves binding to the guanylate cyclase complex, stimulating cyclic guanosine monophosphate (cGMP) synthesis. Which in turn

interacts with protein kinase G and modulates phosphodiesterases involved in nitric oxide (NO) synthesis. Previous population-based studies suggested a protective effect of the NP-cGMP pathway, associating increased circulating cGMP and BNP with cardiovascular metabolic dysfunction, HF and mortality(65,66). Moreover, Within these lines, recent findings from a randomized trial assessing the efficacy of a novel oral soluble guanylate cyclase stimulator in patients with low ejection fraction (less than 45%), highlight the potential benefits of activating this integrated cardiovascular and metabolic pathway in reducing HF decompensations and cardiovascular mortality(67).

As a counterpart, the peptide natriuretic C (CNP) is mainly synthesized within the endothelium and functions as a counterregulatory agent for the RAAS vasoconstrictive, pro-hypertrophic and profibrotic effects (68,69). Similarly, Adrenomedullin (ADM) is a potent vasoactive peptide with a widespread expression in the cardiovascular system. Its main role is attributed its vasodilatory effects, but it also has anti-inflammatory properties and participates in critical cellular processes such as energy metabolism and signal transduction. Finally, ET1 is widely recognized as a principal indicator of endothelial and microvascular function, and the increase of its levels is associated with poor clinical outcomes in HF and other cardiovascular diseases (70,71). Figure 3 summarizes the current perspectives on the interconnections occurring between the different organs and systems within the cardiovascular system.

Figure 3. Comprehensive model of HF within the cardiovascular system. Neurohormonal activation is the main driver of HF, yet its consequences extend beyond its effect in cardiac structure and function through direct and indirect effects. At the myocardium, it promotes increases oxygen consumption leading to oxidative stress, causing hypertrophy and apoptosis. Myocardial cells secrete natriuretic peptides with diverse functions amid the cardiovascular system and specifically in the kidneys, where they compensate the effect of classical neurohormonal activation effects through the RAAS and the sympathetic nervous system. The endothelium at the cardiovascular system is interconnected with the rest of the heart and the kidneys through additional messengers like adrenomedullin and endothelin which also try to compensate the results of classical neurohormonal activation: fluid retention and increased vascular tone. These effector pathways, create a continuous feedback loop that further activates the neurohormonal axis and leading to progressive multiorgan dysfunction



ADM: Adrenomedullin. ANP: A natriuretic peptide. BNP: B natriuretic peptide. CNP: C natriuretic peptide. ET-1: Endothelin 1. Created with BioRender.

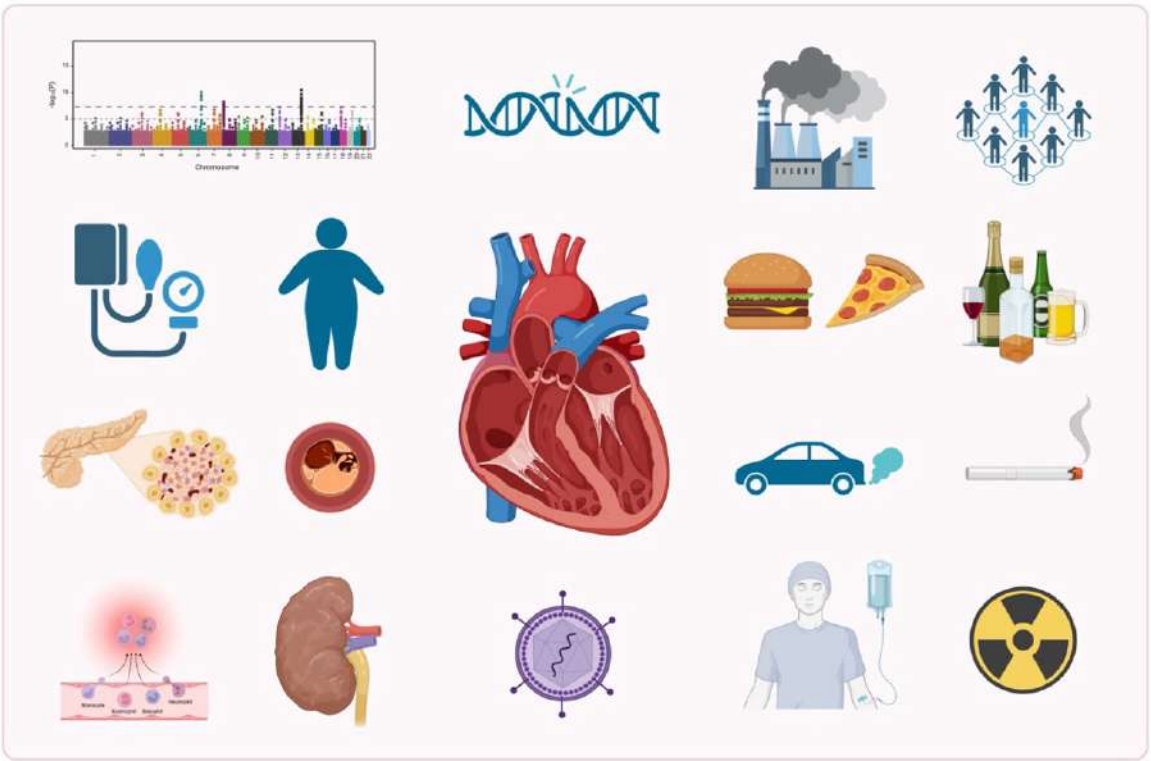
2.4 Towards a more comprehensive model of heart failure

Despite established concept of HF orbits around persistent congestion and sustained maladaptive neurohormonal activation, it falls short in explaining the singular disease phenotypes and trajectories among different individuals with otherwise similar heart structural and functional abnormalities, suggesting there might be additional factors to take into consideration. In this regard, metabolism has become central in the current understanding of HF pathophysiology. Current research highlights the significance of additional individual genetic and environmental risk factors, emphasizing the need for comprehensive phenotypic analyses of patients (72). For instance, in patients with preserved ejection fraction, the phenotypic clustering based on clinical characteristics reveals significant differences in clinical aspects like age, gender, and comorbidity burden that have a profound influence on symptoms burden and disease management and prognosis (73). On the other hand, patients with non-ischemic dilated cardiomyopathies, present with different pathophysiological footprints such as profibrotic or metabolic subtypes, which might become decisive for detecting patients at a higher risk for arrhythmias and disease progression and thus, guide treatment strategies (74,75).

There is a growing recognition on factors such as cardiovascular risk factors such as hypertension or diabetes mellitus and additional elements such as aging and other metabolic disorders linked to lifestyle and environmental pollution, contribute to HF onset and progression (76). In a similar fashion, diverse genetic components related to myocardial structure, cardiovascular function and metabolism are also becoming relevant actors in this interaction (65,77,78). This concept is further supported by evidence of genetic differences affecting distinct hemodynamic phenotypes and the regulation of cytoskeletal development and organization and large-scale longitudinal cohort studies evidencing a shared genetic background between risk factors and different specific HF phenotypes (72,79). This entails that instead of merely acquiring a risk factor accidentally and following a specific trajectory in the disease process, an individual's genetic background could be influencing both the occurrence and the following clinical pathway for this factor in disease phenotype and progression.

Nonetheless, there is significant heterogeneity even among individuals with the same genetic predispositions, indicating that other modifying factors, either epigenetic or microenvironmental, are also at play. In addition, common signaling pathways—such as inflammation, oxidative stress, and apoptotic pathways—are activated in response to different environmental insults, regardless of the precipitating factor (80–82). Thus, irrespective of their origin, these diverse factors collectively contribute to decreased myocardial resilience, increased susceptibility to cardiac injury, and impaired cellular repair mechanisms (Figure 4). As we explore the factors contributing to decreased myocardial resilience and their implications, it is essential to consider the diverse comorbidities associated with HF, given their well-established interconnected roles in disease progression. The consequences of this extend a far the purpose of the present thesis but will be important to be considered when interpreting the results and understanding the potential implications of the newly identified disease pathophysiology mechanisms.

Figure 4. Elements involved in the development of HF: from environmental to individual risk factors. From hemodynamic alterations to metabolic disruptions and inflammatory



responses. During the recent years, there has been a consistent shift in the traditional emphasis on central hemodynamics as the sole determinant of HF, towards a more comprehensive model of multiple interactions between individual genetic, exposure to different cardiovascular toxic compounds, lifestyle and other environmental factors that interact to shape the HF phenotype. Generated with BioRender.

2.5 About comorbidities in heart failure

Patients with HF present with a significant number of both cardiac and non-cardiac concurrent conditions that have an impact clinical symptoms and outcomes. More than half of HF patients concurrently present with common comorbidities, including obesity, diabetes mellitus, liver disease, hypertension, chronic kidney disease, and chronic obstructive pulmonary disease, among others (83,84). These comorbidities possess unique characteristics and can trigger specific adverse effects within the cardiovascular system, ultimately impacting the HF phenotype and trajectory. As a result, the current insight into the pathophysiology of HF involves considering these comorbidities as integral components of the HF syndrome rather than isolated factors trigger metabolic changes that affect the heart.

Obesity and overweight, affect more than a third of the global population today, and are strongly linked to an increased risk of incident HF independent of other cardiovascular risk factors. Obese patients with HF have a higher symptom burden, a worse quality of life and a higher risk of hospitalizations and mortality(85). Rather than being a separate or parallel process, obesity impairs cardiovascular health through multiple mechanisms, impacting several related downstream metabolic pathways promoting the advent of cardiovascular risk factors, including elevated blood pressure, dyslipidemia, and hyperglycemia. Diabetes mellitus is an important predictor of HF, independent of concomitant hypertension or coronary artery disease. In patients with HF, diabetes mellitus exacerbates mechanisms underlying atherosclerosis and contributes significantly to cardiovascular morbidity and mortality. Insulin resistance develops before prediabetes or diabetes mellitus, progressing alongside worsening hyperglycemia, leading to endothelial dysfunction and inflammation (86).

Liver dysfunction is often an overlooked comorbidity in HF. Persistent venous congestion affects the portal and hepatic venous circulation, causing hepatocellular injury, which can progress to fibrosis and ultimately cirrhosis, leading to portal hypertension, bleeding, and thrombosis(87). Additionally, common HF risk factors such as alcohol consumption, obesity, hyperlipidemia, and dysglycemia contribute to the development of both fatty liver and HF. These metabolic pathways and their interactions with metabolic risk factors play a pivotal role in understanding the complexities of the interplay between the liver and the heart. In this context, liver X receptors are involved in the regulation of metabolism and inflammatory pathways. In the myocardium, these receptors are associated with the activation of protective pathways involving neoangiogenesis, metabolic adaptation, and the prevention of hypertrophy and apoptosis (88,89). However, in the presence of prolonged metabolic abnormalities, there seems to be a resistance to the biological pathways that contribute to the progression of both liver disease and the development of myocardial dysfunction leading to HF progression(90). Moreover, the interactions between the liver and other organs and systems include conditions such as hepatorenal syndrome, hepatopulmonary syndrome, or portopulmonary hypertension, with substantial adverse effect on the overall prognosis. Among these lines, the interactions between the heart and lungs are also not limited to anatomical proximity and pulmonary circulation physiology. Patients with chronic lung disease are at a higher risk of hypoxemia and recurrent bronchial infections that leads to chronic systemic inflammation(91). Furthermore, hypoxemia exacerbates right ventricular myocardial dysfunction, worsening symptoms and intensifying systemic congestion, which contributes to progressive multiorgan dysfunction and worsens the overall prognosis (92,93). Again, all these processes collectively contribute to inflammation and endothelial dysfunction (94,95).

Renal dysfunction stands as one of the most significant independent risk factors for poor outcomes and all-cause mortality in patients with HF (96). The cardiorenal syndrome vividly illustrates the concept of interconnected pathophysiological processes in HF. In brief, in the presence of elevated venous pressure and reduced cardiac output, fosters the secretion of renin and the activation of the sympathetic nervous system. This leads to vasoconstriction and glomerular filtration, which activates secondary pathways to increase sodium and water retention, creating further congestion and increased

glomerular stress (96). However, this connection extends beyond this straightforward view. Endothelial cell dysfunction also plays a central role in the cardiorrenal syndrome. However, this transcending the classical understanding of reduced nitric oxide bioavailability and impaired vascular relaxation. The endothelial cell layer of the vasculature acts as a semipermeable barrier, facilitating regulated exchange of fluids, molecules, and cells, and it plays a crucial role in maintaining vascular health. In HF and chronic kidney disease, chronic excessive stretch on blood vessels, induce endothelial permeability, provoke inflammatory responses, and trigger oxidative stress (97). Chronic inflammation and the oxidative stress further contribute to left ventricular hypertrophy, interstitial fibrosis, impaired oxygen delivery, and increased myocardial demand, thereby initiating myocardial remodeling and dysfunction (98). Patients with HF demonstrate dysfunction in both large vessels and microvasculature, evidenced by reduced cardiac microvascular density and upregulation of endothelial adhesion molecules and pro-oxidative regulators in myocardial biopsies(97).

Additionally, anemia, is a frequent comorbidity in patients with HF and chronic kidney disease. Interestingly, ID also frequently coexists with chronic kidney disease and HF. In fact, the prevalence of ID increases with worsening renal function and low hemoglobin levels, and both independently correlate with an increased risk of death (99). However, it is important to note that anemia and ID are distinct yet interconnected processes with common pathophysiological pathways. In HF, anemia stems from various factors such as nutritional deficiencies, bleeding, inflammation, and impaired kidney erythropoiesis, and thus it is more prevalent among elderly and chronic kidney disease patients(100). In fact, chronic cardiovascular disease and inflammation, independently correlate with these syndromes where the endothelium has a crucial role. Indeed, despite anemia correlates with lower iron levels, the functional and prognostic implications of ID extend beyond anemia alone. In this thesis, we will explore how iron ID is intimately linked with pathophysiological processes such as metabolism and microvascular disease and we will challenge the conventional classification of ID as a mere comorbidity. Our focus will be on ID as an active contributor to the pathogenesis and progression of HF, examining its impact on both cardiac function and the broader cardiovascular system.

3. Iron deficiency in heart failure

ID is a prevalent global health disorder, impacting more than 2 billion individuals worldwide. In lower-income regions, it often arises from inadequate dietary intake and predominantly affects children, adolescents, and the elderly. Women, due to increased needs are particularly susceptible to ID due to increased hemoglobin loss, while chronic diseases also play a significant role in the rest of the population. (101,102). Iron is an essential component serving as a vital component in the synthesis of coenzymes that include iron-sulfur (Fe-S) complexes, playing a pivotal role within the mitochondrial electron chain (ETC) and many antioxidant enzymes, because it is a cofactor for redox reactions(103). Additionally, it is integral to hemoglobin and myoglobin, addressing the fundamental aspects of metabolism: oxygen delivery and cellular respiration (103). Therefore, the effects of ID are expected to impact throughout all the organs and systems, particularly those with higher metabolic requirements.

ID is widely recognized as a prevalent condition in HF affecting 40-50% of patients, its correlation with disease severity is well-established, and interventions to increase iron availability have proven effective in improving clinical manifestations and overall prognosis (104–106). However, it is important to acknowledge that the initial insights into the relevance of ID originated from patients with HF, anemia and chronic kidney disease. This is because anemia is also common in HF and just like ID, it is also associated with worse outcomes(107–109). In fact, the clinical consequences of ID persist regardless of the presence of anemia (89,122). Nevertheless, the relevance of anemia in HF begins at the outset of HF treatment, when the use of angiotensin converting enzyme inhibitors was associated with a reduction in the production of red blood cells through the inhibition in the production and the sensitivity to erythropoietin (110,111). However, subsequent population-based studies and clinical trials have consistently highlighted the importance of ID repletion therapies to improve outcomes, while cautioning against the potential harm of erythropoiesis-stimulating agents (112). Consequently, it is the balance of iron, rather than anemia alone, that has assumed a pivotal role in the management of HF and to date, ID is considered an independent pathophysiological factor that can and should be effectively treated with parenteral iron infusion preparations(1,2).

3.1 Iron function and regulation in health and disease

Imbalances in iron levels or dysfunctions in iron regulatory pathways can lead to iron overload or deficiency, both of which have adverse health consequences. Iron plays a critical role in oxygen transport via hemoglobin and myoglobin, in intracellular metabolism and in physiological processes such as DNA synthesis and cellular signaling. Excessive amounts of iron can generate reactive oxygen species (ROS), resulting in oxidative stress, and a lack of iron impairs many physiological processes, such as energy production and substrate use efficiency (113).

Iron overload can derive from several underlying causes, including hereditary conditions like hemochromatosis, frequent blood transfusions, or anemias with increased erythrocyte share stress and loss. In these situations, the imbalance between iron absorption and utilization results in the accumulation of iron within cells and tissues. Iron overload within the cell leads to a phenomenon named ferroptosis, a unique form of cell death characterized by iron overload and elevated levels of lipid peroxidation and generation of ROS, including lipid hydroperoxide (114). The well-known Fenton reaction involves the oxidation process activated by Fe(II) salts in the presence of H_2O_2, generating radical species in solution and oxidizing a wide range of organic substrates with high activity but poor selectivity leading to the oxidation of a broad range of organic substrates (103). As a result, the accumulation of iron leads to an increasing labile iron pool—or free iron— which triggers the production of ROS, overpowering the body's antioxidant defenses and resulting in oxidative damage to lipids, proteins, and DNA. Within the cell, this results in an extensive damage to phospholipids and disruptions to the plasma membrane, activating the caspase and necrosome-dependent cell death pathways (115). From a clinical perspective, the oxidative stress linked with excessive iron levels is present in a wide spectrum of otherwise common pathological conditions such as neurodegenerative diseases, cardiovascular disorders and to certain cancers, highlighting the detrimental health impacts of iron overload (114,116). In the heart, the presence of excessive iron within the cardiac tissue disrupts normal cellular processes through an increased oxidative stress, mitochondrial dysfunction, and subsequent damage to the heart muscle, which might lead the development of systolic or diastolic dysfunction and arrhythmias(117).

On the other hand, ID refers to a state where inadequate iron levels cause dysfunction in various organs, systems, or metabolic pathways. In an iron-deficient state, the activity of several enzymes is compromised, reducing cellular energy production primarily at the mitochondria, inducing metabolic inefficiency and oxidative stress, and leading to apoptosis and inflammation. (103,118). In fact, ID frequently coexists in most of cardiovascular diseases such as hypertension, diabetes mellitus, ischemic heart disease or chronic kidney disease (119,120) and it is also independently associated with disease progression(121). Regardless of the body total iron content, the crucial question is whether this iron is appropriately distributed across organs and tissues in sufficient quantities to support normal biological processes. Indeed, rather than an individual entity, ID consists in a spectrum of iron abnormalities beginning with reduced iron availability advancing towards total depletion of iron in all body (122). For instance, as an illustration, the mismatch between iron stores and availability is that during infections or inflammatory conditions, the body responds by increasing hepcidin production reducing iron absorption and sequestering iron within storage organs. This mechanism serves as a defense strategy, limiting iron accessibility to pathogens and inhibiting their replication. While this response is geared towards protection, it also restricts the iron supply to essential organs under stress and leads to an overload of iron in storage sites, including the liver, spleen, and the reticuloendothelial (or mononuclear phagocyte) system.

3.2 The regulation of systemic iron

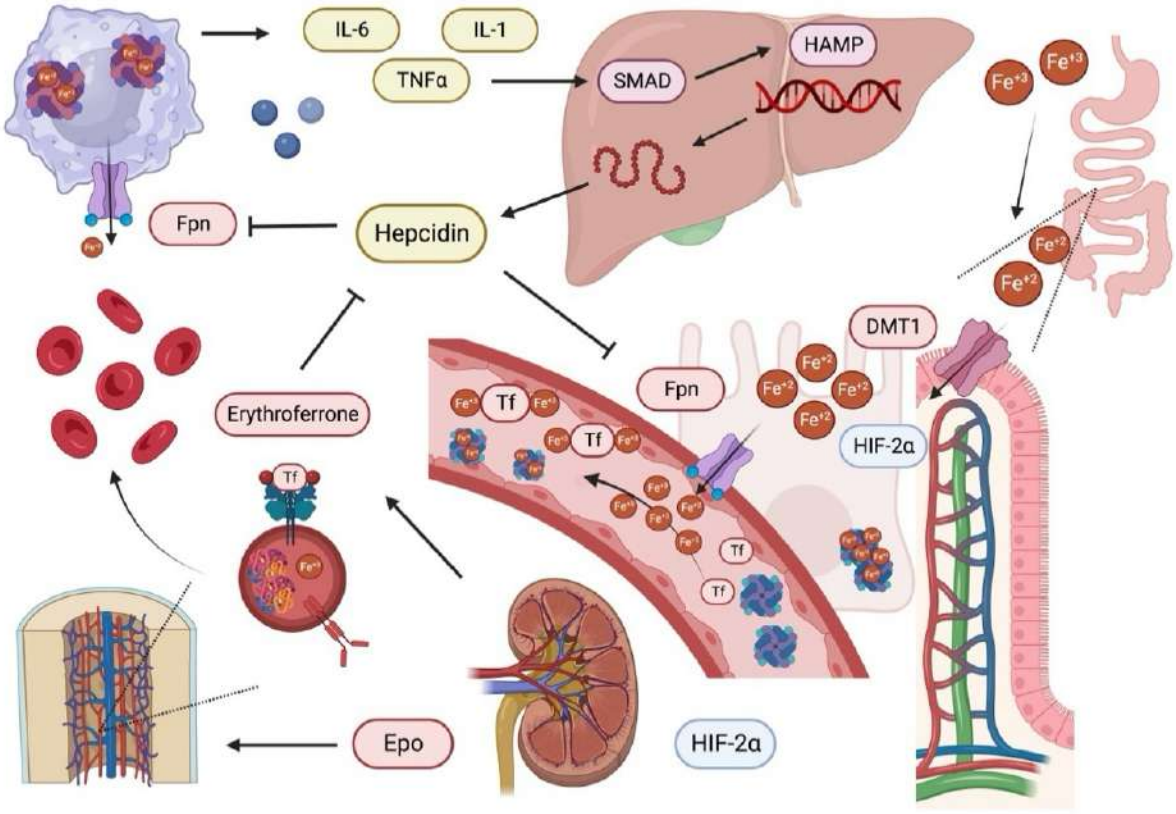
Systemic iron availability is tightly regulated to maintain a delicate balance between its essential functions and potential toxicity, through several mechanisms tackling absorption, transport, storage, delivery, and recycling. Iron is naturally acquired through diet in its ferric form (Fe^{3+}) and absorbed in the duodenum and proximal jejunum. The absorption in the small intestine is done by the enterocytes via divalent metal transporter 1 (DMT1) in its ferrous state (Fe^{2+}), and to a lesser extent, through the haem carrier protein 1 (123). The hepatic hepcidin and hypoxia-inducible factor 2 alfa (HIF2α) regulate iron absorption and export. HIF2α is a transcription factor that mediates the cellular response to hypoxia through hypoxia responsive elements and iron responsive elements 1 and 2 (IRP1 and IRP2), which mediate DMT1 transcription and facilitate iron absorption

(124). Iron is subsequently stored within the cells inside ferritin (FTN), become part of heme groups, or remain as labile iron, which is potentially harmful. FTN is a globular protein composed by a heavy (h) and a light (l) subunit, with ferroxidase activity (the ability of switching iron from ferrous to ferric state). Inside the cells, FTN oxides Fe^{2+} and stores it in its Fe^{3+} state to avoid oxidative damage. Under hypoxic and iron-deficient conditions iron is released to cytoplasm and exported to the bloodstream across the basolateral membrane by ferroportin (FPN) (125).

The FPN synthesis is mainly regulated by a peptide hormone called hepcidin which is coded by the hepcidin antimicrobial peptide (Hamp) gene. Hepcidin acts by inhibiting FPN and thus limits the release of iron into circulation and together constitute the Hepcidin-FPN axis, one of the key regulators of systemic iron(126). Hamp transcription is regulated by an extremely complex interplay that involves hypoxia stimuli, serum iron levels, erythropoiesis, and inflammation(126,127). In addition, erythroferrone, which is released by erythroblasts, inhibits the production of hepcidin through BMP/SMAD signaling pathways to increase the available iron and allow further red blood cell production(128). On the contrary, cytokines can increase hepcidin production under pro-inflammatory environments, leading to decreased iron absorption and increased iron sequestration within cells(129). Iron is transported through the bloodstream bound to a carrier protein called transferrin in Fe^{3+} state and delivers it to cells expressing transferrin receptors by internalizing the iron-transferrin complex through receptor-mediated endocytosis. Iron recycling exclusively takes place when macrophages phagocyte senescent red blood cells to extract iron from their heme groups. Consequently, red pulp macrophages in the spleen, Kupffer cells in the liver, and central nurse macrophages in the bone marrow are essential contributors to the regulation of systemic iron. As a consequence, systemic iron homeostasis is primarily controlled at the point of absorption in the intestinal mucosa and beyond this, there are limited ways to excrete iron except for the shedding of iron-containing cells or through blood loss, making the system heavily reliant on tightly regulated uptake to prevent overload (130).

Figure 5. The iron regulation axis. Systemic iron homeostasis is intricately regulated to balance essential functions and prevent toxicity. Iron absorption, transport, storage, delivery, and recycling involve complex mechanisms. HIF2α, hepcidin, erythroferrone, and cytokines, play crucial roles

in this regulation. Systemic iron homeostasis is primarily maintained at the point of absorption in the intestinal mucosa The Hepcidin-FPN axis is a key regulator of systemic iron, while macrophages in the spleen, liver, and bone marrow contribute significantly to iron recycling and distribution.



3.3 Understanding iron regulation and deficiency in heart failure

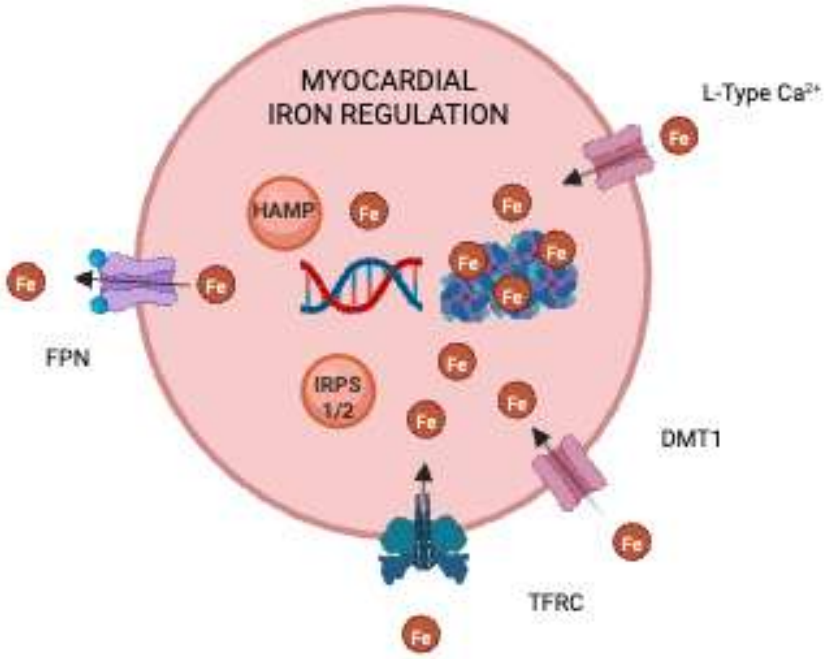
The etiology of ID in HF is complex and is thought to stem from a combination of factors such as diet, gastrointestinal losses, and inflammation (131). In fact, initial studies emphasized the link between iron and anemia with the effect of cytokines like interleukins 1 (IL-1) and 6 (IL-6), tumor necrosis factor alfa (TNF-α), and increased hepcidin levels (132). However, the underlying pathogenesis and its exact role of ID in the progression of HF remain largely unknown. There is a strong correlation between ID and congestion and myocardial iron content is markedly decreased in individuals with advanced HF, suggesting that iron might play a pivotal role in the disease pathophysiology itself (133,134). Moreover, iron status does not solely impact the myocardium but also extends to skeletal muscles and highly energy-demanding organs like the kidneys, the liver and the central nervous system (135).

The challenge lies in establishing the precise threshold at which iron levels become detrimental. Current perspectives suggest a focus on increased iron demand and impaired iron transport rather than solely emphasizing absolute low iron availability (133). Although the gold standard method to analyze iron stores is bone marrow staining, in routine clinical practice iron status is evaluated by measuring peripheral blood biomarkers such as serum iron, ferritin, transferrin, and the transferrin saturation (TSAT). ID in HF is usually defined as a ferritin level below 100µg/L or a ferritin level between 100-299µg/L with TSAT below 20% (1). However, these specific thresholds are currently under debate within the HF community, due to significant clinical discrepancies in expected patient outcomes and the effectiveness of iron treatment when using these definitions (113,136). Nanas et al. conducted a study revealing ID in the bone marrow of 73% of patients with advanced HF (119). Bevetorg et al. evaluated the correspondence of serum iron parameters with bone marrow aspiration in 42 consecutive patients with ejection fraction ≤45% who underwent coronary artery bypass grafting and found a 40% prevalence of bone marrow ID that correlated with a TSAT ≤19.8% or serum iron ≤13 µmol/L(137). Moreover, in a recent study involving more than 4000 HF patients with reduced ejection fraction, a TSAT <20% and serum iron <13 mmol/L but not ferritin <100ng/mL were associated with a higher all-cause and cardiovascular mortality (138).

There is a need to better differentiate between available and non-available iron and raises questions about whether these thresholds truly reflect systemic, tissue, and myocardial iron availability. Initial insights on the relevance of iron distribution in HF derive from extensive cohort-based studies which have demonstrated that aberrant systemic iron transport is a crucial determinant In influencing both the clinical features and patient outcomes (139). Recent studies have demonstrated a strong correlation between elevated levels of soluble transferrin receptor (sTfR) with one-year mortality, impaired exercise capacity and quality of life in HF patients(140,141). Of note, elevated plasma soluble transferrin receptor (sTfR) levels are indicative of an increased cellular need for iron at the tissue level(122).

The regulation of iron within the human body is predominantly centered on the capacity for iron absorption in the gastrointestinal system and the control of iron retention and mobilization from storage. However, the regulation of intramyocardial iron has only been partially unveiled recently and appears to differ significantly. The main factors implicated in iron regulation at a myocardial level are the iron-responsive elements (IRPs) 1 and 2, the transferrin receptor 1 (TFR1), and the hepcidin – ferroportin (FPN) axis (142,143). Iron mostly travels bound to transferrin in the bloodstream and enters the myocardium through TFR1 and to a lesser extent, TFR2. The transcription of TFR1 in cardiomyocytes is highly regulated by iron-regulatory proteins (IRP) 1 and 2, in response to the intracellular levels of iron (142). On the other hand, non-transferrin-bound iron can enter the cardiomyocytes through the DMT1 or via calcium and zinc transporter channels. Intracellular iron levels regulate L-type and T-type calcium transporter channels as well as zinc transporters function through post-transcriptional mechanisms and the synthesis of DMT1 is thought to be regulated by HIF2α transcription factor (144). The main route for iron release from cardiomyocytes involves FPN, and cardiac hepcidin plays a crucial role in regulating this process. (143). As we will explore later, there are notable differences in the regulation of the HAMP gene, the synthesis of hepcidin and FPN between systemic regulation and within cardiomyocytes.

Figure 6. Cardiomyocyte iron regulation. Iron, delivered via transferrin through TFR1, enters the myocardium, and non-transferrin-bound iron accesses cardiomyocytes through DMT1 or calcium and zinc transporter channels. FPN releases iron from cardiomyocytes, a process regulated by hepcidin. Iron-responsive proteins (IRP) 1 and 2 regulate the transcription of TFR1 and DMT1.



3.4 Connecting iron deficiency, metabolism and heart failure

While the historical focus on metabolic dysfunctions in cardiovascular disease has primarily been on atherosclerosis development, there is a growing recognition of the role of alternative metabolic pathways in initiating myocardial and endothelial dysfunction at the core of cardiovascular conditions, particularly HF. At the cardiomyocytes, metabolic dysfunction, involves impaired substrate utilization, increased ROS production and decreased adenosine triphosphate (ATP) biogenesis. Under normal physiological conditions, cardiomyocytes are highly metabolic cells that utilize a variety of substrates for energy production, including fatty acids, glucose, and, to a lesser extent, lactate, ketones, and amino acids. Although the predominant source of energy are fatty acids, metabolic flexibility allows cardiomyocytes to switch from fatty acid oxidation to glucose oxidation under stress conditions (145). HF development is associated with cardiomyocyte hypertrophy and a swift towards myocardial glucose uptake that induces

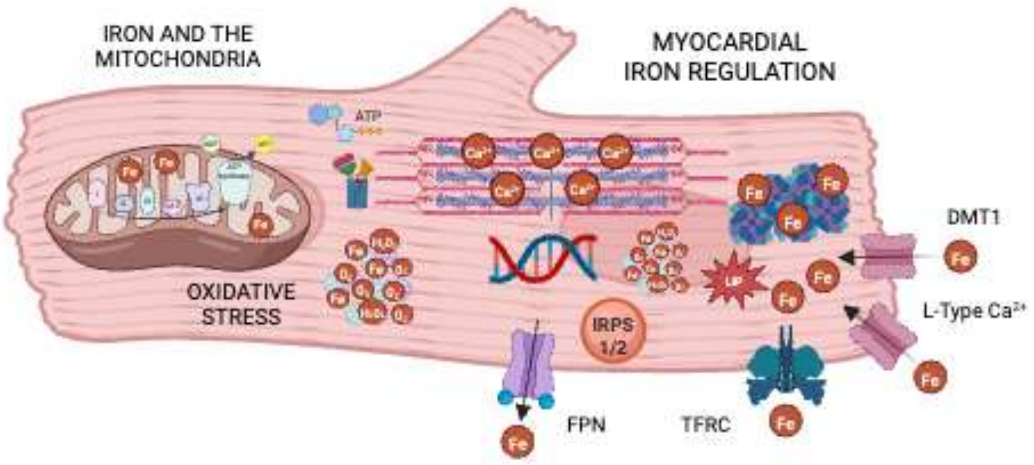
transcriptional changes affecting the ion coupling and inhibiting antiapoptotic pathways (146).

Understanding the relationship between iron regulation and myocardial function is crucial in interpreting the pathophysiology and identifying potential therapeutic strategies. Insufficient intracellular iron levels in the cardiomyocytes results impaired contractility and relaxation(147). Depriving cells of iron leads to a swift downregulation of mitochondrial protein levels and oxidative capacity compromising the cellular respiration and leading to increased radical oxygen species toxicity to the rest of organelles and cellular structures. The mitochondria are the energy suppliers of the cardiomyocytes and have the capacity to continuously adapt to shifting external environments and cellular requirements. Mitochondrial enzymes participating in oxidative phosphorylation, antioxidative defense, and oxygen transport necessitate iron either bound in heme or within Fe–S clusters and participate in the regulation of mitochondrial biogenesis (148). Of note, ID induced mitochondrial damage has been found to be fully reversible within 2–3 days upon the reintroduction of iron(148). Consequently, iron becomes crucial for the optimal functioning of cardiomyocytes and in fact, several preclinical models have consistently exhibited progressive cardiac remodeling and systolic dysfunction under induced iron deprivation (120–122).

While this may appear as a distinctive feature of ID, these metabolic dysfunctions and their resulting consequences are frequently observed in other types of HF, and more particularly in patients with HF and preserved LVEF (149,150). Systemic metabolic disorders induced by conditions such as diabetes or obesity lead to structural and functional cardiac abnormalities similar to those seen in patients with ID such as metabolic inflexibility, lipotoxicity, and extracellular fibrosis (151,152). In fact, metal ion metabolism dysregulation has shown to contribute to myocyte metabolic dysfunction, oxidative stress including lipid peroxidation and cardiac fibrosis(153). This shift leads to impaired relaxation, akin to diastolic dysfunction, accompanied by a decrease in force-generating capacity, causing impaired contractility (82). The structural integrity of sarcomeres is crucial for normal contractile and relaxation functions. Myofibrillar proteins such as actin, desmin, tropomyosin, and troponins are highly susceptible to oxidative

damage. Recent data indicate that oxidative modification of these sarcomeric proteins significantly contributes to dysfunction in both skeletal and cardiac muscle. In the absence of ATP, the head domains of myosin cross-bridges in muscle bind to actin filaments in a rigor conformation, resembling the state following the working stroke during active contraction, resulting in reduced muscle contractility and relaxation. In the cardiomyocytes, Under severe oxidative stress, ROS target sarcomere proteins, causing a shift towards a rigor-like conformation of myosin heads domains leading to decreased enzymatic activity of myosin and actomyosin (73,154). Of note, in preclinical models, the adverse effects of ID have been shown to be reversible upon correcting it through iron supplementation(142,147,155).

Figure 7. The interplay between iron regulation, mitochondrial function, and contractility. This connection involves the incorporation of iron through TFRC, DMT1, and Ca2+ channels, with storage inside ferritin. Non-ferritin-bound iron creates a labile iron pool, elevating oxidative stress. ID hampers mitochondrial ATP production by disrupting the electron chain reaction, subsequently influencing calcium handling at the sarcomere, thereby impacting contractility and relaxation. Iron-responsive proteins (IRP) 1 and 2, predominantly responsive to intracellular iron levels, intricately regulate the transcription of TFR1 and DMT1.



3.5 Iron repletion as a treatment for heart failure

The primary objective of iron supplementation in common clinical practice has traditionally been the correction of iron-deficient anemia; however, iron supplementation therapies are now considered a standard of care in HF, irrespective of anemia (1,2). Intravenous iron supplementation offers the potential for rapid and substantial correction of iron levels, transferrin saturation, and blood ferritin. From a historical standpoint, iron repletion therapies in HF were first evaluated in patients with end-stage chronic kidney disease and persistent anemia despite the use of erythropoietin-stimulating agents. These therapies subsequently resulted in improvements in hemoglobin levels, functional class, LVEF, and reduced hospitalizations. The rationale of intravenous infusions, was to overcome the limitations of gastrointestinal absorption due to elevated hepcidin levels due to chronic inflammation, yet in patients with HF the effect of systemic low iron levels inhibit hepcidin production and facilitate the absorption in the intestines and delivery to the bloodstream from stores(156). While proven safe, the use of repetitive infusions may present logistical challenges and significant uncertainty remains regarding the optimal approach to intravenous iron supplementation and the specific patient profiles that benefit most (110)(157,158).

Current evidence supporting iron repletion therapies in HF originates from various sources, spanning from laboratory research to clinical practice. For example, studies involving iron-depleted genetically modified cardiomyocytes demonstrate mitochondrial dysfunction and impaired contractility, which can be restored with a single dose of iron carboxymaltose(142). Of note, the same study demonstrated that iron supplementation specifically targeted myocardial efficiency by improving mitochondrial respiration, which led to improved cardiomyocyte contractility. Additionally, iron supplementation prevented remodeling in a post-myocardial infarction HF mouse model and enhanced LVEF. In the clinical setting, intravenous iron has been shown to improve HF symptoms, exercise capacity and quality of life, and to reduce decompensations and cardiovascular mortality (105,106,159–161) (Table 1). Similar to findings in preclinical models, patients treated with intravenous iron demonstrate signs of myocardial iron repletion and improved cardiac contractile performance parameters(162,163), and decreases neurohormonal activation and systemic inflammation (164,165).

Table 1: Main clinical trials involving iron supplementation in HF(104,106,136,160,166,167).

Trial and Patients	Drug	Inclusion Criteria	Primary Outcomes	Conclusion
FAIR-HF (2009) **n=459**	Ferric carboxymaltose 200mgr iv	NYHA class II and LVEF <40% or NYHA class III and LVEF <45%; ferritin <100 µg/l or ferritin 100–299 µg/l and transferrin saturation <20%	Quality of life and NYHA	Improves quality of life and NYHA functional class
CONFIRM-HF (2015) **n=304**	Ferric carboxymaltose 500–1000mg iv	NYHA class II–III and LVEF <45%; NTproBNP >400 pg/ml; ferritin <100 µg/l or ferritin 100–299 µg/l and transferrin saturation <20%	∆6MWT HF hospitalizatio n	Improves functional capacity and HF admission
IRONOUT-HF (2017)	Iron polysaccharide 150mgr bid oral	NYHA class II-IV and LVEF <40%; ferritin <15-100µg/l or ferritin 100–299 µg/l and transferrin saturation <20%	∆V02 in cardiopulmon ary exercise test	Does not improve peak VO2
AFFIRM-HF (2020) **n=1132**	Ferric carboxymaltose 500–1000mg iv	Admitted to hospital for acute HF and LVEF <50%; ferritin <100 µg/l or ferritin 100–299 µg/l and transferrin saturation <20%	HF hospitalizatio n and CV death	Reduces the risk of HF hospitalizatio n
IRONMAN (2021) **n=1869**	Ferric derisomaltose 20 mg/kg iv*	Ferritin<100mg/dL or TSAT<20% and Ferritin <400mg/dL; LVEF <45%; HF hospitalization or elevated natriuretic peptides	HF admissions and CV death	Reduces CV death and HF recurrent admissions
HEART-FID (2023) **n=3065**	Ferric carboxymaltose iv*	LVEF <40%; ferritin <100 µg/l or ferritin 100–299 µg/l and transferrin saturation	Death or HF admission or ∆6MWT**	Does not prevent death or HF

	<20%; HF hospitalization or elevated natriuretic peptides.		hospitalization and did not improve 6MWT

*Body weight and hemoglobin corrections applied **Hierarchical composite study outcome including 12-month death and HFH and 6MWT at 6 months

∆V02: change in peak oxygen uptake; 6MWT: 6-minute walking test; CV; cardiovascular; HF: heart failure; LVEF: left ventricular ejection fraction; NYHA: New York Heart Association; NTproBNP: N-terminal pro b-type natriuretic peptide.

Nevertheless, additional studies have yielded differing results regarding specific outcomes, particularly in the prevention of cardiovascular and all-cause mortality, leading to significant criticism (136,166). These discrepancies may arise from variations in iron repletion strategies, patient selection, HF phenotypes, and definitions of systemic ID. Nevertheless, it clearly states that it is crucial to improve our understanding of the mechanisms driving ID development in HF patients, its impact on myocardial function, and its role in the development of multiorgan dysfunction. ID is emerging as a crucial element in understanding HF pathophysiology, while the primary driver remains sustained maladaptive neurohormonal stimuli within the cardiovascular system. Patients with more severe neurohormonal activation and advanced HF tend to exhibit higher degrees of ID and preclinical models have shown the impacts of ID within the myocardium and the positive effect of iron repletion on cardiomyocyte energetics and contractility. However, to date, no study has fully elucidated the potential interrelation between neurohormonal activation and ID in HF.

This led us to hypothesize that neurohormonal activation may contribute to ID and play a significant role in HF pathophysiology. That neurohormonal activation itself may disrupt intramyocardial iron regulation through cellular iron regulation systems, potentially lowering intracellular iron levels and impairing mitochondrial function. Moreover, we hypothesized patients with systemic ID presented changes in the transcriptional footprint, linking HF with systemic metabolism and cardiovascular function, and have an influence disease progression.

Hypothesis

1. The high prevalence of ID in HF patients suggests that it might be an intrinsic component of the HF syndrome rather than a consequence of it.
2. HF may induce changes in the expression of genes involved in myocardial iron regulation, leading to ID and promoting disease progression.
3. Neurohormonal activation in HF may be a key factor responsible for the disruption of intramyocardial iron regulation.
4. The effect of ID on myocardial function might be mediated through mitochondrial dysfunction and increased oxidative stress.
5. ID might induce changes in the expression of specific genes involved in systemic metabolism, iron regulation, and cardiovascular function, independent of anemia.
6. Alterations in myocardial and systemic metabolism due to ID might play a significant role in the progression of HF.

Objectives

1. Analyze the impact of HF on iron status and regulation in the myocardium.
2. Investigate the impact of neurohormonal stimuli in HF on cardiac cell iron levels and regulatory pathways.
3. Evaluate the influence of HF neurohormonal stimuli on cardiac cell mitochondrial iron function.
4. Study alterations in mitochondrial iron regulation under neurohormonal stimuli intracellular ID.
5. Examine the systemic transcriptomic profile in HF patients with systemic ID but without anemia and identify systemic biological pathways linking HF and ID, in the absence of anemia.
6. Discover candidate genes associated with iron regulation, cardiovascular function, and metabolism within these shared pathways and analyze the gene expression patterns of the identified genes related to cardiovascular disease and metabolism in the context of ID without anemia.
7. Evaluate the clinical implications of variations in genetic expression profiles of selected biological pathways and candidate genes in blood samples from HF patients.
8. Correlate biological pathways observed in mice and cellular models with gene expression patterns in transcriptomic analyses of patient blood samples.

Material, Methods and Results

1. Neurohormonal activation induces intracellular iron deficiency and mitochondrial dysfunction in cardiac cells.

In this study, we aimed to investigate the impact of neurohormonal activation in HF on iron homeostasis and mitochondrial function in cardiac cells. We induced HF in mice with isoproterenol and challenged H9c2 cells to angiotensin 2 and noradrenalin, as major components of HF characteristic neurohormonal stimuli. We measured the gene expression and the amount of protein of essential elements related to intracellular iron metabolism and assessed myocardial and intracellular iron levels within the myocardium and in the cardiac cells. Additionally, we examined mitochondrial function by analyzing mitochondrial membrane potential, the accumulation of ROS and ATP production in H9c2 cells.

The results showed that in HF mice and the stimulated cardiac cells, there were decreases in the expression of key iron regulatory proteins and a depletion of intracellular iron levels. We observed a disruption in intramyocardial iron regulation mechanisms, particularly related to iron uptake, along with decreased iron levels in mice treated with isoproterenol. We demonstrated that neurohormonal activation promotes the disruption of intracellular iron regulation, affecting iron uptake and lowering intracellular iron levels. We observed alterations in the mitochondrial membrane structure, a reduction in ATP production, and an increase in ROS levels that paralleled with a reduction in the mitochondrial ferritin gene expression and protein levels.

These findings suggest that neurohormonal activation in HF can disrupt iron regulation, lower intracellular iron levels, and impair mitochondrial function, potentially implicating iron in HF pathophysiology.

Tajes et al. Cell Biosci (2021) 11:89
https://doi.org/10.1186/s13578-021-00605-5

Cell & Bioscience

RESEARCH Open Access

Neurohormonal activation induces intracellular iron deficiency and mitochondrial dysfunction in cardiac cells

M. Tajes[1,2], C. Díez-López[1,2,3,7], C. Enjuanes[1,2,4], P. Moliner[1,2,4], J. L. Ferreiro[1,2], A. Garay[1,2,4], S. Jiménez-Marrero[1,2,4], S. Yun[1,4,9], S. G. Sosa[1,2], L. Alcoberro[1,2,4], J. González-Costello[1,2,3,7], E. García-Romero[1,2,3], L. Yañez-Bisbe[5], B. Benito[5,6,8] and J. Comín-Colet[1,2,4,7*]

Abstract

Background: Iron deficiency (ID) is common in patients with heart failure (HF) and is associated with poor outcomes, yet its role in the pathophysiology of HF is not well-defined. We sought to determine the consequences of HF neurohormonal activation in iron homeostasis and mitochondrial function in cardiac cells.

Methods: HF was induced in C57BL/6 mice by using isoproterenol osmotic pumps and embryonic rat heart-derived H9c2 cells were subsequently challenged with Angiotensin II and/or Norepinephrine. The expression of several genes and proteins related to intracellular iron metabolism were assessed by Real time-PCR and immunoblotting, respectively. The intracellular iron levels were also determined. Mitochondrial function was analyzed by studying the mitochondrial membrane potential, the accumulation of radical oxygen species (ROS) and the adenosine triphosphate (ATP) production.

Results: Hearts from isoproterenol-stimulated mice showed a decreased in both mRNA and protein levels of iron regulatory proteins, transferrin receptor 1, ferroportin 1 and hepcidin compared to control mice. Furthermore, mitoferrin 2 and mitochondrial ferritin were also downregulated in the hearts from HF mice. Similar data regarding these key iron regulatory molecules were found in the H9c2 cells challenged with neurohormonal stimuli. Accordingly, a depletion of intracellular iron levels was found in the stimulated cells compared to non-stimulated cells, as well as in the hearts from the isoproterenol-induced HF mice. Finally, neurohormonal activation impaired mitochondrial function as indicated by the accumulation of ROS, the impaired mitochondrial membrane potential and the decrease in the ATP levels in the cardiac cells.

Conclusions: HF characteristic neurohormonal activation induced changes in the regulation of key molecules involved in iron homeostasis, reduced intracellular iron levels and impaired mitochondrial function. The current results suggest that iron could be involved in the pathophysiology of HF.

Keywords: Neurohormonal activation, Heart failure, Iron deficiency, Cardiac cell, Mitochondria function

Background

Heart failure (HF) is a devastating condition and represents a challenge for public healthcare systems [1]. The current understanding of its pathophysiology is based on the "neurohormonal hypothesis", which states that HF progression is promoted by the long-term maladaptive

*Correspondence: josepcomin@gmail.com; jcomin@bellvitgehospital.cat
[1] Bio-Heart Cardiovascular Diseases Research Group, Bellvitge Biomedical Research Institute (IDIBELL), L'Hospitalet de Llobregat, Barcelona, Spain
Full list of author information is available at the end of the article

and deleterious effects of sustained neurohormonal activation in the heart and in rest of the cardiovascular system [2, 3]. Many of classical neurohormones such as norepinephrine (Nor) and angiotensin II (Ang II) are known to be synthesized directly within the myocardium and, therefore, act in an autocrine or paracrine manner [3, 4]. In fact, the sustained activation of these pathways lead to an increased plasma Ang II, epinephrine and Nor levels [5, 6]. The inhibition of these neurohormonal systems has demonstrated a consistent reduction in morbidity and mortality in patients with systolic HF and is the basis of modern pharmacological treatment [7, 8].

However, these effective therapies have failed to promote a complete remission of symptoms and restore life expectancy in many patients. This has motivated an increasing interest in emerging therapeutic targets. In this regard, iron deficiency (ID) is present in up to 50% of HF patients [9, 10] and is associated with a higher risk of mortality and hospitalization [10, 11], reduced functional capacity, and impaired health-related quality of life [12], independent of the presence of anemia [10, 12, 13]. Interestingly, intravenous iron supplementation has been recommended by European Society of Cardiology (ESC) HF guidelines as a potential therapeutic approach, since the correction of ID with intravenous iron alleviates HF symptoms, reduces risk of hospitalization and improves quality of life [14–16].

Although ID has been mainly considered as an extracardiac co-morbidity complicating the course of HF, recent data suggest that it may actually interact directly with the mechanisms involved in HF initiation and progression [17–20]. Previous studies from our group suggested an association between HF neurohormonal activation and systemic ID [17]. It has been shown that in chronic HF patients, the increase in the sympathetic activity (measured by Nor systemic levels) was associated with ID, independently of anemia [17]. In addition, Maeder et al. suggested that the sympathetic activation found in HF patients may contribute to iron depletion, specifically in heart, since Nor stimulation may reduce the intracellular iron levels in cardiac cells by downregulating the transferrin receptor 1 (*Tfr1*) expression [18]. Other studies have shown that iron content in myocardial was reduced by 20–30% in patients with advanced HF [21]. Apart from its role in oxygen transport, iron plays an important role in cellular metabolism, impacting on the regulation of oxidative stress and ATP synthesis, as an enzymatic cofactor in the mitochondria [19, 22]. Radical oxygen species (ROS) directly impacts on the mitochondrial Ca^{2+} homeostasis contributing to an altered contractibility and increasing the cardiac wall stiffness [23]. Hence, iron homeostasis is crucial for cells with high energy demand, such as cardiomyocytes [10, 19].

In spite of all the accumulating data, the interplay of iron in the pathophysiology of HF is not well-defined. We hypothesized that neurohormonal activation could be involved in the generation of ID in cardiac cells, directly impacting on the mitochondria function. Therefore, the aim of this study was to analyze the relationship between neurohormonal stimuli and iron homeostasis, as well as their role regulating the mitochondrial function in cardiac cells.

Results

We designed a two-step experimental approach. First, the effect of HF over several molecules involved in iron regulation was evaluated in hearts from a well-established isoproterenol-induced HF mice [24]. Second, the mechanisms linking iron regulation, energy metabolism and mitochondrial function, were further explored in depth in heart-derived cell cultures H9c2 exposed to the neurohormonal activation typically found in HF.

Changes in the gene expression and protein levels of iron regulatory molecules in the heart of isoproterenol-induced HF mice

First, we analyzed the cardiomyocyte area on histological sections from mice hearts (Fig. 1a). As it is shown in the Fig. 1, isoproterenol induced cellular hypertrophy, compared to control animals (Fig. 1a, b).

Both, the mRNA and protein levels of the main molecules related to cellular iron uptake, release and storage, were analyzed in HF and control mice. First, it was determined the two iron regulatory proteins (Irp1 and

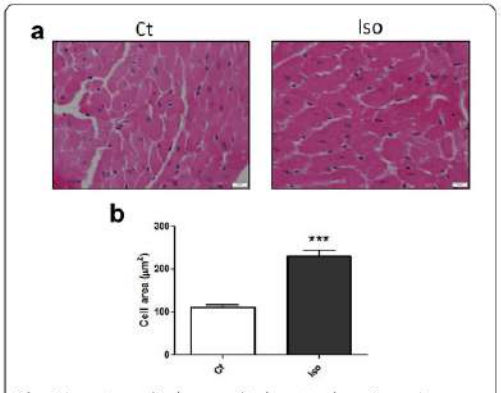


Fig. 1 Isoproterenol induces cardiac hypertrophy. **a** Masson's Trichrome staining of sections from ventricular myocardial of control (Ct) and isoproterenol (Iso) stimulated mice. **b** Quantification of cardiomyocytes area. Data were expressed as mean ± SEM (n = 8 per group) (***p < 0.001 vs control)

2), which regulate the translation of several proteins involved in iron metabolism [21]. Irp1 (Fig. 2a) and 2 (Fig. 2b) were down-regulated in mice stimulated with isoproterenol compared to control animals. Next, the levels of the main iron transporters into the cell, Tfr1 and divalent metal transporter 1 (Dmt1), were also determined. Whereas Dmt1 was unaltered (Fig. 2c), Tfr1 was reduced in the hearts of HF mice (Fig. 2d).

In order to look deeper into the role of the molecules related to the cellular iron release, ferroportin 1 (Fpn1) (Fig. 3a), and its inhibitor, hepcidin antimicrobial peptide (Hamp) (Fig. 3b) were analyzed. In the isoproterenol-stimulated mice, the levels of both molecules were down-regulated when compared to control animals. Finally, the cytoplasmic iron storage molecule ferritin, encoded by ferritin heavy chain 1 (*Fth1*) and ferritin light chain 1 (*Ftl1*), was analyzed. No changes were found between the experimental groups in the *Fth1* and *Ftl1* mRNA (Fig. 3c), neither in the Ferritin protein levels (Fig. 3d).

Regulation of mitochondrial iron metabolism molecules in the heart of HF mice

Due to the role of iron in mitochondrial function, the mitochondrial iron uptake transporters Mitoferrin 1 (Mfrn1) (Fig. 4a) and 2 (Mfrn2) (Fig. 4b), and the mitochondrial iron storage molecule Mitochondrial ferritin (Ftmt) (Fig. 4c) were analyzed. Whereas the Mfnr1 was not modified, the Mfrn2 was down-regulated in the hearts from the HF animals. In addition, Ftmt was also down-regulated in hearts from isoproterenol-stimulated mice.

Intracellular iron depletion in the heart of isoproterenol-induced HF mice

Finally, the total heart iron (Fig. 5a), as well as the iron ions Fe^{2+} (Fig. 5b) and Fe^{3+} (Fig. 5c), were analyzed in mice. As it is shown in Fig. 5, the iron ions were reduced in the hearts from the animals stimulated with isoproterenol compared to control ones.

Neurohormonal stimulation induced hypertrophy in H9c2 cells

To further explore the role of neurohormonal activation in iron metabolism at cellular level, an in vitro cardiac cell model was studied. Since myocardial hypertrophy is a hallmark of HF [3], some of the classical markers of cardiac hypertrophy (Bnp and Myh7) [25] were assessed in the H9c2 cells stimulated with the neurohormonal stimuli (Ang II and/or Nor). As it is shown in Fig. 6, both Ang II and Nor induced an increase in the mRNA levels of *Bnp* and *Myh7* (Fig. 6a). Similar results were found when the MYH7 protein levels were analyzed (Fig. 6b).

Neurohormonal activation induced changes in the intracellular iron metabolism molecules in H9c2 cells

The same molecules studied in mice were subsequently assessed in the in vitro neurohormonal activation model. Irp1 and 2 were significantly reduced when cells were exposed to all the experimental conditions (Fig. 7a, b). In addition, Tfr1 and Dmt1 were down-regulated in the setting of any of the neurohormonal challenges (Fig. 8a, b).

With respect to cellular iron release, whereas *Fpn1* mRNA levels were reduced under Ang II or Nor treatment alone, there were no changes in its expression when cells were simultaneously treated with both stimuli (Fig. 9a, left). Fpn1 protein levels did not change in any experimental condition (Fig. 9b, left). On the other hand, while Hamp was induced by Ang II treatment, its levels were reduced under the influence of Nor and simultaneous treatment with Nor and Ang II (Fig. 9a, right and Fig. 9b right).

Finally, although the stimulation with Nor produced a slight reduction in the *Fth1* expression, there were no significant changes when exposing cardiac cells to Ang II or both stimuli at the same time (Fig. 10a, right). Also, no significant changes in *Ftl1* expression were found in any experimental condition (Fig. 10a, left). Accordingly, no significant changes were found in the cytoplasmic Ferritin protein levels (Fig. 10b).

Neurohormonal activation depleted intracellular iron in H9c2 cells

Beyond the effects of neurohormonal activation on the molecules involved in iron metabolism, its effect on the intracellular iron levels was assessed in H9c2 cells. As it is shown in Fig. 11, the total iron (Fig. 11a), as well as the iron ions Fe^{2+} (Fig. 11b) and Fe^{3+} (Fig. 11c), were reduced in the cells challenged with Nor, Ang II, or their combination, when compared to control cells.

Neurohormonal activation promoted down-regulation of Ftmt levels in H9c2 cells

The exposure to Nor and/or Ang II did not induce changes in the levels of the mitochondrial iron transporters Mfrn1 and Mfrn2 (Fig. 12a, b). Interestingly, the mRNA and protein levels of the Ftmt were reduced in all the experimental conditions (Fig. 12c, d).

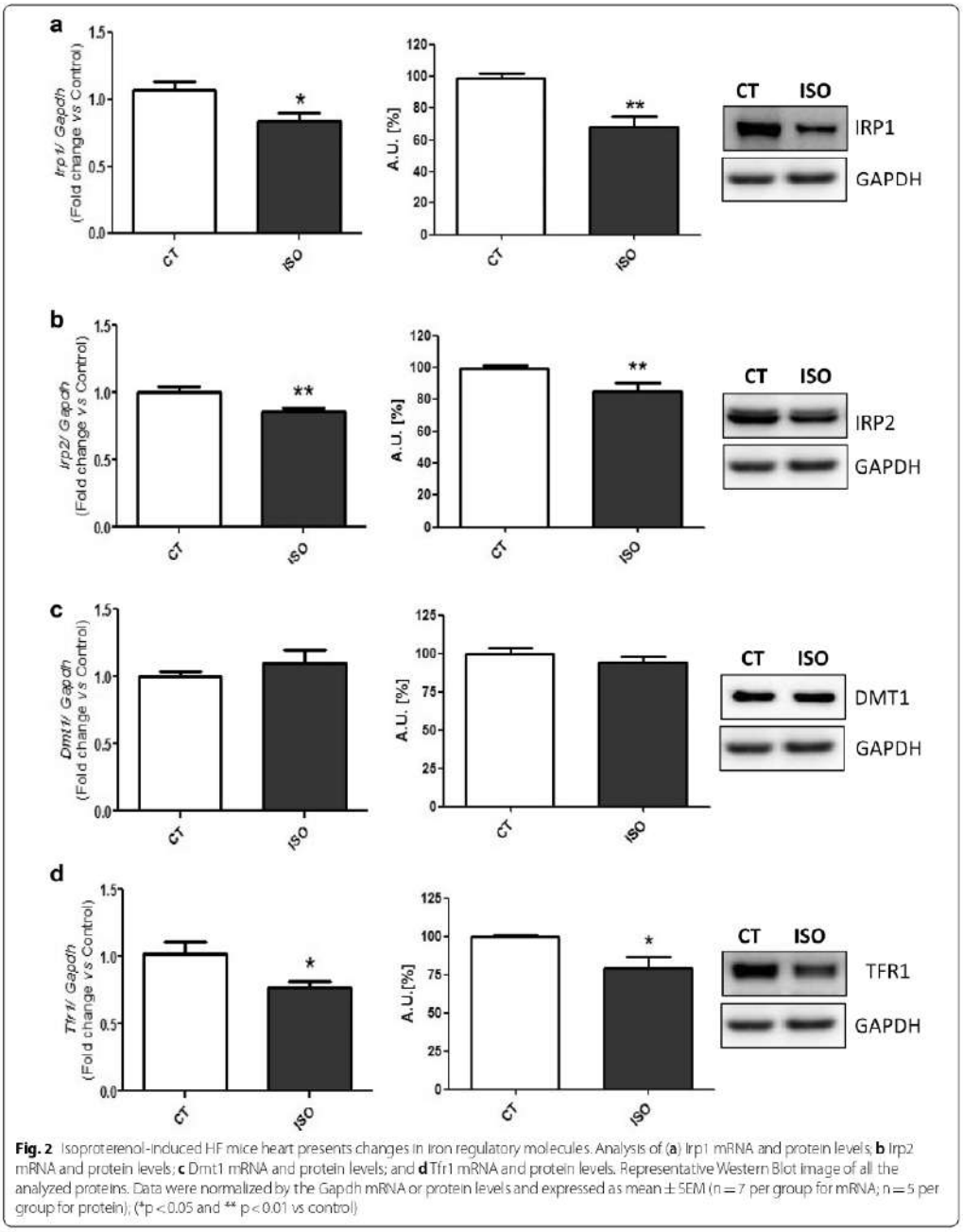


Fig. 2 Isoproterenol-induced HF mice heart presents changes in iron regulatory molecules. Analysis of (**a**) Irp1 mRNA and protein levels; **b** Irp2 mRNA and protein levels; **c** Dmt1 mRNA and protein levels; and **d** Tfr1 mRNA and protein levels. Representative Western Blot image of all the analyzed proteins. Data were normalized by the Gapdh mRNA or protein levels and expressed as mean ± SEM (n = 7 per group for mRNA; n = 5 per group for protein); (*$p < 0.05$ and **$p < 0.01$ vs control)

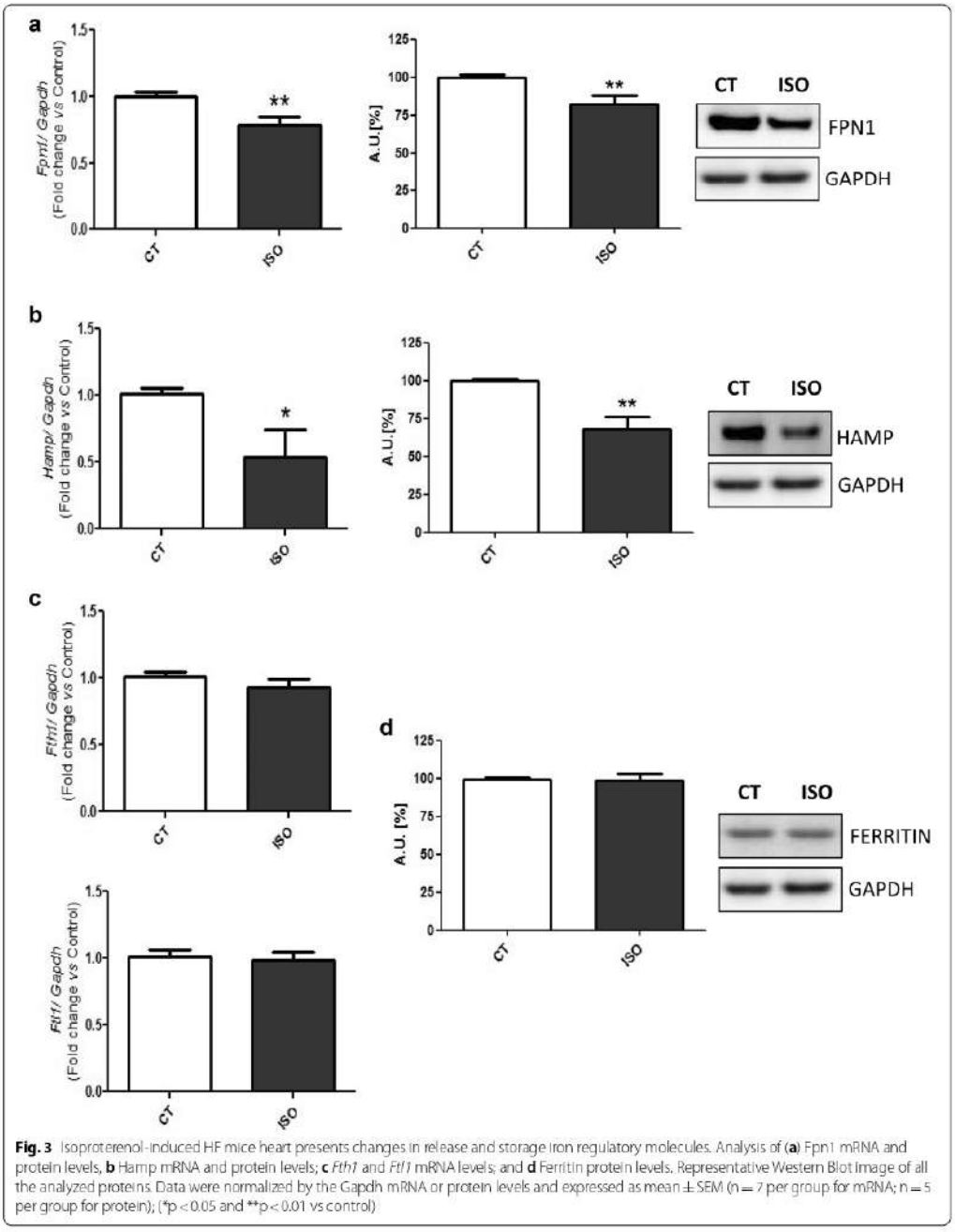


Fig. 3 Isoproterenol-induced HF mice heart presents changes in release and storage iron regulatory molecules. Analysis of (**a**) Fpn1 mRNA and protein levels, **b** Hamp mRNA and protein levels; **c** *Fth1* and *Ftl1* mRNA levels; and **d** Ferritin protein levels. Representative Western Blot image of all the analyzed proteins. Data were normalized by the Gapdh mRNA or protein levels and expressed as mean ± SEM (n = 7 per group for mRNA; n = 5 per group for protein); (*$p < 0.05$ and **$p < 0.01$ vs control)

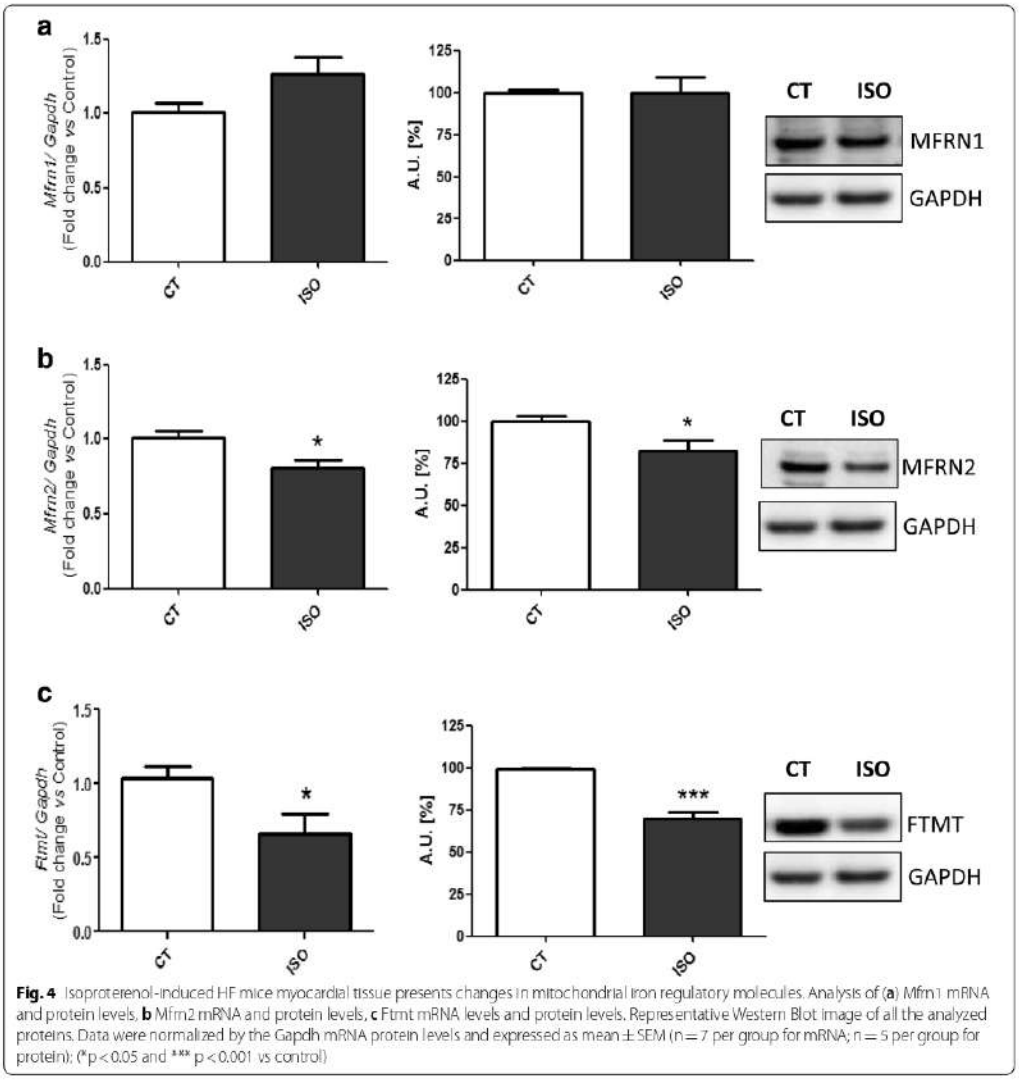


Fig. 4 Isoproterenol-induced HF mice myocardial tissue presents changes in mitochondrial iron regulatory molecules. Analysis of (**a**) Mfrn1 mRNA and protein levels, **b** Mfrn2 mRNA and protein levels, **c** Ftmt mRNA levels and protein levels. Representative Western Blot image of all the analyzed proteins. Data were normalized by the Gapdh mRNA protein levels and expressed as mean ± SEM (n = 7 per group for mRNA; n = 5 per group for protein); (*$p < 0.05$ and ***$p < 0.001$ vs control)

Neurohormonal activation impaired mitochondrial function in H9c2 cells

Several parameters were assessed to characterize mitochondrial function in the neurohormonal activation in vitro model. In the stimulated cells, the mitochondrial membrane potential was reduced (Fig. 13a). Additionally, there was an increase in the ROS production (Fig. 13b), along with a decrease in the ATP levels (Fig. 13c), regardless of the type of stimuli.

Discussion

ID has been associated with adverse outcomes in HF [26, 27]. Despite the clinical association between HF and ID, it is currently unknown whether intracellular

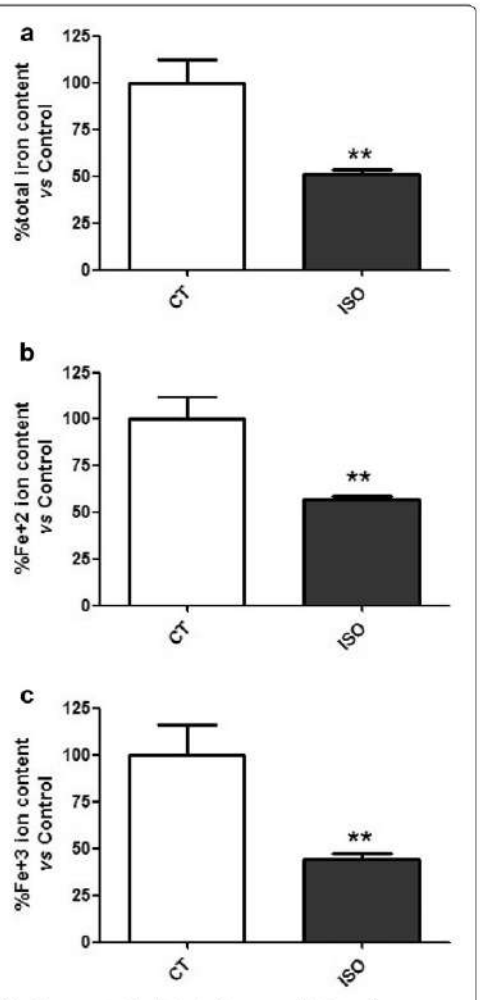


Fig. 5 Iron content depletion in the myocardial tissue of isoproterenol-induced HF mice. Analysis of intracellular iron levels in hearts: (**a**) total iron content, **b** Fe^{2+} ion content and **c** Fe^{3+} ion content. Data were normalized by protein levels and expressed as mean ± SEM (n = 5 per group); (**p < 0.01 vs control)

ID in HF-cardiac cells is a consequence of the disease or, otherwise, directly takes part in the cellular alterations leading the pathology. In the present study we demonstrated that neurohormonal activation related to HF promoted significant abnormalities in the iron metabolism regulatory systems, reduced intracellular iron levels and impaired mitochondrial function in cardiac cells.

Although the unequivocal benefits of intravenous iron may be related to a peripheral effect [14, 28], recent studies support a cardiac-centered hypothesis. First, ferric carboxymaltose administration was associated with cardiac iron repletion in cardiac magnetic resonance T2* and T1 mapping sequences [29]. Second, Doppler and strain rate echocardiography parameters were significantly improved after intravenous iron administration in patients with stable systolic HF and ID without anemia [30]. Third, recent data in patients with HF have correlated ID and reduced peak exercise capacity with impaired myocardial contractile reserve [31].

We set up a study with both in vivo and in vitro approaches. First, we used a well-established HF mice model induced by isoproterenol (β-adrenoceptor agonist) osmotic pumps [24, 32]. Interestingly, these animals showed an increase of Ang II in blood and heart [33], thereby being a good model to study the effect of neurohormonal activation. Moreover, the results found in the in vivo model were validated in H9c2 cardiac cells challenged with AngII, Nor or both, in order to mimic the neurohormonal activation with more than just on stimuli [3, 6].

To validate the neurohormonal activation cell model, we analyzed the expression of cardiac hypertrophy molecules (Bnp and Myh7) as hallmarks of HF [3, 34]. Our neurohormonal activation in vitro model induced both mRNA and protein levels of cardiac hypertrophy markers in all the experimental conditions. Nevertheless, no synergistic effect was observed in cells stimulated with both stimuli simultaneously, possibly because the stimulation with each of them separately already achieved the maximum effect. Similarly, isoproterenol also induced cardiomyocyte hypertrophy in mice.

Our data showed that the neurohormonal activation was related to a reduction of the intracellular iron levels (both Fe^{2+} and Fe^{3+}), thereby suggesting that neurohormonal stimuli may contribute to cardiac alteration through intracellular ID. Interestingly, previous studies reported iron depletion in myocardial tissue of HF patients [18, 22] and highlighted the relevance of neurohormonal activation leading to iron depletion.

At a cardiac cell level, ID may be due to several mechanisms involving iron uptake, release and storage. Interestingly, Irps are able to regulate the translation of different molecules related to these processes depending on iron levels [35]. As a matter of fact, Haddad et al. showed that Irps are crucial to maintain the iron supply in cardiomyocytes and to prevent HF in mice [21]. Furthermore, the same study showed a decrease in the Irp activity from HF

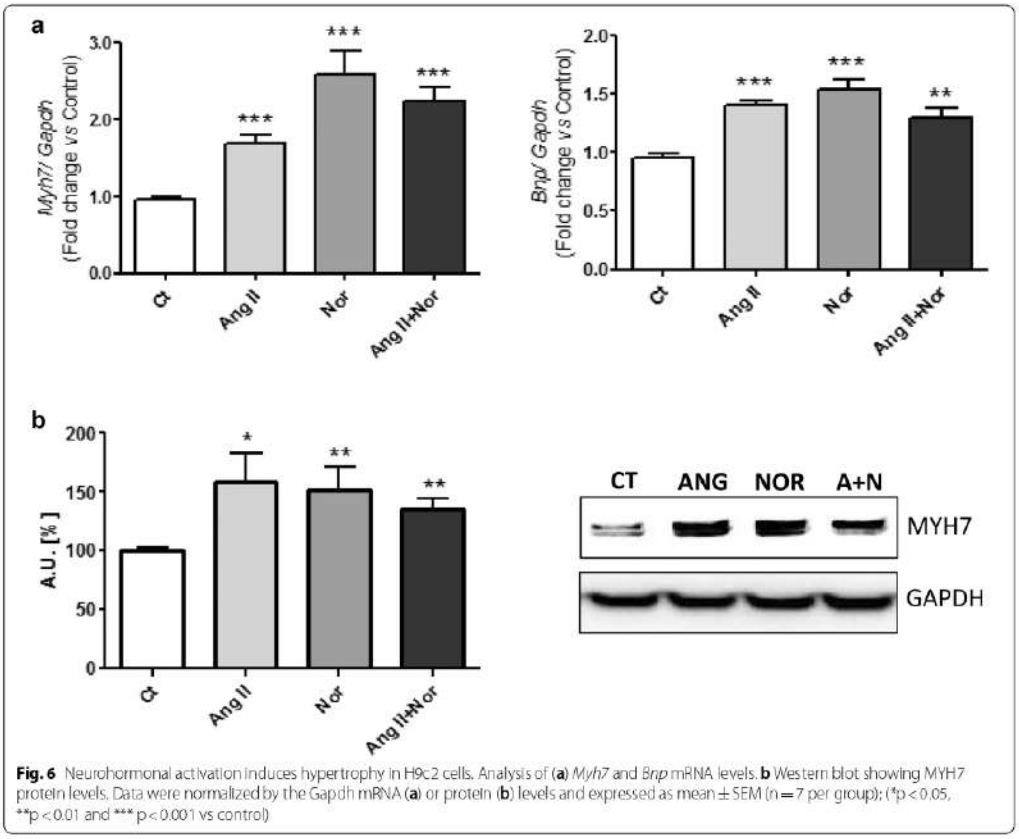


Fig. 6 Neurohormonal activation induces hypertrophy in H9c2 cells. Analysis of (**a**) *Myh7* and *Bnp* mRNA levels. **b** Western blot showing MYH7 protein levels. Data were normalized by the Gapdh mRNA (**a**) or protein (**b**) levels and expressed as mean ± SEM (n = 7 per group); (*p < 0.05, **p < 0.01 and *** p < 0.001 vs control)

patients, related to a depletion of iron in the heart [21]. Accordingly, our study evidenced a down-regulation of the Irps in both of our experimental models. Next, we assessed the levels of Tfr1, which is involved in the transferrin-bound iron intake [36]. Xu et al. showed that the lack of Tfr1 in knock-out mice produced a lethal cardiomyopathy by diminishing the intracellular iron content and impairing mitochondrial function [37]. Besides, the levels of Tfr1 were found reduced in the myocardial tissue of HF patients [18, 21]. Interestingly, Maeder et al. demonstrated that cardiomyocytes treated with aldosterone or Nor showed a decrease in the Tfr1 levels [18]. In line with these studies, we found that Tfr1 was decreased in both hearts of HF mice and in the cardiac cells, in the setting of any of the neurohormonal challenges. Finally, although the Dmt1, related to the non-transferrin bound iron uptake [38], was slightly down-regulated in the neurohormonal-stimulated cells, we found no modifications in hearts from isoproterenol-induced HF mice. As Kobak et al. recently reviewed, Dmt1 uptake could act as a compensatory mechanism of the Tfr1-mediated iron import [39].

Neurohormonal stimuli also regulate the molecules involved in the cellular iron release, Fpn1 and Hamp. In the heart, Hamp participates in the local iron homeostasis regulation by inhibiting Fpn1 function [40]. Fpn1 exports intracellular iron outside the cell [41]. Deregulations that affect the homeostasis of the Fpn1/Hepcidin axis are known to produce iron overload or anemia, depending on the direction of the functional changes [42]. In the studied models we found different behaviors when analyzing these components. Whereas *Fpn1* mRNA levels were reduced under AngII and Nor stimulation, the combination of both stimuli not induces changes in the expression of this gene in cardiac cells. In contrast, in the isoproterenol treated mice, we

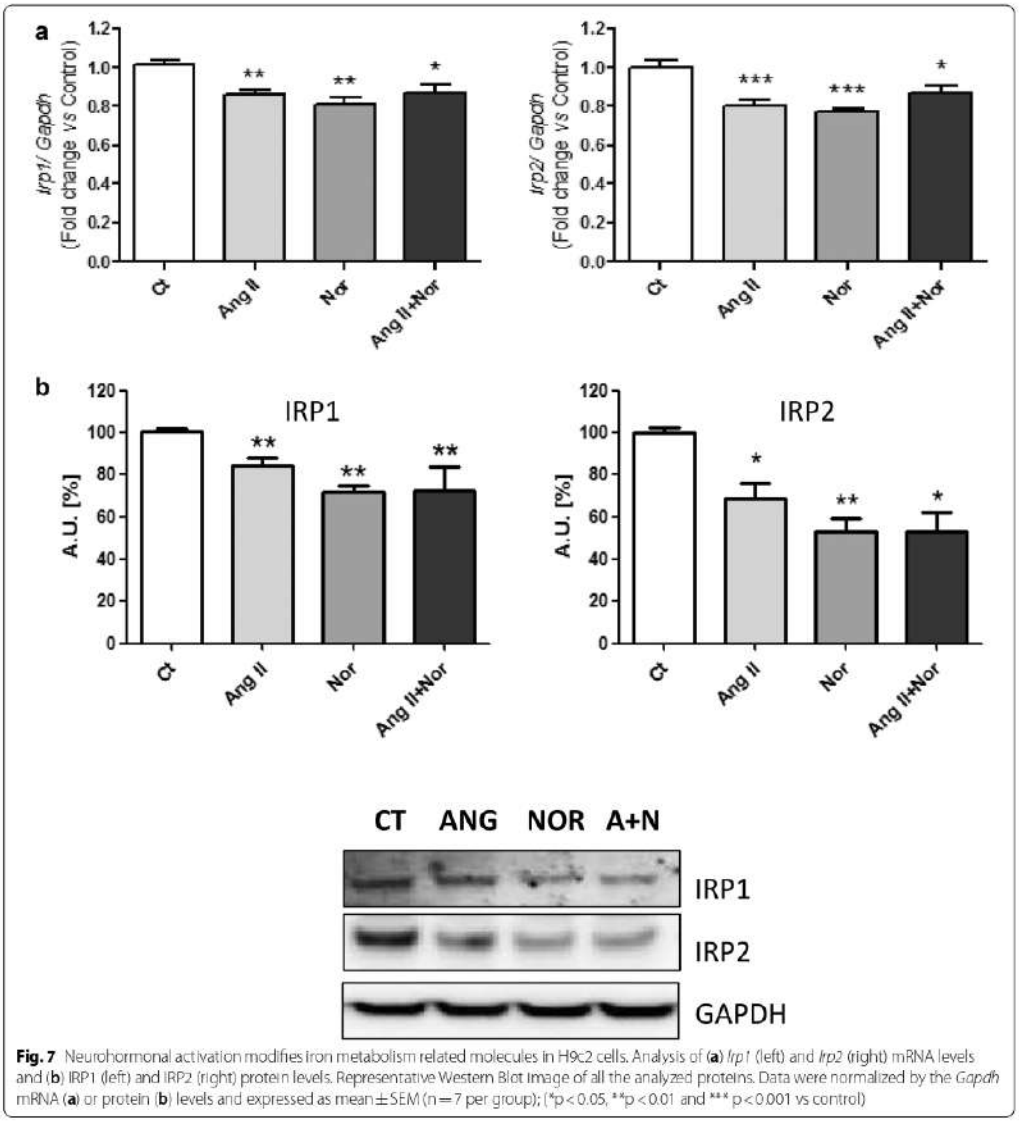


Fig. 7 Neurohormonal activation modifies iron metabolism related molecules in H9c2 cells. Analysis of (**a**) *Irp1* (left) and *Irp2* (right) mRNA levels and (**b**) IRP1 (left) and IRP2 (right) protein levels. Representative Western Blot image of all the analyzed proteins. Data were normalized by the *Gapdh* mRNA (**a**) or protein (**b**) levels and expressed as mean ± SEM (n = 7 per group); (*p < 0.05, **p < 0.01 and *** p < 0.001 vs control)

observed a decreased of Fpn1 compared to the control mice. On the other hand, Hamp was downregulated in isoproterenol-stimulated mice, as well as in cells challenged with Nor and both stimuli together. Therefore, although the Fpn1 levels decrease in the HF mice model, probably as an attempt to stop the iron release, the reduced Hamp levels could favor the release of iron by Fpn1. Nevertheless, Hamp was upregulated in the cells challenged only with Ang II. Interestingly, the expression of Hamp is up-regulated in iron-deprived environments to secure the intracellular iron content by exerting a negative feed-back on Fpn1 in the cardiomyocytes [40]. Our data suggest that while Ang II-stimulated cells would try to retain the intracellular

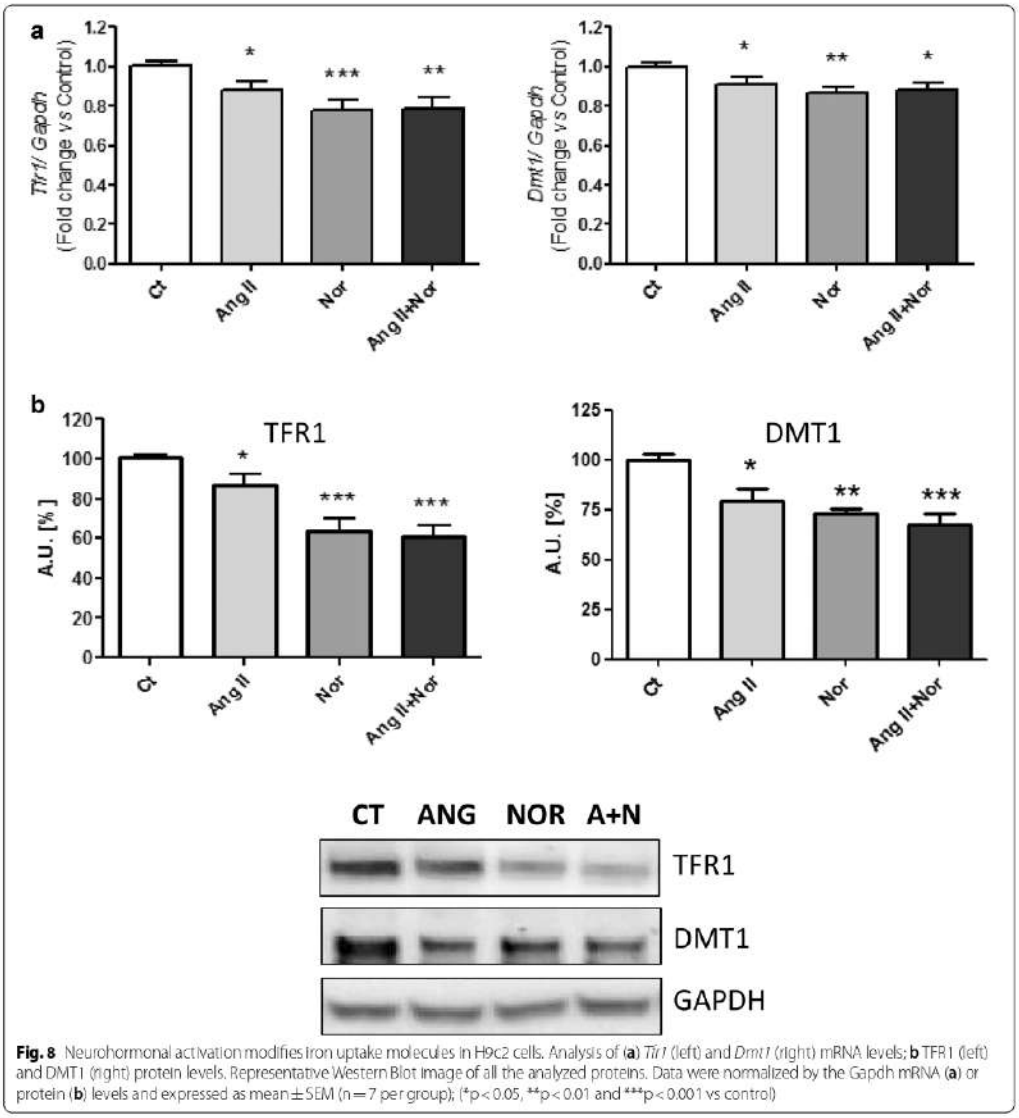


Fig. 8 Neurohormonal activation modifies iron uptake molecules in H9c2 cells. Analysis of (**a**) *Tfr1* (left) and *Dmt1* (right) mRNA levels; **b** TFR1 (left) and DMT1 (right) protein levels. Representative Western Blot image of all the analyzed proteins. Data were normalized by the Gapdh mRNA (**a**) or protein (**b**) levels and expressed as mean ± SEM (n = 7 per group); (*p < 0.05, **p < 0.01 and ***p < 0.001 vs control)

iron by increasing the levels of Hamp, the exposure to a more intense neurohormonal insult would counteract this compensatory mechanism, and could favored the iron release. To note, the *Hamp* knock-out mice induce myocardial ID and dilated cardiomyopathy due to an increase of iron release [42].

On the other hand, our study did not evidence changes in the cytoplasmic iron storage molecule ferritin by the neurohormonal activation stimuli (in any experimental condition). It is worth to note that ferritin may be further regulated by mechanisms others than Irps, such as oxidative stress [43].

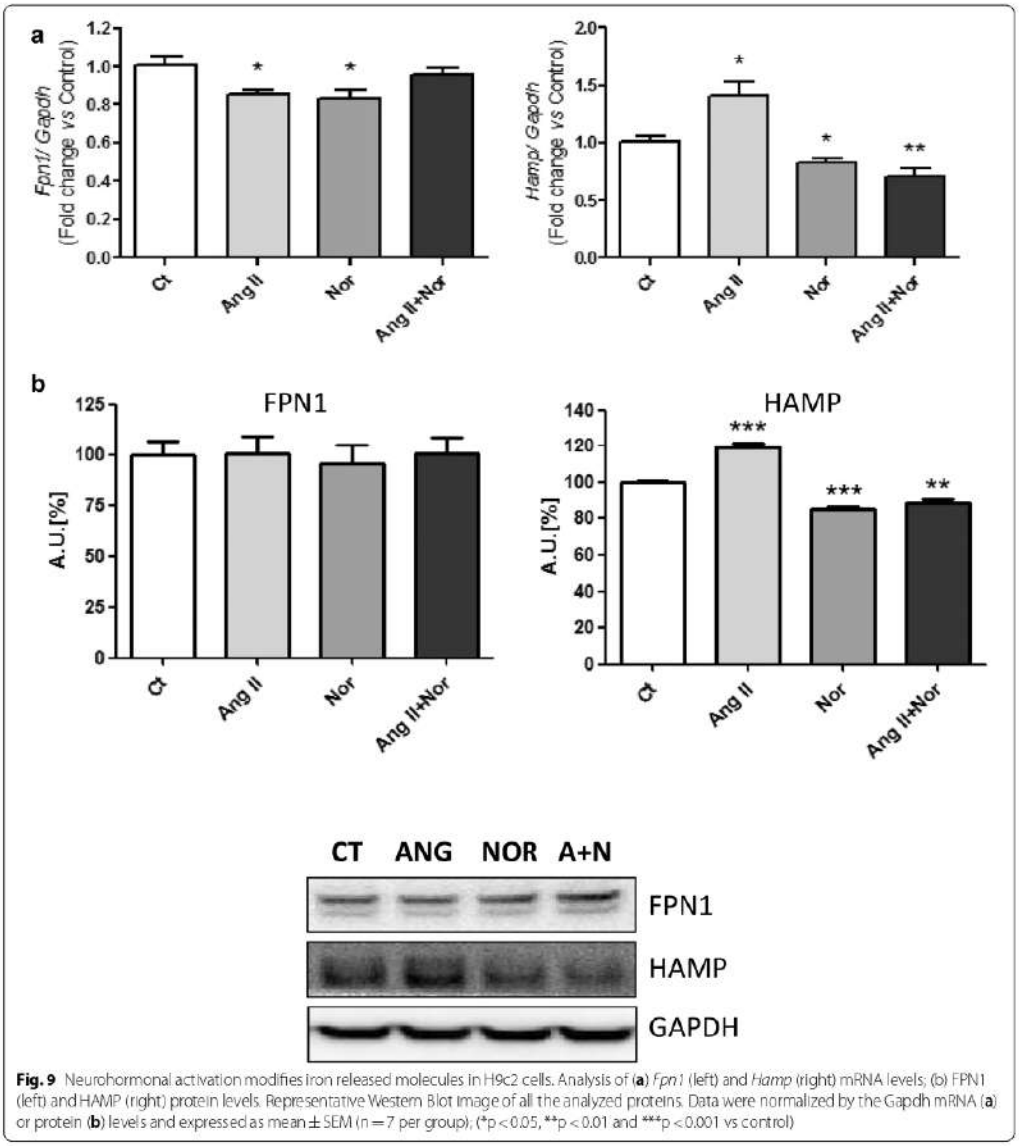


Fig. 9 Neurohormonal activation modifies iron released molecules in H9c2 cells. Analysis of (**a**) *Fpn1* (left) and *Hamp* (right) mRNA levels; (b) FPN1 (left) and HAMP (right) protein levels. Representative Western Blot image of all the analyzed proteins. Data were normalized by the Gapdh mRNA (**a**) or protein (**b**) levels and expressed as mean ± SEM (n = 7 per group); (*p < 0.05, **p < 0.01 and ***p < 0.001 vs control)

Altogether, our results suggest that neurohormonal activation may contribute to intracellular iron depletion by both increasing intracellular iron release and reducing extracellular iron uptake in cardiac cells. It means that the neurohormonal activation observed in HF patients could participate in disease progression through intracellular iron deprivation. However, further studies should be conducted to show the exact mechanisms underlying the neurohormonal regulation of these components in the failing heart.

The role of iron in cardiac function is closely related to the mitochondria because of its role as an enzymatic

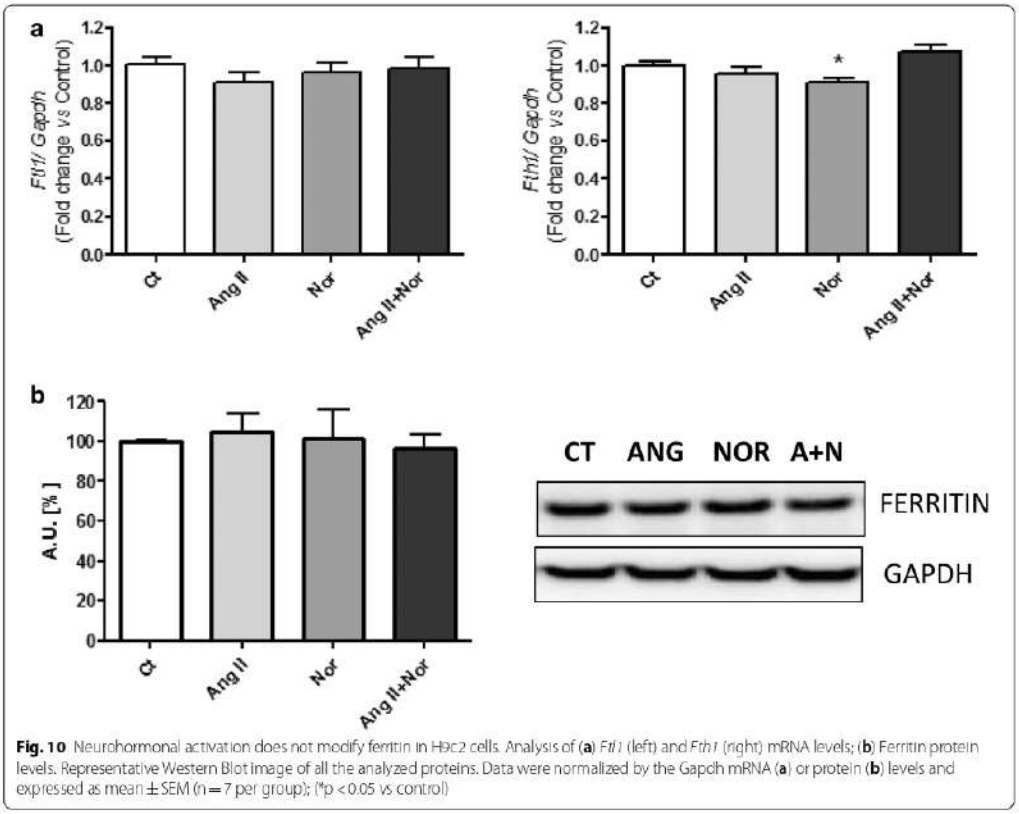


Fig. 10 Neurohormonal activation does not modify ferritin in H9c2 cells. Analysis of (**a**) *Ftl1* (left) and *Fth1* (right) mRNA levels; (**b**) Ferritin protein levels. Representative Western Blot image of all the analyzed proteins. Data were normalized by the Gapdh mRNA (**a**) or protein (**b**) levels and expressed as mean ± SEM (n = 7 per group); (*p < 0.05 vs control)

cofactor, and the role of mitochondrial dysfunction in HF is being increasingly recognized [44, 45]. Due to the importance of mitochondria in cardiomyocytes, we assessed the levels of the mitochondrial iron uptake transporter Mfrn and the iron storage molecule Ftmt. Mfrn1 and Mfrn2 were not altered in the neurohormonal-stimulated cells, although in hearts from isoproterenol-induced mice the Mfrn2 was down-regulated. Paradkar et al. showed that the reduction in *Mfrn1* and *Mfrn2* by RNA interference resulted in a decreased mitochondrial iron accumulation, heme synthesis and iron-sulfur cluster synthesis [46]. Interestingly, Mfrn2 is more ubiquitously expressed than Mfrn1. Besides, we observed a down-regulation of the Ftmt levels in all the experimental conditions. Our data are in line with those reported in hearts from Ftmt$^{-/-}$ mice, since these animals showed mitochondrial damage and fibril disorganization [40]. Moreover, given the antioxidant role of Ftmt [47], neurohormonal activation may directly contribute to make cardiac cells more sensitive to oxidative stress. This hypothesis is further supported by other studies showing the decrease of antioxidant enzymes in hearts from HF patients [22].

Our data revealed that neurohormonal activation increased ROS production and reduced mitochondrial membrane potential in cardiac cells, showing impairment in the mitochondria. Mitochondria are crucial to maintain the high-energy demand of the heart [10, 22]. Actually, mitochondrial function has been found impaired in myocardial tissue of advanced HF patients [10]. The role of myocardial and cardiomyocyte iron depletion leading to mitochondrial malfunction has been highlighted as one of the drivers impairing contractile function [19]. Accordingly, mitochondrial dysfunction found in the cells challenged with the neurohormonal stimuli was related to a decrease in the ATP levels. Altogether, our results are in line with the data reviewed by Brown

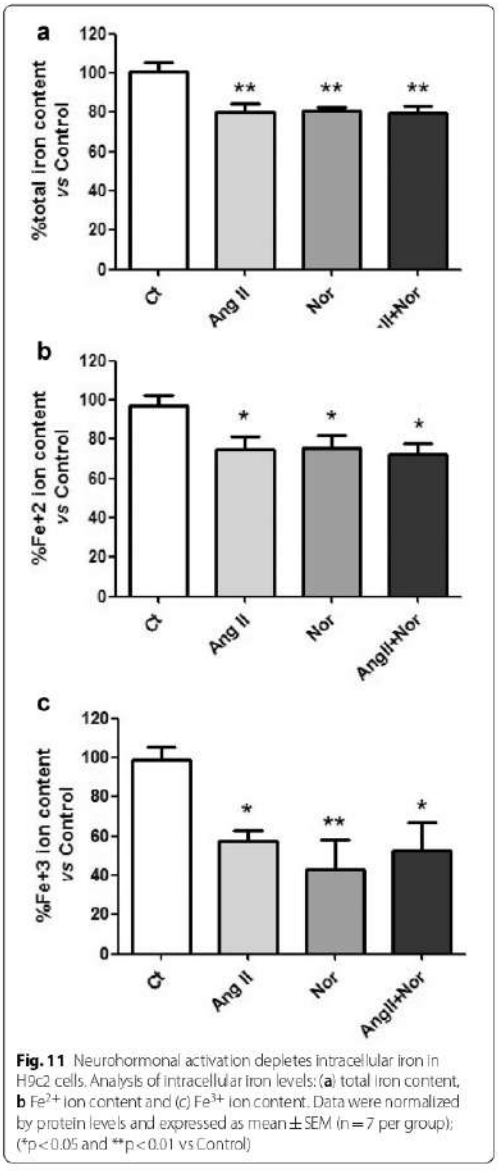


Fig. 11 Neurohormonal activation depletes intracellular iron in H9c2 cells. Analysis of intracellular iron levels: (**a**) total iron content, **b** Fe^{2+} ion content and (c) Fe^{3+} ion content. Data were normalized by protein levels and expressed as mean ± SEM (n = 7 per group); (*p < 0.05 and **p < 0.01 vs Control)

et al. suggesting that mitochondrial impairment could be linked to cardiomyocyte injury and HF progression [45].

Finally, Fig. 14 summarized the key results obtained in this work, showing the effect of neurohormonal activation over the main iron metabolism molecules, the intracellular iron deficiency and the mitochondrial dysfunction at cardiac cell level (Fig. 14).

Conclusions

In conclusion, our data provide evidences that the neurohormonal activation observed in the setting of HF promotes a reduction in intracellular iron levels within cardiac cells and hampers mitochondrial function. The molecular mechanisms mediating this relationship may involve a transcriptional regulation of several iron metabolism genes in the cell. The current results suggest that ID could be a key element in the pathophysiological sequence that leads to the progression of HF. Nevertheless, further research is necessary to fully characterize the role of iron in the pathophysiology of HF.

Materials and methods

Animal model

Sixteen 10-week-old male C57BL/6 mice received a continuous infusion of isoproterenol (ISO group; n = 8) or vehicle (saline) (Control group; n = 8) at a rate of 30 mg/kg/day for 28 days using a subcutaneously implanted osmotic mini-pump (Alzet, model 1004), to generate a validated model of experimental HF in mice [24]. The animals were anesthetized by isoflurane inhalation during the implantation pump. Buprenorphine (0.3 mg/kg, i.p.) was administered 10 min before surgery and after 24 h. Mice were euthanized by an i.p. injection of pentobarbital sodium (100 mg/kg). The experimental protocol was approved by the local Institutional Ethics Committee of the Institut Municipal d'Investigacions Mèdiques-Universitat Pompeu Fabra (CEEA-PRBB 16-1814I) and all animal procedures performed according to the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes.

Heart samples were fixed in buffered 4% paraformaldehyde, embedded in paraffin and cut into 4 µm-thick slices. Heart sections were deparaffinized and rehydrated with xylene, etanol (100%, 90%, 70%) and water, and stained with Masson's Trichrome Stain Kit (Polyscience) to assess cardiomyocyte hypertrophy. Representative ventricular photomicrographs (5–10 per animal) were acquired at 400 × magnification with a light microscope (BX61, Olympus) and a mounted digital camera (DP72, Olympus). LV cardiomyocyte area was measured in at least 30 random cardiomyocytes by outlining round to cuboidal-shaped nucleated cardiomyocites using the ImageJ software.

Cell culture model

Embryonic rat heart-derived cells (H9c2 cells) were maintained in a high-glucose Dulbecco's modified Eagle's medium (4.5 g/l glucose) (DMEM; supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/

ml streptomycin) at 37 °C and 5% CO_2. Cells were seeded in multi-well plates and serum deprived in DMEM supplemented with 1% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin for 16 h, and then stimulated with 1 µM of Ang II (Sigma Aldrich, Spain) or/and 2 µM of Nor (Sigma Aldrich, Spain) for 48 (n = 7 in each experimental group).

RNA preparation and quantitative real time reverse transcription-polymerase chain reaction (RT-PCR) analysis

Briefly, levels of mRNA were assessed by RT-PCR. Total RNA was isolated from H9c2 cells and mice heart using Nucleospin RNA II kit (Macherey–Nagel, Spain). RNA was quantified by a NanoDrop 1000 Spectrophotometer (Thermo Scientific, Spain). TaqMan gene expression assays-on-demand (Thermo Scientific, Spain) were used for rat myosin heavy chain 7 (Myh7) (Rn00691731_m1), rat natriuretic peptide B (*Bnp*) (Rn00580641_m1), rat iron regulatory protein 1 (*Irp1*) (Rn00569045_m1) and mouse *Irp1* (Mm01223514_m1), rat iron regulatory protein 2 (*Irp2*) (Rn00575852_m1) and mouse *Irp2* (Mm01179595_m1), rat ferroportin (*Fpn1*) (Rn00591187_m1) and mouse *Fpn1* (Mm01254822_m1), rat hepcidin antimicrobial peptide (*Hamp*) (Rn00584987_m1) and mouse *Hamp* (Mm00519025_m1), rat transferrin receptor 1(*Tfr1*) (Rn01474701_m1) and mouse *Tfr1* (Mm01344485_m1), rat divalent metal transporter 1 (*Dmt1*) (Rn01533109_m1) and mouse *Dmt1* (Mm01308330_s1), rat ferritin heavy chain 1 (*Fth1*) (Rn00820640_g1) and mouse *Fth1* (Mm00850707_g1), rat ferritin light chain 1 (*Ftl1*) (Rn04341729_g1) and mouse *Ftl1* (Mm03030144_g1), rat mitochondrial ferritin (*Ftmt*) (Rn01492073_s1) and mouse *Ftmt* (Mm01268428_s1), rat mitoferrin 1 (*Mfrn1*) (Rn01753423_m1) and mouse *Mfrn1* (Mm00471133_m1), rat mitoferrin 2 (*Mfrn2*) (Rn01411393_m1) and mouse *Mfrn2* (Mm01199497_m1). Glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) was used as the endogenous control, rat (Rn01775763_g1) and mouse (Mm99999915_g1). The results were normalized to *Gadph*, and relative quantification was performed using the comparative Ct (2-DDCt) method. mRNA levels were expressed as fold induction over control.

Immunoblotting

Whole protein extracts were collected from H9c2 cells in RIPA buffer (0.1% SDS, 150 mM NaCl, 1% Nonidet P40, 50 mM Tris–HCl, 0.5% deoxycholate) containing phosphatase and protease inhibitors (Roche Diagnostics; Basel, Switzerland) and western blot analyses were performed using antibodies against MYH7, TFR1, FT, HAMP, FTMT and GAPDH from Abcam (USA), and FPN1, IRP1, IRP2, MFRN1, MFRN2 and DMT1 from Thermo Scientific (Spain). Detection was performed using the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody (Dako; Glostrup, Denmark). The bands were visualized using ClarityTM Western ECL Substrate (BioRad, Spain) with the Quantity One software (BioRad, Spain). Differences in the protein levels were expressed as arbitrary units (A.U.) percentage induction over control.

Intracellular iron determination

The intracellular iron ions content was determined in hearts and H9c2 cells using the colorimetric Iron Assay kit (Abcam, Spain), following the manufacturer's instructions. All assays were performed in duplicate, and measured on a Tecan Infinite F200 microplate reader. Levels of total iron, as well as the ions Fe^{2+} and Fe^{3+}, were expressed relative to untreated controls.

Mitochondrial membrane potential

The mitochondrial membrane potential was determined using the reagent TMRE-Mitochondrial Membrane Potential Assay Kit (Abcam; Spain). All assays were performed in duplicate, and measured on a Tecan Infinite F200 microplate reader. Levels of fluorescence were expressed relative to untreated controls.

Intracellular radical oxygen species (ROS) determination

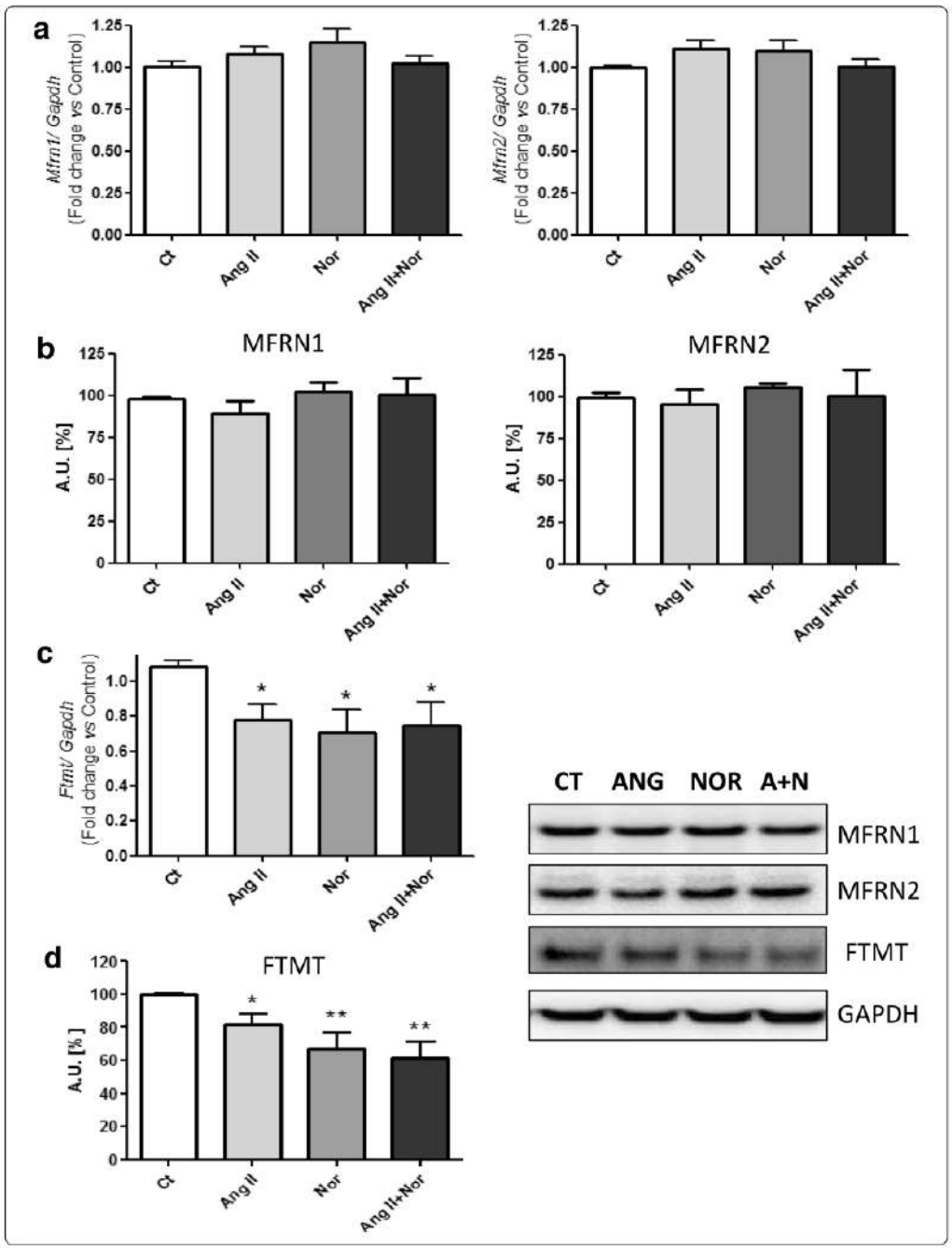
Intracellular ROS levels were determined using Cellular Reactive Oxygen Species Detection Assay Kit (Abcam; Spain). All assays were performed in duplicate, and measured on a Tecan Infinite F200 microplate reader. Levels of ROS were expressed relative to untreated controls.

Detection of adenosine triphosphate (ATP) content

Intracellular ATP content was determined by the ATP Assay kit (Abcam; Spain). All assays were performed in duplicate, and measured on a Tecan Infinite F200 microplate reader. Levels of ATP were expressed relative to untreated controls.

(See figure on next page.)

Fig. 12 Neurohormonal activation modifies mitochondrial iron storage related molecules in H9c2 cells. Analysis of (**a**) *Mfrn1* and *Mfrn2* mRNA levels, (**b**) MFRN1 and MFRN2 protein levels, (**c**) *Ftmt* mRNA levels and (d) FTMT protein levels. Representative Western Blot image of all the analyzed proteins. Data were normalized by the Gapdh mRNA (**a**, **c**) or protein (**b**, **d**) levels and expressed as mean ± SEM (n = 7 per group); (*p < 0.05 and **p < 0.01 vs Control)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b
MFRN1
MFRN2
A.U. [%]
125
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75
50
25
0
c
Ftmt/ Gapdh (Fold change vs Control)
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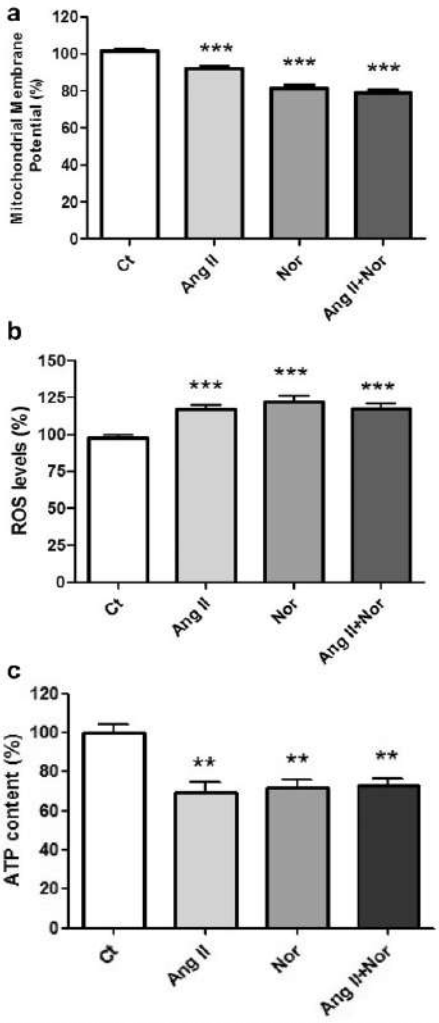

Fig. 13 Neurohormonal activation impaires mitochondrial function. Quantification of different parameters: (**a**) Mitochondrial membrane potential, **b** intracellular ROS production, and (**c**) ATP levels. Data were normalized by the cellular protein content and expressed as mean ± SEM (n = 7 per group); (**p < 0.01 and *** p < 0.001 vs control)

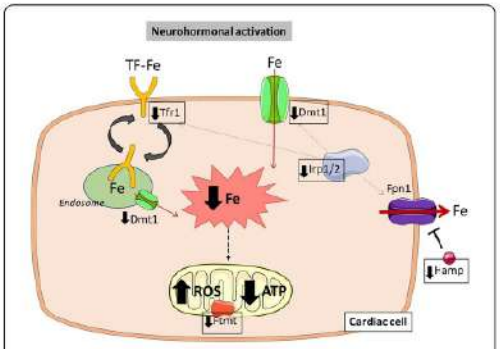

Fig. 14 Changes in iron metabolism due to neurohormonal activation in cardiac cells. This illustration describes the key iron molecules modified under neurohormonal activation in cardiac cells. It is also defined the iron deficiency and the mitochondrial dysfunction present under the neurohormonal stimuli. *ATP* adenosine triphosphate, *Dmt1* divalent metal transporter 1, *Fe* iron, *Fpn1* ferroportin 1, *Ftmt* mitochondrial ferritin, *Hamp* hepcidin, *Irp1/2* iron regulatory proteins ½, *ROS* reactive oxygen species, *Tf-Fe* transferrin-bound iron, *Tfr1* transferrin receptor 1

Statistical analysis

Data are expressed as the mean ± standard error of the mean (SEM). Significant differences were established using Student's t-test or one-way analysis of variance (ANOVA), followed by Bonferroni's post hoc test, as appropriate. Data were analysed by using the GraphPad Instat programme (GraphPad Prism 6.01 Software Inc., USA). Differences were considered statistically significant at P-value < 0.05.

Abbreviations

HF: Heart failure; Bnp: Brain natriuretic peptide; Irps: Iron regulatory proteins; Fth1: Ferritin heavy chain 1; Ftl1: Ferritin light chain 1; Hamp: Hepcidin antimicrobial peptide; Tfr1: Transferrin receptor 1; Myh7: Myosin heavy chain 7; Dmt1: Divalent metal transporter 1; AngII: Angiotensin II; Nor: Norepinephrine; ID: Iron deficiency; RNA: Ribonucleic acid; ROS: Radical oxygen species; Fpn1: Ferroportin 1; Ftmt: Mitochondrial ferritin; Mfrn: Mitoferrin; ATP: Adenosin triphosphate.

Acknowledgements

We thank CERCA Programme/Generalitat de Catalunya for institutional support.

Authors' contributions

Conceptualization, MT, CDL, CE, PM and JCC; methodology, MT, CDL, LY, SGS, JLF, and JCC; validation, MT, CE, PM, SGS, LY, BB and JCC; formal analysis, MT, CDL, CE, PM and JCC; investigation, MT, CDL, CE, PM, JGC and JCC; resources, MT, CE and JCC; writing—original draft preparation, MT, CDL and JCC; writing—review and editing, MT, CDL, CE, PM, AG, SJM, SY, LA, JGC, EGR, BB and JCC; supervision, MT, CE and JCC; project administration, MT, CE and JCC; funding acquisition, MT, CE, PM and JCC. All authors read and approved the final manuscript.

Funding

This work was funded by the following Grants: unrestricted grant from Vifor Pharma and Basic Research Competitive Grant in Cardiology from the Spanish Society of Cardiology 2015.

Availability of data and materials

All relevant data are included in this published article.

Declarations

Ethics approval and consent to participate

The experimental protocol was approved by the local Institutional Ethics Committee of the Institut Municipal d'Investigacions Mèdiques-Universitat Pompeu Fabra (CEEA-PRBB 16-1814I) and all animal procedures performed according to the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes.

Consent for publication

Not applicable.

Competing interests

Dr. Josep Comin-Colet has received honoraria for speaking for Vifor Pharma and consultancy fees from Vifor Pharma.

Author details

[1]Bio-Heart Cardiovascular Diseases Research Group, Bellvitge Biomedical Research Institute (IDIBELL), L'Hospitalet de Llobregat, Barcelona, Spain. [2]Cardiology Department, Bellvitge University Hospital, L'Hospitalet de Llobregat, Barcelona, Spain. [3]Advanced Heart Failure and Heart Transplant Unit, Cardiology Department, Bellvitge University Hospital, L'Hospitalet de Llobregat, Barcelona, Spain. [4]Community Heart Failure Program, Cardiology Department, Bellvitge University Hospital, L'Hospitalet de Llobregat, Barcelona, Spain. [5]Vascular Biology and Metabolism Program, Vall d'Hebron Research Institute (VHIR), Barcelona, Spain. [6]Cardiology Department, Hospital Vall d'Hebron Hospital, Barcelona, Spain. [7]Department of Clinical Sciences, School of Medicine, University of Barcelona, Barcelona, Spain. [8]Department of Medicine, Universitat Autònoma de Barcelona, Barcelona, Spain. [9]Department of Internal Medicine, Bellvitge University Hospital, L'Hospitalet de Llobregat, Barcelona, Spain.

Received: 31 December 2020 Accepted: 6 May 2021
Published online: 17 May 2021

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Publisher's Note

Supplemental material

All relevant data are included in this published article

2. Neurohormonal activation induces intracellular iron deficiency and mitochondrial dysfunction in cardiac cells.

In this study we aimed to uncover the biological connections between HF and ID to identify additional components of disease progression and discover new potential prognostic biomarkers. We analyzed the gene expression patterns of patients from the DAMOCLES study, a cohort of chronic HF patients with reduced ejection fraction. We performed comprehensive transcriptomic analyses on a subset of patients without anemia and with or without systemic ID. Subsequently, we assessed the differential gene expression of selected candidate genes representatives of iron regulation, metabolism, and microvascular function relative to the presence of ID, in an independent cohort.

In the whole transcriptome analyses, we identified 1128 differentially expressed transcripts related to iron status in our exploratory cohort. The analysis indicated that ID in HF induces alterations in key biological pathways, encompassing iron metabolism, mitochondrial function, intracellular energy production, oxidative stress, calcium ion signaling, platelet aggregation, and transforming growth factor pathway signaling; these collectively serve as surrogate markers for cardiovascular function, systemic inflammation, and elements involved in key mechanisms to maintain cellular stability and adaptability under stress conditions such as autophagy, DNA repair, and apoptosis. Next, we validated our results in a larger cohort of 71 patients, focusing on 22 genes with statistical significance and physiological relevance.

The gene ontology enrichment analysis corroborated that the functions of these selected genes align with the key pathophysiological pathways previously identified in the whole-transcriptome analysis, including cardiovascular homeostasis, metabolism, and intracellular homeostasis. Patients with systemic ID exhibited lower mRNA levels of mitochondrial ferritin, sirtuin-7, small integral membrane protein 20, adrenomedullin, and endothelin converting enzyme-1. Importantly, intermediate expression of mitochondrial ferritin and varying levels of sirtuin-7 and small integral membrane protein 20 mRNA are associated with increased risks of all-cause mortality and HF admission.

This study reveals that there are distinct blood gene expression patterns in chronic HF patients depending on their systemic iron status. These patterns involve crucial genes related to iron regulation, mitochondrial function, endothelial health, and cardiovascular physiology. Importantly, the genetic expression trends of certain genes correlated with adverse clinical outcomes.

Journal of *Clinical Medicine*

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Blood Differential Gene Expression in Patients with Chronic Heart Failure and Systemic Iron Deficiency: Pathways Involved in Pathophysiology and Impact on Clinical Outcomes

Carles Díez-López [1,2,3,4], Marta Tajes Orduña [1], Cristina Enjuanes Grau [1,2,5], Pedro Moliner Borja [1,2,5], José González-Costello [1,2,3,4], Elena García-Romero [1,2,3], Josep Francesch Manzano [1], Sergi Yun Viladomat [1,5,6], Santiago Jiménez-Marrero [1,2,4,5], Raul Ramos-Polo [1,2,5], Maria del Mar Ras Jiménez [1,5,6] and Josep Comín-Colet [1,2,4,5,*]

1 Bio-Heart Cardiovascular Diseases Research Group, Bellvitge Biomedical Research Institute (IDIBELL), L'Hospitalet de Llobregat, 08907 Barcelona, Spain; cdiez@bellvitgehospital.cat (C.D.-L.); mtajes@idibell.cat (M.T.O.); cenjuanes@bellvitgehospital.cat (C.E.G.); pmoliner@bellvitgehospital.cat (P.M.B.); jgonzalez@bellvitgehospital.cat (J.G.-C.); e.garcia.r@bellvitgehospital.cat (E.G.-R.); jfrancesch@idibell.cat (J.F.M.); syunvi@gmail.com (S.Y.V.); sjimenezm@bellvitgehospital.cat (S.J.-M.); raulramospolo@gmail.com (R.R.-P.); mras@bellvitgehospital.cat (M.d.M.R.J.)
2 Community Heart Failure Unit, Cardiology Department, Bellvitge University Hospital, L'Hospitalet de Llobregat, 08907 Barcelona, Spain
3 Advanced Heart Failure and Heart Transplant Unit, Cardiology Department, Bellvitge University Hospital, L'Hospitalet de Llobregat, 08907 Barcelona, Spain
4 Department of Clinical Sciences, School of Medicine, University of Barcelona, 08907 Barcelona, Spain
5 Community Heart Failure Program, Cardiology Department, Bellvitge University Hospital, L'Hospitalet de Llobregat, 08907 Barcelona, Spain
6 Department of Internal Medicine, Bellvitge University Hospital, L'Hospitalet de Llobregat, 08907 Barcelona, Spain
* Correspondence: jcomin@bellvitgehospital.cat; Tel.: +34-932-607-078

Abstract: Background: Iron deficiency is a common disorder in patients with heart failure and is related with adverse outcomes and poor quality of life. Previous experimental studies have shown biological connections between iron homeostasis, mitochondrial metabolism, and myocardial function. However, the mechanisms involved in this crosstalk are yet to be unfolded. Methods: The present research attempts to investigate the intrinsic biological mechanisms between heart failure and iron deficiency and to identify potential prognostic biomarkers by determining the gene expression pattern in the blood of heart failure patients, using whole transcriptome and targeted TaqMan® low-density array analyses. Results: We performed a stepwise cross-sectional longitudinal study in a cohort of chronic heart failure patients with and without systemic iron deficiency. First, the full transcriptome was performed in a nested case-control exploratory cohort of 7 paired patients and underscored 1128 differentially expressed transcripts according to iron status (cohort1#). Later, we analyzed the messenger RNA levels of 22 genes selected by their statistical significance and pathophysiological relevance, in a validation cohort of 71 patients (cohort 2#). Patients with systemic iron deficiency presented lower mRNA levels of mitochondrial ferritin, sirtuin-7, small integral membrane protein 20, adrenomedullin and endothelin converting enzyme-1. An intermediate mitochondrial ferritin gene expression and an intermediate or low sirtuin7 and small integral membrane protein 20 mRNA levels were associated with an increased risk of all-cause mortality and heart failure admission ((HR 2.40, 95% CI 1.04–5.50, p-value = 0.039), (HR 5.49, 95% CI 1.78–16.92, p-value = 0.003), (HR 9.51, 95% CI 2.69–33.53, p-value < 0.001), respectively). Conclusions: Patients with chronic heart failure present different patterns of blood gene expression depending on systemic iron status that affect pivotal genes involved in iron regulation, mitochondrial metabolism, endothelial function and cardiovascular physiology, and correlate with adverse clinical outcomes.

Keywords: heart failure; iron deficiency; cardiac metabolism; mitochondria

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Citation: Díez-López, C.; Tajes Orduña, M.; Enjuanes Grau, C.; Moliner Borja, P.; González-Costello, J.; García-Romero, E.; Francesch Manzano, J.; Yun Viladomat, S.; Jiménez-Marrero, S.; Ramos-Polo, R.; et al. Blood Differential Gene Expression in Patients with Chronic Heart Failure and Systemic Iron Deficiency: Pathways Involved in Pathophysiology and Impact on Clinical Outcomes. *J. Clin. Med.* **2021**, *10*, 4937. https://doi.org/10.3390/jcm10214937

Academic Editor: Andrea Frustaci

Received: 8 September 2021
Accepted: 22 October 2021
Published: 26 October 2021

1. Introduction

Heart failure (HF) is a world-wide epidemic caused by functional and structural myocardial abnormalities that result in congestion, functional impairment and eventually, multiorgan dysfunction [1]. Contemporary medical treatment is based on the prevention of disease progression by extensive pharmacological neurohormonal blockade and device therapies [2]. Yet, despite the recent advances in the understanding of the pathophysiology of HF and the advent of new pharmacologic and device-based treatments, these patients present progressive clinical deterioration, limited life expectancy and great limitations in quality of life [3].

In the last years, iron deficiency (ID) has emerged as a key element in the current understanding of HF pathophysiology. Derangements in iron homeostasis are frequent in HF patients [4] and come along with a negative clinical impact regarding functional capacity, disease progression and mortality, independent of the hemoglobin levels [5–8]. Moreover, patients with impaired iron transport capacity (functional ID), have worse prognosis, independent of the presence of absolute ID [9,10]. Indeed, the treatment with intravenous iron has demonstrated to have a positive impact in functional capacity, quality of life and HF-related hospitalizations [11–13].

Nonetheless, little is known about the mechanisms by which ID occurs in HF patients, the interplay between systemic and myocardial iron status with the HF syndrome itself and the precise role of iron in myocardial performance. A combination of different clinical factors such as a reduced iron uptake, increased iron loss and impaired iron utilization explains at least part of the etiologies of ID in HF patients [14]. Recent clinical data demonstrate that patients with increased neurohormonal sympathetic activation present an impaired iron transport and increased iron demand [15], and myocardial iron content is reduced, and mitochondrial function is impaired in patients with end-stage disease [16]. Also, experimental data have disclosed part of the potential role of iron in heart function and myocardial metabolism. Iron regulation is impaired in the cardiomyocytes of patients with HF [17] and cardiomyocyte contractility is reduced in the presence of myocardial ID [18]. Moreover, recent experimental data suggest that HF neurohormonal activation induces myocardial iron depletion leading to impaired mitochondrial inducing structural and functional modifications in biological pathways in cardiomyocyte iron regulating elements that could provoke an increase in iron release and a reduction in iron uptake [19].

Despite the accumulating data, the intrinsic pathophysiological mechanisms involved in the interplay between ID and HF progression are yet to be defined. The study of 'omics' allows collection and characterization of diverse biological processes at different levels [20]. In this regard, the study of RNA expression (transcriptome) using high throughput technology offers a unique opportunity to massively investigate the genetic transcription and discover new mechanisms of disease [21]. Data investigating the proteomics in chronic HF patients have disclosed a few insights on the multifactorial origin of ID in patients with HF [22].

In the present study we aimed to define the biological pathways connecting ID and HF by using transcriptome analysis in a cohort of HF patients with and without systemic ID, and to explore the potential impact of genetic modifications with clinical outcomes. We hypothesized that our work might help to unveil new regulation pathways involved in intracellular and systemic iron homeostasis, to clarify its interrelation with neurohormonal activation, mitochondrial function and oxidative stress, and to identify new potential biomarkers and therapeutic targets to be used in the management of HF patients.

2. Materials and Methods

2.1. Study Design, Study Population and Ethics

The definition of the neurohormonal activation, myocardial function, genomic expression and clinical outcomes in heart failure patients (DAMOCLES) study was a single-center, observational, prospective cohort study of 1236 consecutive patients diagnosed with chronic HF recruited between January 2004 and January 2013.

The methodology of the DAMOCLES study has been published previously by our group [6,15,23–29]. Briefly, for inclusion, patients had to be diagnosed with chronic HF according to the European Society of Cardiology diagnostic criteria, have had at least one recent acute decompensation of HF requiring intravenous diuretic therapy (either hospitalized or in the day-care hospital), and had to be in stable condition at the time of study entry. Exclusion criteria were significant primary valvular disease, clinical signs of fluid overload, pericardial disease, restrictive cardiomyopathy, hypertrophic cardiomyopathy, hemoglobin (Hb) levels < 8.5 g/dL, active malignancy, and chronic liver disease. The patients were recruited regardless of the percentage of left ventricular ejection fraction (LVEF). In the DAMOCLES study, HF with reduced LVEF (HFrEF) was defined as LVEF ≤ 45%. The study was approved by the local committee of ethics for clinical research and was conducted in accordance with the principles of the Declaration of Helsinki. All patients gave written informed consent before study entry.

The objectives of the present study were (1) to define the pathophysiological pathways and its key biological components involved in ID in patients with chronic HFrEF and (2) explore the association of these components with clinical outcomes. First, we planned to perform a full transcriptome analysis of peripheral blood in a first cohort of patients (cohort #1) with HFrEF and ID (cases) and compare the blood RNA expression profiles of these patients with the patterns of expression in matched patients with HFrEF without ID (controls). As a second step, the differences of blood gene expression observed in the transcriptome analysis of cohort #1 were planned to be explored and replicated in a second nested case-control (ID vs. non-ID) cohort (cohort #2) of patients with HFrEF using real-time PCR. Finally, we explored the association between the gene expression of those genes significantly associated with iron status and clinical outcomes including the composite endpoint of all-cause death or HF hospitalization (primary endpoint) and all-cause death (secondary endpoint).

2.2. Selection Criteria and Definition of Study Cohorts

For the purpose of the present analysis, we selected 2 different nested case-control samples of patients with HFrEF. The first cohort (cohort #1) consisted in a nested-case control sample of 14 non-anemic (hemoglobin ≥ 12.5 g/dL) patients with HF (7 cases with ID and 7 matched controls without ID) from the overall DAMOCLES cohort study. The second cohort (cohort #2) was a larger nested case-control sample consisting in 71 patients with HF (35 cases with ID and 36 matched controls without ID) from the DAMOCLES study irrespective of hemoglobin levels (Hb > 9 g/dL). In both cohorts, ID and non-ID subsamples were matched according to the following variables: sex, LVEF, estimated glomerular filtration rate, etiology, hemoglobin, age and BMI.

2.3. Baseline Clinical Assessment

A detailed baseline evaluation was performed for all participants at study entry. This included collection of information about demographic characteristics, exhaustive medical history to gather clinical and disease related factors such as New York Heart Association (NYHA) functional class, comorbidities, laboratory information, medical treatments, and the most recent LVEF. Sources of information were the medical history and standardized questionnaires.

2.4. Blood Sample Management and Laboratory Assessments

Laboratory data and blood sample management methods have been previously reported by our group [6,10,15,24,25]. Briefly, patients were resting in a supine position in a quiet room for 30–60 min after venous cannulation. Blood samples were collected using Vacutainer® (BD, Franklin Lakes, NJ, USA) and 10 mL EDTA tubes and immediately processed as follows: 4 plasma aliquots 250–500 µL each from one 10 mL EDTA tube, 4 serum aliquots 250–500 µL each from 5 mL serum Vacutainer® tubes. Resulting plasma and serum aliquots were frozen and stored at −80 °C using the Micronics® (Micronics

Ltd., High Wycombe, Bucks, UK) system. Blood for DNA and/or RNA studies was stored in 10 mL EDTA tubes at −80 °C until being processed for extraction. Additional blood samples collected on EDTA and Vacutainer® tubes were immersed in melting ice and frozen until they were processed for routine local laboratory analyses.

Serum N-terminal pro b-type natriuretic peptide (NT-proBNP) levels were measured in pg/mL using an immunoassay based on chemiluminescence using Elecsys System (Roche®, Basel, Switzerland). Serum iron (mg/dL) was measured using spectrophotometry; serum ferritin (ng/mL) and transferrin (mg/dL) were measured using immunoturbidimetry. Transferrin saturation (TSAT) was estimated using the formula: TSAT = serum iron (mg/dL)/(serum transferrin (mg/dL) × 1.25). Iron status was also assessed by measuring serum soluble transferrin receptor (sTfR in mg/L) levels using an enzyme immunoassay. Hb (g/dL) was measured with impedance laser colorimetry. The glomerular filtration rate (GFR) was estimated from serum creatinine using the formula of Modification of Diet in Renal Disease Study Group (MDRD equation). Iron deficiency was defined using the Kidney Disease Outcomes Quality Initiative (KDOQI) criteria which categorizes ID as ferritin < 100 ng/mL and/or TSAT < 20% [10,15].

2.5. RNA Extraction and Blood Gene Expression Analyses

First, total RNA was isolated from whole blood samples using Nucleospin RNA II kit (Macherey-Nagel©, Düren, North Rhine-Westphalia, Germany) according to the manufacturer's recommendations. RNA was quantified by a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific©, Waltham, MA, USA).

The transcriptome analyses were undertaken in the blood samples of patients from cohort#1. Transcriptome processing protocol was performed as previously described by García-Díez et al. [30] Briefly, sample processing, amplification, labeling, and hybridizations were performed according to the protocol for the GeneChip WT PLUS Reagent kit and then hybridized to GeneChip Human Gene 2.0 ST Array (Affymetrix) in a GeneChip Hybridization Oven 640 (Thermo Fisher Scientific©, Waltham, MA, USA). Washing and scanning were performed using the Expression Wash, Stain and Scan Kit and the GeneChip System of Affymetrix (GeneChip Fluidics Station 450 and GeneChip Scanner 3000 7G). After quality control of raw data, they were background corrected, quantile normalized and summarized to a gene level using the robust multichip average (RMA) [31], resulting in a total of 48,144 transcript clusters, which roughly corresponded to genes or other mRNAs as miRNAs or lncRNAs. Linear Models for Microarray (LIMMA) was used to detect paired differentially expressed genes between the two different samples, with a p-value less than 0.05 and an absolute fold change above 1.2 considered as significant. The omics data generated in this study have been deposited in NCBI's Gene Expression Omnibus [20] and are available through GEO Series accession number GSE175739.

Based on transcriptome analyses, the 1128 transcripts that were differentially expressed in ID samples compared to controls were classified in 20 main ontology clusters using Gene Ontology (GO) and Kyoto encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis performed by Metascape [32]. Based on that classification, statistical significance and pathophysiology, 22 genes were selected to be explored in cohort #2. The 22 candidate genes were also classified in 8 main ontology clusters using GO and KEGG pathway enrichment analysis performed by Metascape.

As a second step, the RNA expression levels of the 22 candidate genes selected from transcriptome analysis were further explored in the blood samples of cohort #2 using real time (rt) quantitative Polymerase Chain Reaction (PCR) analysis by TaqMan® Low Density Array (TLDA) technology (Thermo Fisher Scientific©, Waltham, MA, USA). Briefly, gene expression was evaluated by generating a custom-made Applied Biosystems TaqMan® Low Density Array (TLDA). Accordingly, the gene expression of selected genes was assessed by TLDA in a nested case-control sample of 71 patients with HFrEF (cohort #2) consisting of 35 patients with ID and 36 patients with normal iron status. Data were normalized to 18s, and relative quantification was performed using the comparative Ct (cycle threshold)

method (2-DDCt). According to this, mRNA levels of ID patients (cases) were expressed as fold change vs. control (samples without ID). In addition, we explored the relationship between individual gene expression, expressed as delta Ct values of individual genes, and clinical outcomes. Delta Ct values corresponded to the difference between CtSOI (sequence of interest) and Ct of gene 18s, our reference housekeeping gene sequence (CtRS). Delta Ct was calculated subtracting CtRS from CtSOI. This approach has been used in previous research [33–35].

2.6. Follow Up and Major Heart Failure Events Ascertainment

Follow-up in DAMOCLES lasted until November 2015. Study participants were followed for a median of 2.93 years (mean 3.3 years). Follow-up was conducted by trained study personnel. Specifically, data on mortality and on cause of death were obtained from hospital and primary care electronic medical records, and/or by direct interview with the patients' relatives.

2.7. Statistical Methods

Using the baseline and follow-up data from the DAMOCLES cohort, cross-sectional and longitudinal descriptive analyses were performed. Demographic and clinical characteristics, as well as laboratory tests results were summarized using basic descriptive statistics according to iron status strata in both cohorts.

For categorical variables, number and percentage were reported, and for continuous variables, mean (standard deviation) or median (interquartile range) were used, depending on the distribution of the variables. χ^2, Student's T, and non-parametric tests were used to compare characteristics across strata.

In the PCR-Real time analysis, relative gene expression data from blood samples were expressed as the mean ± standard error of mean (SEM) and significant differences between strata were established by Student's t-test.

To explore the association between RNA expression of these genes and the primary composite (all-cause death or HF hospitalization) and secondary (all-cause death) endpoints, we developed several generalized additive models (GAM). These models were designed to explore parametric and non-parametric relationships between blood gene expression, expressed as delta Ct values of individual genes, and outcomes, expressed as the beta estimates of primary and secondary endpoints. Ct levels are inversely proportional to the amount of target nucleic acid in the sample (i.e., the lower the Ct level the greater the amount of target nucleic acid in the sample). All models were adjusted for important prognostic variables.

Furthermore, these adjusted models were replicated using adjusted Cox proportional hazards analyses exploring associations of gene expression with outcomes. In these models, gene expression expressed as delta Ct values of individual genes was divided in tertiles and tertiles were grouped considering the predominance of linear or non-linear patterns of association for each gene.

All statistical tests and confidence intervals (CI) were constructed with a type I error alpha level of 5%. For transcriptome analyses, adjusted p-value for multiple hypothesis testing was evaluated using the Benjamini-Hochberg method. p-Values below 0.05 were considered statistically significant. All analyses were performed using SPSS software (version 25.0; IBM, Armonk, NY, U.S.), InStat GraphPad, and R software (versions 4.0.2 and 3.1.1; R Foundation for Statistical Computing, Vienna, Austria). Bioinformatic analyses of the transcriptome were conducted using the R packages aroma Affymetrix, Biobase and Limma [30].

3. Results

In total, a nested case-control sample of 14 patients with HF (7 cases with ID and 7 matched controls without ID) from the DAMOCLES study defined cohort #1 (transcriptome cohort). Equally, a larger nested case-control sample of 71 patients with HF (35 cases

with ID and 36 matched controls without ID) from the DAMOCLES study defined cohort #2 (rt-PCR evaluation cohort).

As shown in Table 1, study groups in both cohorts were well balanced according to baseline characteristics other than iron status. One third of patients were female, most of patients where in NYHA functional class I or II, mean LVEF was below 40%, and the most common HF etiology was ischemic cardiomyopathy. No between-groups differences were found regarding the use of beta-blockers, angiotensin receptor antagonists or diuretics in any of the cohorts. Finally, the prevalence of common HF comorbidities was similar between ID and non-ID patients, although in cohort 2# patients with ID where more frequently diabetic when compared to patients without ID (28 (77.8%) vs. 18 (51.5%); p: 0.002). A similar trend was found in Cohort 1# (5 (71.4% vs. 2 (28.6%) p: 0.286)).

Table 1. Characteristics of cohorts included into the study. Cohort #1 (n = 14, transcriptome analysis), Cohort #2 (n = 71, TLDA-PCR).

	Cohort #1 (Iranscriptome) n = 14			Cohort #2 (TLDA PCR) n = 71		
	ID	Non-ID		ID	Non-ID	
	N = 7	N = 7	P-Value	N = 35	N = 36	p-Value
Demographic and Clinical Factors						
Age, years	70 (9)	71 (10)	0.762	70 (12)	72 (11)	0.472
Sex (male), n (%)	6 (86)	6 (86)	1.0	23 (64)	22 (63)	1.0
Systolic blood pressure, mmHg	135 (19)	124 (22)	0.309	123 (20)	120 (17)	0.495
NYHA functional class						
I-II	5 (71)	5 (71)	1.0			
III-IV	2 (29)	2 (29)				
LVEF, %	36 (5)	35 (6)	0.772	33 (7)	34 (6)	0.908
Ischemic etiology of HF, n (%)	5 (71)	5 (71)	1.0	22 (61)	18 (51)	0.477
6 mwt distance	248 (138)	287 (155)	0.618	230 (141)	257 (172)	0.488
Comorbidities						
Hypertension, n (%)	6 (86)	6 (86)	1.0	29 (81)	27 (77)	0.778
Diabetes Mellitus, n (%)	5 (71)	2 (29)	0.286	28 (78)	18 (51)	0.026
Previous MI, n (%)	2 (29)	2 (29)	1.0	11 (31)	9 (26)	0.793
CKD [a], n (%)	2 (29)	2 (29)	1.0	16 (44)	17 (49)	0.814
Anemia WHO, n (%)	3 (43)	2 (29)	1.0	23 (64)	21 (60)	0.809
Anemia, Hb < 12 g/dL, n (%)	0 (0)	0 (0)	1.0	16 (44)	13 (37)	0.631
Comorbidity index	3 (1)	3 (1)	1.0	4 (1)	3 (1)	0.266
Treatments (%)						
ACEI or ARBs	5 (71)	5 (71)	1.0	24 (67)	27 (77)	0.430
Beta-blockers	6 (86)	5 (71)	1.0	34 (94)	32 (91)	0.674
Diuretics	7 (100)	6 (86)	1.0	32 (89)	31 (89)	1.0
Laboratory						
Hemoglobin, g/dL	13.6 (1.2)	13.4 (0.6)	0.645	11.9 (1.5)	12.2 (1.3)	0.321
eGFR, ml/min/1.73 m^2	65 (21)	65 (19)	0.975	67 (28)	65 (33)	0.839
NT-proBNP, pg/mL	1574 (497–1028)	536 (481–1178)	0.318	1892 (832–4776)	1450 (536–5901)	0.890
hs-CRP	0.4 (0.2–1.4)	0.5 (0.2–6.2)	0.628	0.6 (0.3–1.4)	0.3 (0.2–0.9)	0.086

Table 1. *Cont.*

	Cohort #1 (Transcriptome) $n = 14$			Cohort #2 (TLDA PCR) $n = 71$		
	ID	Non-ID		ID	Non-ID	
	$N = 7$	$N = 7$	P-Value	$N = 35$	$N = 36$	p-Value
Iron status and hematinic						
Serum iron	50.9 (9.2)	85.3 (29.3)	0.012	45.8 (17.2)	89.6 (32.8)	<0.001
Serum ferritin	57.3 (32.2)	262.0 (132.8)	0.002	45.4 (26.9)	322.9 (238.8)	<0.001
TSAT	13.2 (2.0)	25.4 (6.3)	<0.001	11.4 (3.6)	27.1 (10.7)	<0.001
sTfR	2.1 (0.4)	1.5 (0.8)	0.095	2.0 (0.6)	1.2 (0.4)	<0.001

Data presented as mean ± SD, *N* (%) or median (interquartile range). ACEI (angiotensin converting enzyme inhibitor), ARB (angiotensin receptor blocker), BMI (body mass index), BP (blood pressure), CKD (chronic kidney disease), HF (heart failure), Hb (Hemoglobin), COPD (chronic obstructive pulmonary disease), eGFR (estimated glomerular filtration rate), hs-CPR (high sensitive reactive c protein), LVEF (left ventricular ejection fraction), MRA (mineralocorticoid receptor antagonist), NT-proBNP (N-terminal pro-B type natriuretic peptide), NYHA (New York Heart Association), sTfR (serum soluble transferrin receptor), TSAT (transferrin saturation), WHO (world health organization). [a] CKD was defined as eGFR < 60 (MDRD).

The peripheral blood samples from cohort #1 underwent a full transcriptome analysis to study the differential transcripts levels between both populations. The first analysis of the whole transcriptome highlighted 1128 differentially expressed transcripts. From all these transcripts, we performed an enriched gene analysis of the differentially expressed genes from the transcriptome study using Metascape (Supplementary Figure S1). Statistical tests for multiple hypothesis testing were not significant. Among the unadjusted analyses of the transcripts, 22 genes involved in deranged metabolic pathways in HF, cellular iron regulation and metabolism were selected for further evaluation according to statistical significance and/or pathophysiological relevance.

Table 2 presents the description of the 22 candidates genes selected to from transcriptome analysis of cohort #1. Enriched gene ontology of the 22 selected candidate genes is presented in Figure 1. Briefly, the selected genes were important components of HF neurohormonal axis, iron homeostasis and mitochondrial metabolism.

Table 2. General function of the 22 candidate genes analyzed by TLDA (TaqMan® Low Density Array) in cohort #2.

Gene Name	Symbol	Function
Aconitase 1	ACO1	Essential enzyme in the TCA cycle and interacts with mRNA to control de levels of iron inside cells
Adrenomedullin	ADM	Vasodilation, regulation of hormone secretion, promotion of angiogenesis and antimicrobial activity
ATPase H+ transporting accessory protein 2	ATP6AP2	Associated with ATPases (fundamental roles in energy conservation, secondary active transport . . .)
Cytochrome C oxidase assembly factor 3	COA3	Localized to mitochondria and essential for cytochrome c oxidase function. Component of MITRAC
Cytochrome C oxidase copper chaperone 17	COX17	Copper metallochaperone essential for the assembly of the mitochondrial respiratory chain complex IV (cytochrome c oxidase)
C-X-C motif chemokine ligand 8	CXCL8	Member of C-X-C chemokine family and is a major mediator of the inflammation response
Endothelin-converting enzyme 1	ECE1	Proteolytic processing of endothelin precursors to biologically active peptides
Mitochondrial Ferritin	FTMT	Stores iron in a soluble, non-toxic, readily available form (ferric iron binding) and ferroxidase activity

Table 2. *Cont.*

Gene Name	Symbol	Function
Hydroxyacyl-coA dehydrogenase trifunctional multienzyme complex subunit alpha	HADHA	Subunit of the mitochondrial protein which catalyzes the last 3 steps of mitochondrial beta-oxidation of long chain fatty acids
Hypoxia inducible domain family member 1A	HIGD1A	Subunit of cytochrome c oxidase, may play a role in the assembly of respiratory super complexes in mitochondria
Hemopexin	HPX	Plasma glycoprotein that binds heme with high affinity and may be involved in protecting cells from oxidative stress
Lipocalin-2	LCN2	Iron-trafficking protein involved in multiple processes (apoptosis, innate immunity and renal development)
DNA Ligase 3	LIG3	Member of the DNA ligase family, involved in excision repair and is in mitochondria and nucleus
Mitochondrial calcium uniporter regulator 1	MCUR1	Key regulator of MCU (Mitochondrial calcium uniporter) required for calcium entry into mitochondria
Metallothionein 2A	MT2A	Member of the metallothionein family, act as anti-oxidant, important in homeostatic control of metal in cell
Myosin heavy chain 7B	MYH7B	Encodes a heavy chain of myosin II, involved in muscle contraction
Sirtuin 7	SIRT7	NAD-dependent protein-lysine deacylase; functions of huma sirtuins have not yet been well determined
Small integral membrane protein 20	SMIM20	Component of MITRAC complex, that regulates cytochrome c oxidase assembly in mitochondria
STEAP3 metalloreductase	STEAP3	Multipass membrane protein that functions as an iron transporter
Transferrin Receptor	TFRC	Cell surface receptor necessary for cellular iron uptake by receptor-mediated endocytosis
ATP-dependent Zinc metalloprotease	YME1L1	ATP-dependent metalloprotease that catalyzes the degradation of folded and unfolded proteins in mitochondria
Zinc finger protein 260	ZNF260	Transcription factor that acts as a cardiac regulator and an effector of alpha1-adrenergic signaling

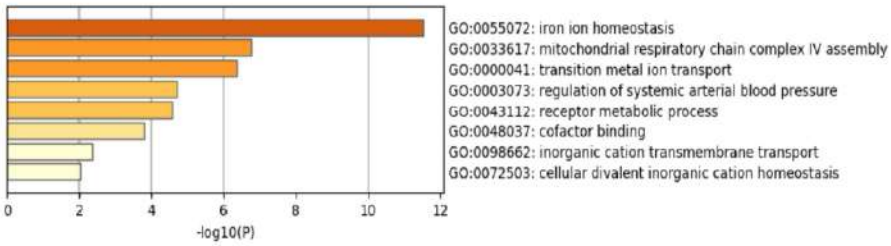


Figure 1. Enriched gene ontology (GO) terms of the 22 candidate genes evaluated in cohort #2. Analysis was carried out by Metascape. The x-axis denotes −log10 (*p*) values based on the cumulative hypergeometric distributions. The colors denote de relative value of −log10 (*p*): darker colors indicate a greater value of −log10 (*p*).

Finally, to deepen our understanding of these metabolic pathways and corroborate our previous results, we used TaqMan low-density array (TLDA) cards for real-time PCR

detection (TLDA PCR) tests, to measure the RNA expression of these 22 genes and evaluated their association with ID status in a larger cohort of patients from the DAMOCLES study (cohort 2#).

As shown in Table 3 and Figure 2, only 6 genes showed significant differences in gene expression depending on iron status: transferrin receptor (TFRC), mitochondrial ferritin (FTMT), sirtuin 7 (SIRT7), small integral membrane protein 20 (SMIM20), adrenomedullin (ADM) and endothelin converting enzyme 1 (ECE1). The RNA expressions of FTMF, SIRT7, SMIM20, ADM and ECE1 were significantly reduced in blood samples of patients with systemic ID compared to control patients with normal iron status. Fold changes in mRNA levels comparing ID vs. non-ID patients ranged from 0.62 to 0.70 (all p-values < 0.05). On the other hand, patients with systemic ID showed an increased ex-pression of TFRC with a statistically significant (p-value 0.03) 1.31 increased fold change in RNA expression compared to control patients without ID.

Table 3. Fold-change of candidate genes in a chronic HF population with systemic ID versus no-ID.

Symbol	ID vs. Ct (Fold Change)	p-Value
ACO1	0.76	0.18
ADM	0.62	0.03
ATP6AP2	0.98	0.87
COA3	0.85	0.42
COX17	0.66	0.08
CXCL8	1.18	0.53
ECE1	0.70	0.03
FTMT	0.26	0.02
HADHA	0.95	0.74
HIGD1A	1.01	0.95
HPX	1.04	0.85
LCN2	0.86	0.36
LIG3	0.71	0.09
MCUR1	0.76	0.15
MT2A	1.05	0.79
MYH7B	0.87	0.56
SIRT7	0.63	0.02
SMIM20	0.68	0.04
STEAP3	1.02	0.92
TFRC	1.31	0.03
YME1L1	0.82	0.28
ZNF260	0.51	0.13

To further explore the prognostic clinical implications of these 6 differentially expressed genes, we evaluated the associations between their mRNA expression and the primary composite (all-cause death or HF hospitalization) and secondary (all-cause death) endpoints, by developing several generalized additive models (GAM). These models were designed to explore parametric and non-parametric relationships between blood gene expression, expressed as delta Ct values of individual genes, and outcomes, expressed as the beta estimates of primary and secondary endpoints. Models were adjusted for important prognostic variables including age, gender, left ventricular ejection fraction, use of disease-modifying drugs, comorbidities, functional capacity, natriuretic peptide levels and renal function. As a result of these multivariable GAM, smooth spline estimates of the primary composite endpoint (all-cause death or HF hospitalization, Figure 3) and the secondary endpoint (all-cause death, Supplementary Figure S2) according to blood gene expression of ADM, ECE1, FTMT, SIRT7, SMIM20 and TFRC were plotted. Significant linear and non-linear relationships between gene expression and the primary endpoint were found for ADM, ECE1, FTMT, SIRT7, SMIM20 but not for TFRC. These associations were also confirmed for the secondary endpoint only for FTMT and SMIM20.

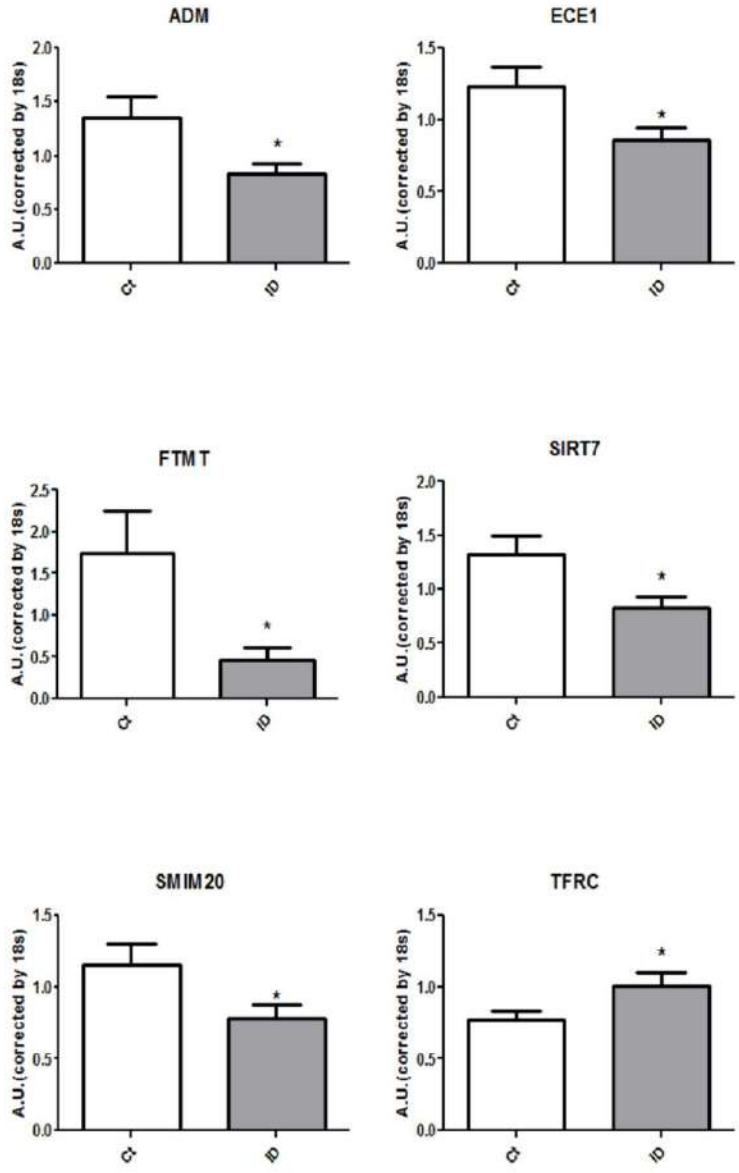
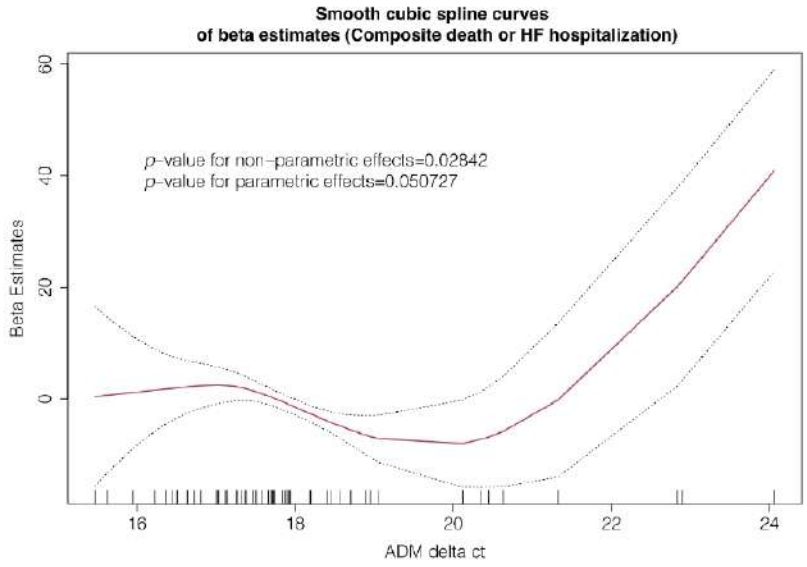
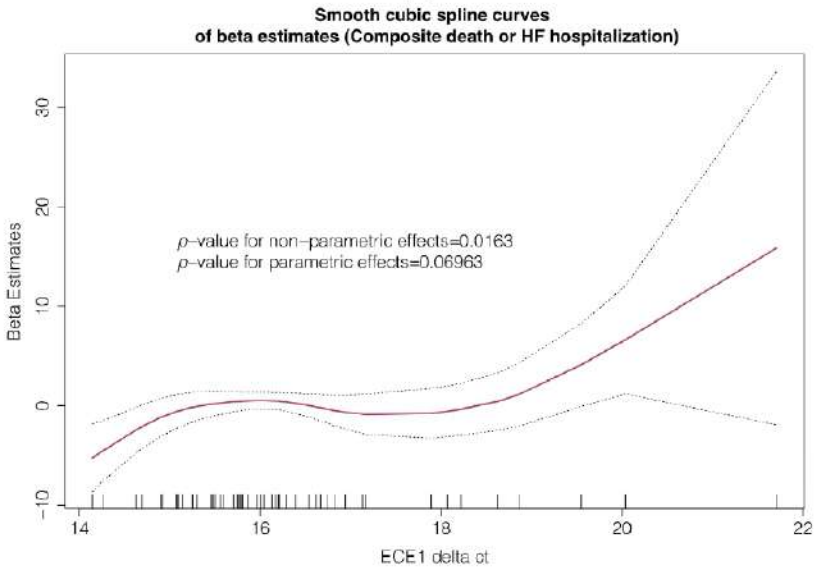


Figure 2. *ADM*, *ECE1*, *FTMT*, *SIRT7*, *SMIM20* and *TFRC* genes are differentially expressed in the whole blood of ID (iron deficiency) patients versus the control ones. Graph representation of the 6 mRNA levels from blood in both studied populations. Data were normalized by the 18 s and expressed as mean ± SEM (* $p < 0.05$ vs. Control).

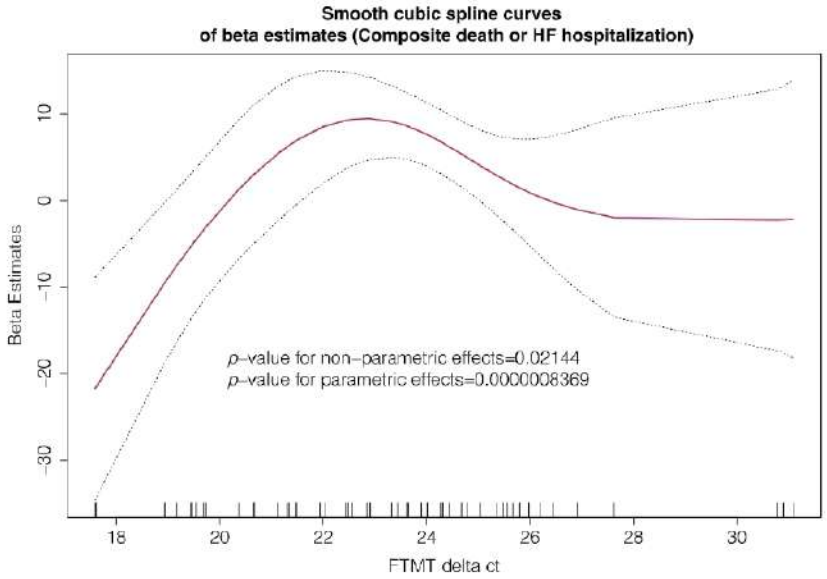


(A)



(B)

Figure 3. *Cont.*



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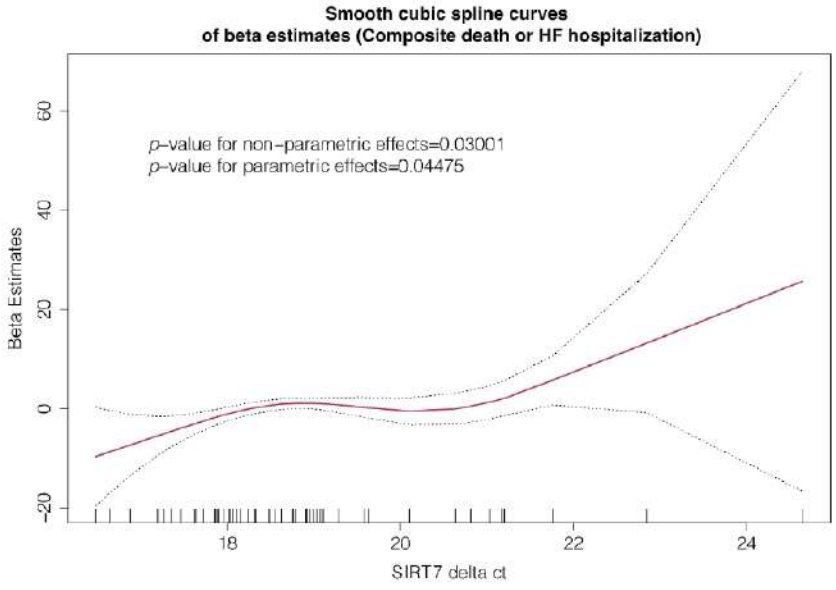
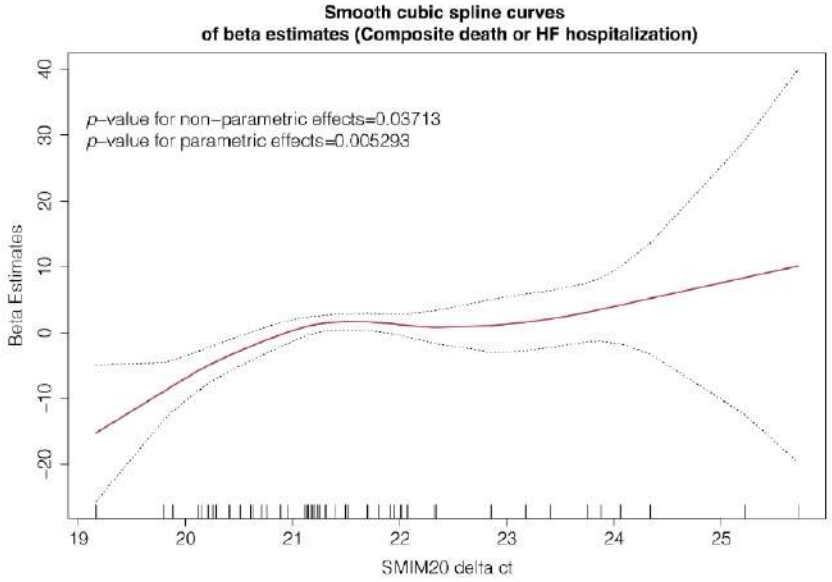
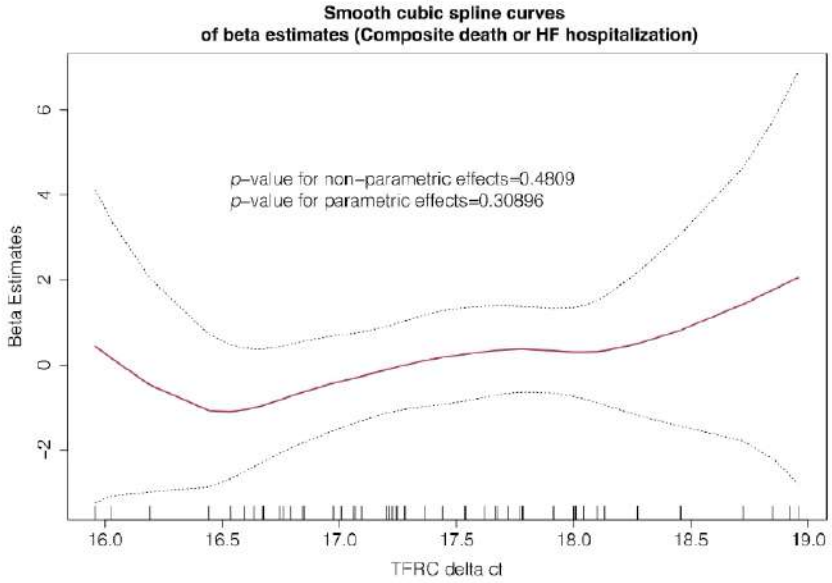


(D)

Figure 3. *Cont.*



(E)



(F)

Figure 3. Smooth spline estimates of the composite endpoint (all-cause death or HF hospitalization) according to gene expression of *ADM* (**A**), *ECE1* (**B**), *FTMT* (**C**), *SIRT7* (**D**), *SMIM20* (**E**) and *TFRC* (**F**). Δct: change in gene expression cycle threshold). Ct levels are inversely proportional to the amount of target nucleic acid in the sample (i.e., the lower the Ct level the greater the amount of target nucleic acid in the sample). Plots show the beta estimates of the outcome.

Furthermore, these models were replicated using adjusted Cox proportional hazards analyses exploring associations of gene expression with outcomes. Gene expression was divided in tertiles and tertiles were grouped in categories. To improve clinical interpretation of the results, grouping was conducted considering the predominance of linear or non-linear patterns of association for each gene. As shown in Table 4 and Supplementary Figure S3, intermediate FTMT gene expression compared to high or low FTMT gene expression was associated with and increased risk of the primary endpoint (HR 2.40, 95% CI 1.04–5.50, p-value = 0.039). Similar results were observed for intermediate or low SIRT7 gene expression compared to high SIRT7 gene expression (HR 5.49, 95% CI 1.78–16.92, p-value = 0.003) and for intermediate or low SMIM20 gene expression compared to high SMIM20 gene expression (HR 9.51, 95% CI 2.69–33.53, p-value < 0.001).

Table 4. Adjusted Cox proportional hazards analyses exploring associations of gene expression with outcomes.

	Composite Primary Endpoint		
	HR	**95% CI**	***p*-Value**
FTMT Δct, T2 vs. T1 + T3	2.396	1.043–5.502	0.039
SIRT71 Δct, T2 + T3 vs. T1	5.495	1.784–16.923	0.003
SMIM20 Δct, T2 + T3 vs. T1	9.511	2.698–33.530	0.000459
TFRC Δct, T3 vs. T1 + T2	1.042	0.347–3.126	0.942
ADM Δct, T2 + T3 vs. T1	1.296	0.456–3.683	0.626
ECE1 Δct, T2 + T3 vs. T1	1.489	0.588–3.767	0.401
	All-Cause Death		
	HR	**95% CI**	***p*-Value**
FTMT Δct, T2 vs. T1 + T3	4.448	1.497–13.216	0.007
SIRT71 Δct, T2 + T3 vs. T1	7.122	1.848–27.438	0.004
SMIM20 Δct, T2 + T3 vs. T1	7.44	1.882–27.930	0.03
TFRC Δct, T3 vs. T1 + T2	2.056	0.700–6.036	0.19
ADM Δct, T2 + T3 vs. T1	1.497	0.460–4.869	0.502
ECE1 Δct, T2 + T3 vs. T1	1.083	0.310–3.783	0.9

Adjusted Cox proportional hazards models using backwards methods. Δct (change in change in gene expression cycle threshold) gene expression tertiles according to generalized additive models of mRNA levels: T1 (lower expression), T2 (intermediate expression), T3 (higher expression). ADM (adrenomedullin), ECE1 (endothelin converting enzyme 1), FTMT (mitochondrial ferritin), TFRC (transferrin receptor), SMIM20 (small integral membrane protein 10), SIRT7 (sirtuin-7).

Moreover, as presented in Table 4 and Supplementary Figure S4, the association of intermediate FTMT gene expression and intermediate or low SIRT7 or SMIM20 gene expressions with all-cause death was also confirmed in multivariable analyses. Cox proportional hazards model analyses did not confirm associations with outcomes for the remaining genes studied for any of the outcomes.

4. Discussion

This is the first study to find consistent gene transcription differences in key functional pathways that connect iron homeostasis with mitochondrial function, oxidative stress and cardiovascular physiology, in patients with chronic HF. Moreover, we found an association between these transcriptomic signals and prognosis.

HF is a complex multisystemic disease involving numerous biological processes that influence the myocardial structure and function [36,37]. Despite optimal gold-standard treatment, a significant number of patients present higher clinical deterioration [3], suggesting that there are unknown biologic components playing a role in disease progression beyond the typical neurohormonal activation. In this scenario, systemic and myocardial iron homeostasis have emerged as relevant factors that participate in the HF syndrome given that: (1) a significant number of HF patients have systemic ID [4], (2) patients with ID have a worse clinical course when compared with non-ID patients [5,6,8,25], and

(3) treatment of ID patients with intravenous iron results in significant clinical improvements [12,13,38,39].

Limited investigational data have disclosed a few of the mechanisms that connect HF and ID. From a pathophysiological point of view, clinical data indicate that ID in HF may be related to low intake, gut losses, persistent congestion, chronic kidney disease, malnourishment and inflammation [22]. In this regard, functional ID has been shown to be one of the main mechanisms that explain ID at a tissue level and to correlate with prognosis [9,10]. Our group has been long investigating the relationship between HF neurohormonal activation and systemic and myocardial iron homeostasis. We have previously demonstrated that patients with ID presented higher noradrenaline levels and that this increased neurohormonal activation was related with impaired iron homeostasis [15]. Moreover, we have recently demonstrated that key iron regulating elements were altered in mice with HF, leading to myocardial ID, and we have shown that the stimulation of cardiac cells with norepinephrine and angiotensin, altered critical iron regulation components, generating intracellular ID and mitochondrial dysfunction [19].

In the present study, we exposed some of these biological crosstalks by analyzing the transcriptomic footprint in a two-phase stepwise cross-sectional study using two nested case control matched cohorts with and without ID: the exploratory cohort (cohort1 #) and the validation cohort (cohort 2#). Full transcriptome results underscored clusters of genes related to myocardial metabolism, iron homeostasis and cardiovascular physiology that were subject to the iron status. Among these, we selected 22 candidate genes according to the highlighted biological pathways by the enriched gene ontology analyses, statistical significance and biological interest, whose expression levels were subsequently analyzed by rt-PCR analyses in the validation cohort 2#. In this cohort, we found that patients with ID presented a lower expression of FTMT, SIRT7, SMIM20, ADM and ECE1 genes compared to non-ID patients, confirming the initial insights outlined in the exploratory transcriptome. Furthermore, in multivariable regression analyses, we found a statistically significant correlation between the levels of FTMT, SIRT7 and SMIM20 gene expression, with the risk of all-cause death and HF hospitalizations.

Transcriptome analyses constitute both a challenge and an opportunity to better understand the mechanistic insights of diseases and a gateway to discover new biomarkers and treatments to improve the management of patients. Indeed, the dysregulation of both coding and non-coding RNA are important elements in cardiac physiology [40–42]. In HF, RNA high throughput analyses have underscored the importance of mRNA in cardiac homeostasis tackling the control of the vascular system, inflammation and cardiac regeneration [21], and previous works have shown that the analysis of specific mRNA might have prognostic implications in HF patients [43]. Similarly, the analysis of proteomic profiles in HF patients have emphasized the importance of these metabolic pathways [44,45].

In our cohort of chronic HF patients, we found that HF and ID are indeed connected in a way that patients with systemic ID have a significant dysregulation in the ex-pression of pivotal genes related with iron regulation, mitochondrial metabolism, endothelial function and neurohormonal activation. Moreover, we found that an intermediate FTMT gene expression and intermediate or low SIRT7 or SMIM20 gene expressions were related with worse outcomes. Cardiac metabolism and mitochondrial function are critical elements in HF progression and a matter of study for future treatments [46,47]. In this regard, SIRT7 gene is a member of the mammalian sirtuins, a group of NAD + -dependent deacylases with roles in genes transcription and DNA repair, which are responsible for the control of oxidative stress, energy metabolism and cellular senescence [48]. From a cardiovascular point of view, sirtuins are known to participate in lipid metabolism and endothelial function, and to protect from inflammation, the development of atherosclerosis, left ventricular hypertrophy and fibrosis [49–51]. SMIM20 is situated in the inner mitochondrial membrane and takes part of the regulation of the cytochrome c oxidase complex [52] and in the heart, it is thought to play a cardioprotective role [53]. Finally, FTMT acts as an antioxidant

in the mitochondrial matrix and has a critical role in the management of iron and DNA repair [54].

Together, the results of our study point to genetic transcription differences in ID patients that affect critical elements of pathophysiological pathways that involve iron regulation, mitochondrial function, oxidative stress and cardiovascular physiology. These findings demonstrate the biological interplay between ID, HF typical neurohormonal activation and mitochondrial metabolism as key elements that might help in the understanding of the underlaying mechanisms that participate in HF progression. Moreover, the prognostic relevance of our findings regarding these gene transcription modifications paves the way to the potential use of these critical metabolic pathways as biomarkers of mitochondrial dysfunction and oxidative stress, and its use as a target for future treatments.

Our study has several limitations to be acknowledged. First, this study was a single-center study and hence, our findings may not be representative of all HF patients. Second, we performed a cross-sectional study and therefore, the statistical correlations that we found merely point to an association between ID and the differences in transcriptomics. Indeed, although our statistical findings suggest that patients with ID presented derangements in the metabolic pathways that implicate mitochondrial function, oxidative stress and iron regulation, it could also be possible that such gene transcription differences caused systemic ID and that the prognostic implications found indicated that patients were in a sicker condition. Third, as we have demonstrated previously, the pathophysiological pathways involving ID, mitochondrial function and HF are multiple and therefore, the genes underscored by our analyses are only a part of a wider complex network. Finally, by using the standard definition of ID we indirectly estimated the true iron status of patients, yet this is the most common method used daily practice.

5. Conclusions

In patients with chronic HF, we determined differences in the transcription of genes according to the presence of ID that affect the pathophysiological pathways of mitochondrial metabolism, iron regulation and cardiovascular physiology. Future research may confirm the prognostic implications found in the transcription levels of FTMT, SIRT7 and SMIM20 genes. These new insights underscore the importance of iron homoeostasis in the interplay between heart metabolism and myocardial dysfunction.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/jcm10214937/s1. Supplementary Figure S1: Enriched gene ontology (GO) analysis of differential expression genes observed in the transcriptome analyses of blood samples in cohort #1. Supplementary Figure S2: Smooth spline estimates of all-cause death according to gene expression. Supplementary Figure S3: Adjusted survival curves of significant associations between gene expression and primary composite endpoint (all-cause death or HF hospitalization). Supplementary Figure S4. Adjusted survival curves of significant associations between gene ex-pression and all-cause death.

Author Contributions: Conceptualization, C.D.-L., M.T.O. and J.C.-C.; methodology, C.D.-L., M.T.O., C.E.G. and J.C.-C.; software, C.D.-L., M.T.O., J.F.M. and J.C.-C.; validation, C.D.-L., M.T.O., J.C.-C., P.M.B., J.G.-C. and J.C.-C.; formal analysis, C.D.-L., M.T.O. and J.C.-C.; investigation, C.D.-L., M.T.O., E.G.-R., S.Y.V. and J.C.-C.; resources, J.C.-C.; data curation, J.C.-C.; writing—original draft preparation, C.D.-L., M.T.O. and J.C.-C.; writing—review and editing, J.G.-C., E.G.-R., S.J.-M., R.R.-P. and M.d.M.R.J.; visualization, S.J.-M., E.G.-R. and R.R.-P.; supervision, M.T.O., J.G.-C. and J.C.-C.; project administration, M.T.O. and J.C.-C.; funding acquisition, J.C.-C. All authors have read and agreed to the published version of the manuscript.

Funding: This work was funded by a Basic Research Competitive Grant in Cardiology from the Spanish Society of Cardiology 2015.

Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Institutional Review Board (or Ethics Committee) of the local institution (approval code: 2004/1788/I).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: All relevant data are included in this published article.

Acknowledgments: We would like to thank Lara Nonell and MARGenomics from IMIM for their support in data analysis.

Conflicts of Interest: Josep Comín-Colet has received honoraria for speaking for Vifor Pharma and consultancy fees from Vifor Pharma.

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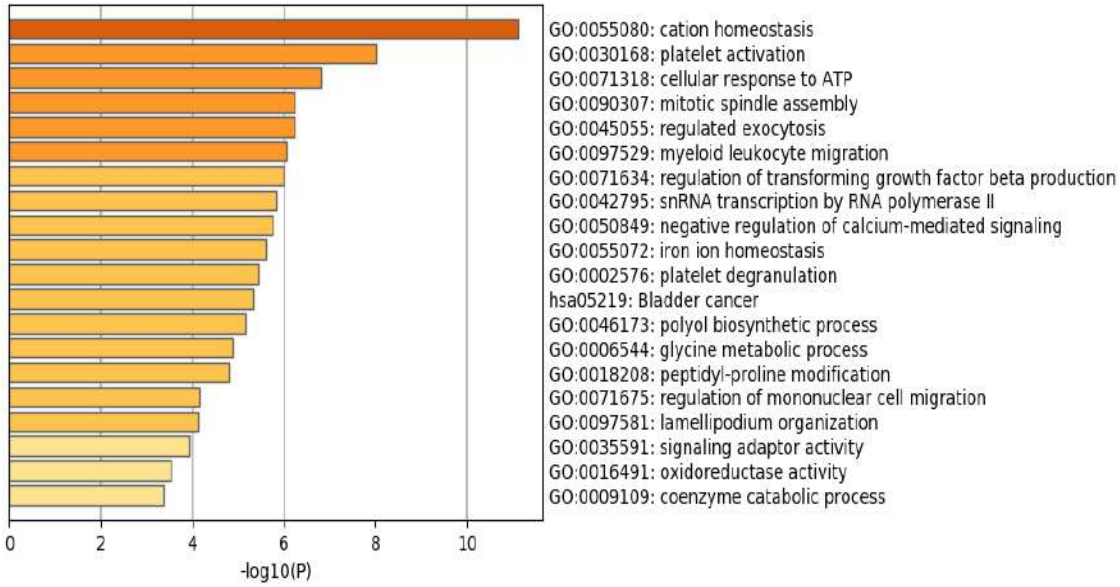
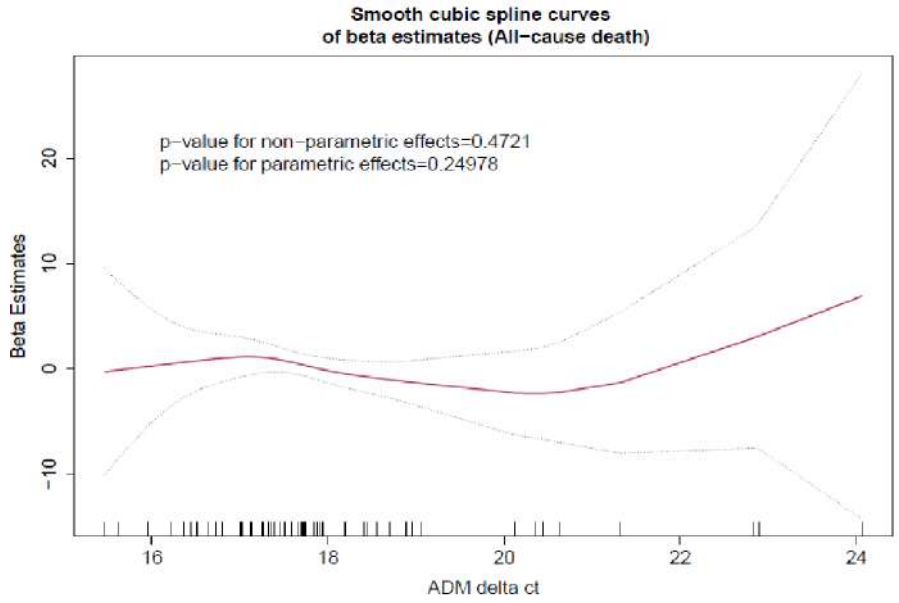
Supplemental material

Supplementary Figure 1. Enriched gene ontology (GO) analysis of differential expression genes observed in the transcriptome analyses of blood samples in cohort #1. Analysis was carried out by Metascape. The x-axis denotes –log10 (P) values based on the cumulative hypergeometric distributions. The colors denote de relative value of –log10 (P): darker colors indicate a greater value of –log10 (P).

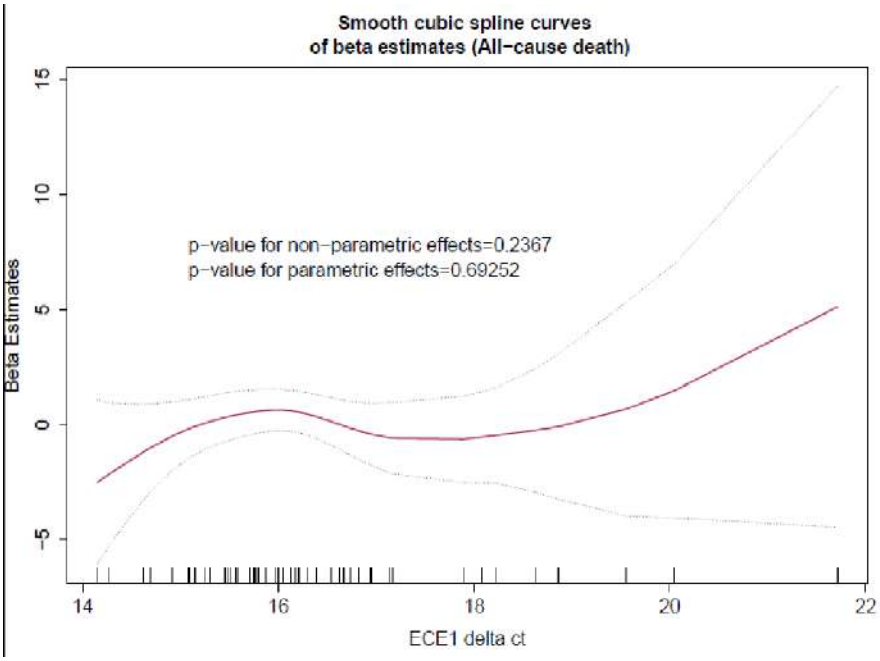


Supplementary Figure 2. Smooth spline estimates of all-cause death according to gene expression of *ADM* (A), *ECE1* (B), *FTMT* (C), *SIRT7* (D), *SMIM20* (E) and *TFRC* (F).

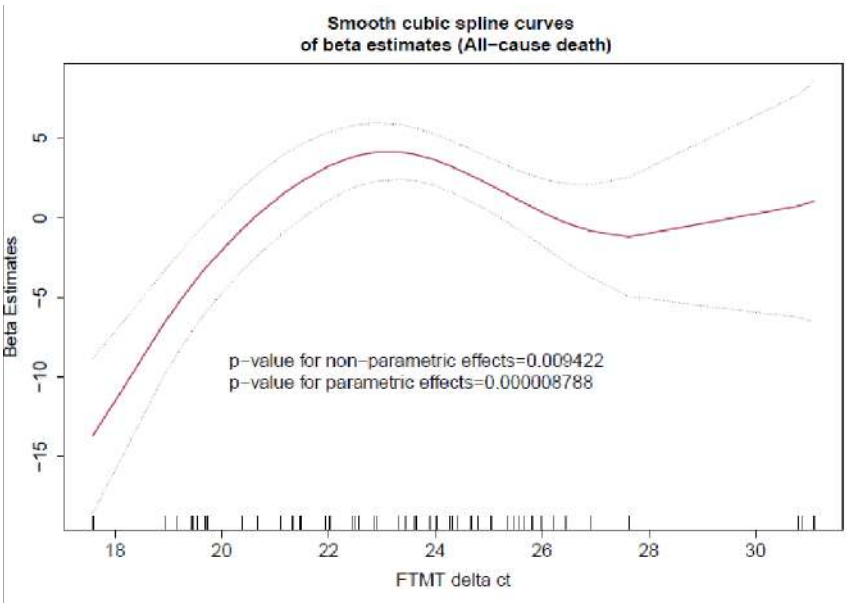
[A]



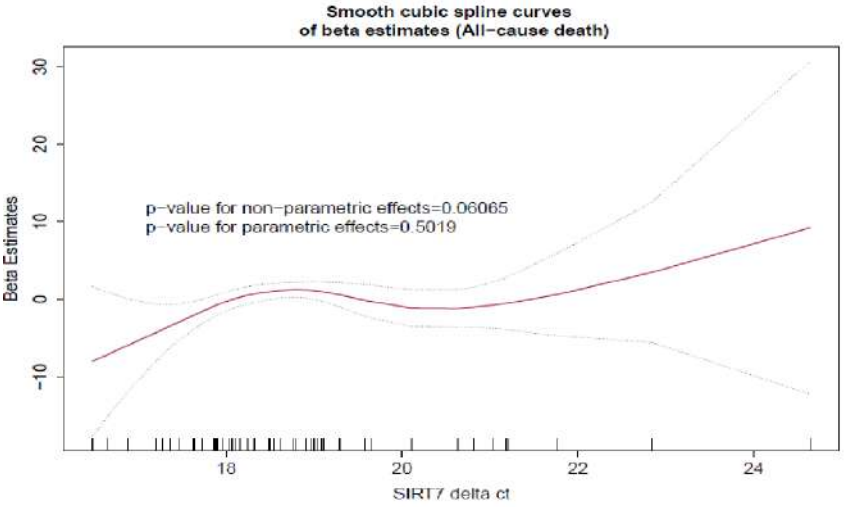
[B]



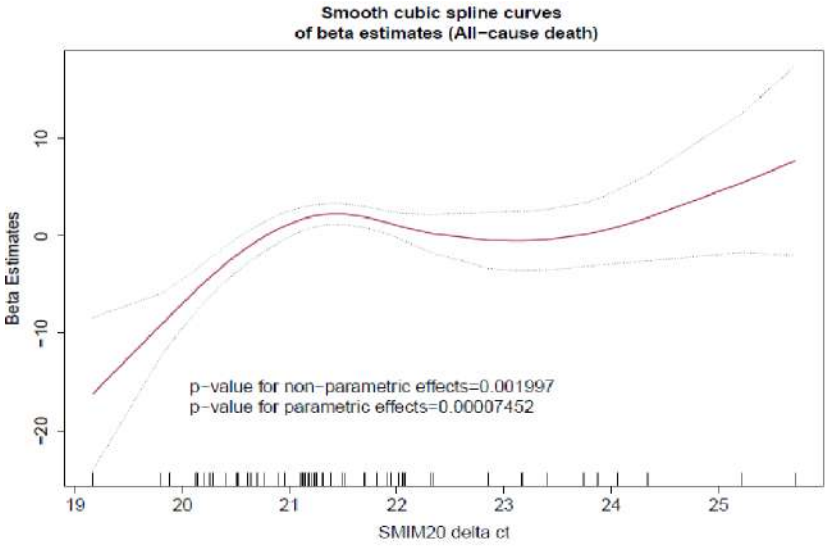
[C]


Smooth cubic spline curves
of beta estimates (All−cause death)
Beta Estimates
5
0
−5
−10
−15
p−value for non−parametric effects=0.009422
p−value for parametric effects=0.000008788
18
20
22
24
26
28
30
FTMT delta ct

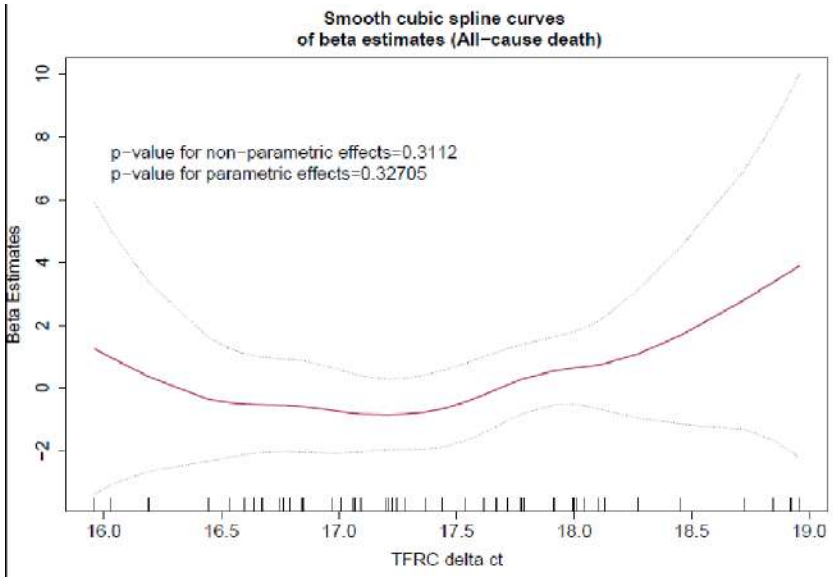
[D]


Smooth cubic spline curves
of beta estimates (All−cause death)
Beta Estimates
30
20
10
0
−10
p−value for non−parametric effects=0.06065
p−value for parametric effects=0.5019
18
20
22
24
SIRT7 delta ct

[E]

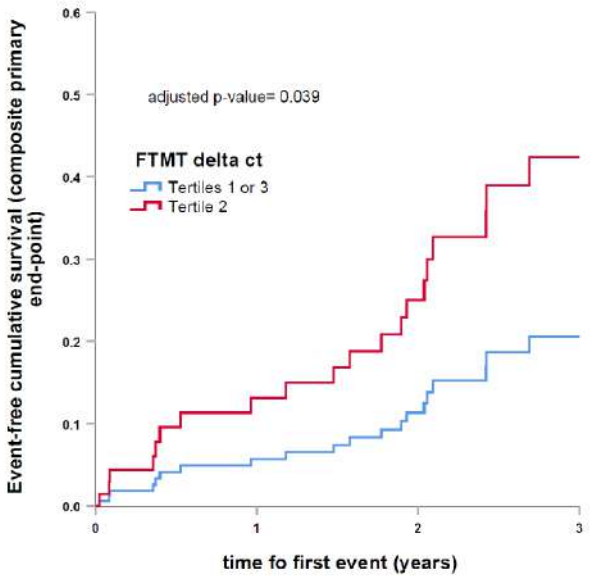

Smooth cubic spline curves
of beta estimates (All−cause death)
Beta Estimates
10
0
−10
−20
p−value for non−parametric effects=0.001997
p−value for parametric effects=0.00007452
19
20
21
22
23
24
25
SMIM20 delta ct

[F]

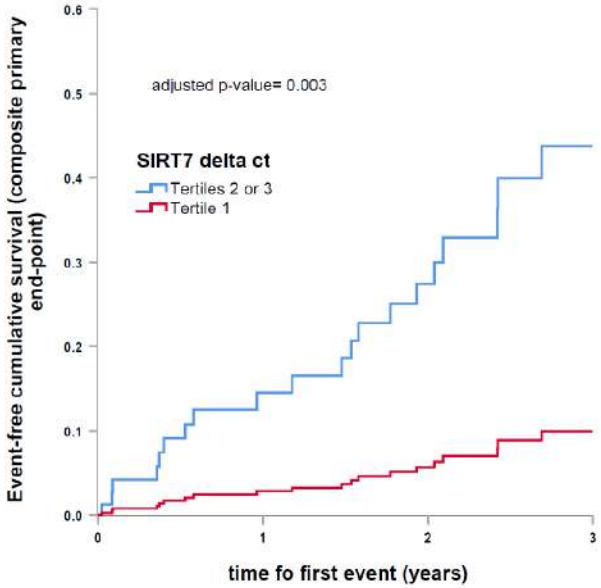

Smooth cubic spline curves
of beta estimates (All−cause death)
Beta Estimates
10
8
6
4
2
0
−2
p−value for non−parametric effects=0.3112
p−value for parametric effects=0.32705
16.0
16.5
17.0
17.5
18.0
18.5
19.0
TFRC delta ct

Supplementary Figure 3. Adjusted survival curves of significant associations between gene expression and primary composite endpoint (all-cause death or HF hospitalization)

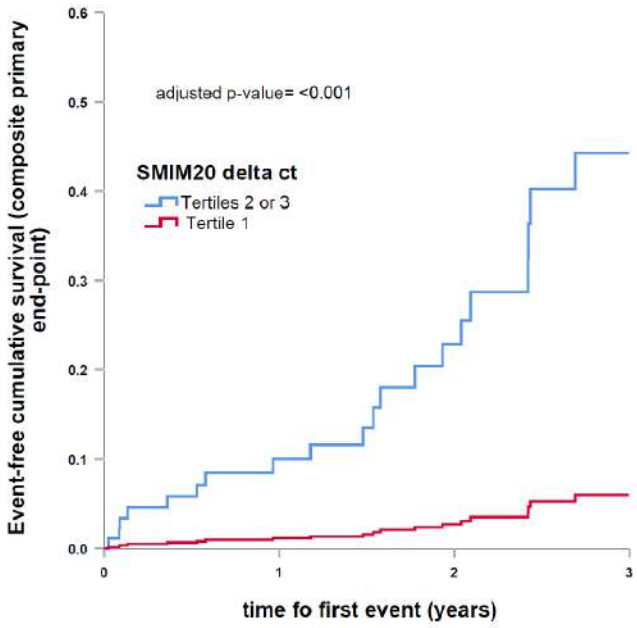
Panel A: *FTMT* mid-range expression (tertile 2 of ∆ct) compared to high expression (lower tertile of ∆ct –tertile 1) or low expression (higher ∆ct –tertile 3) of *FTMT*.



Panel B: *SIRT7* mid-range expression (tertile 2 of ∆ct) or low expression (higher ∆ct – tertile 3) compared to high expression (lower tertile of ∆ct –tertile 1) of *SIRT7*.

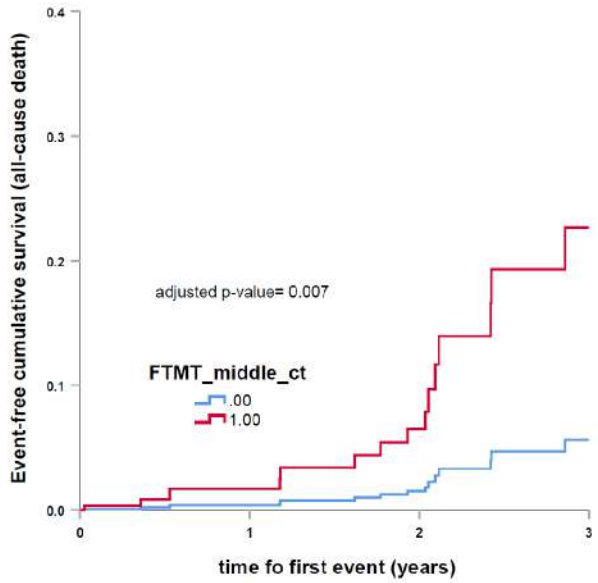


Panel C: *SMIM20* mid-range expression (tertile 2 of ∆ct) or low expression (higher ∆ct –tertile 3) compared to high expression (lower tertile of ∆ct –tertile 1) of *SMIM20*.

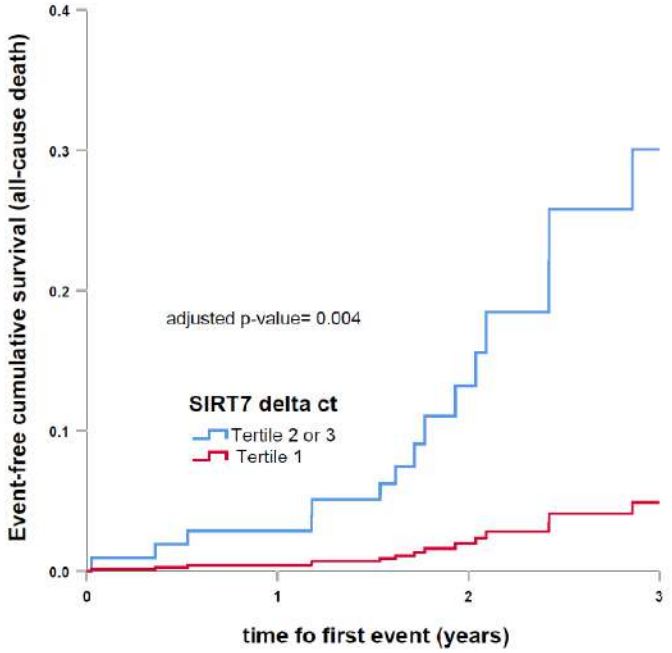


Supplementary Figure 4. Adjusted survival curves of significant associations between gene expression and all-cause death.

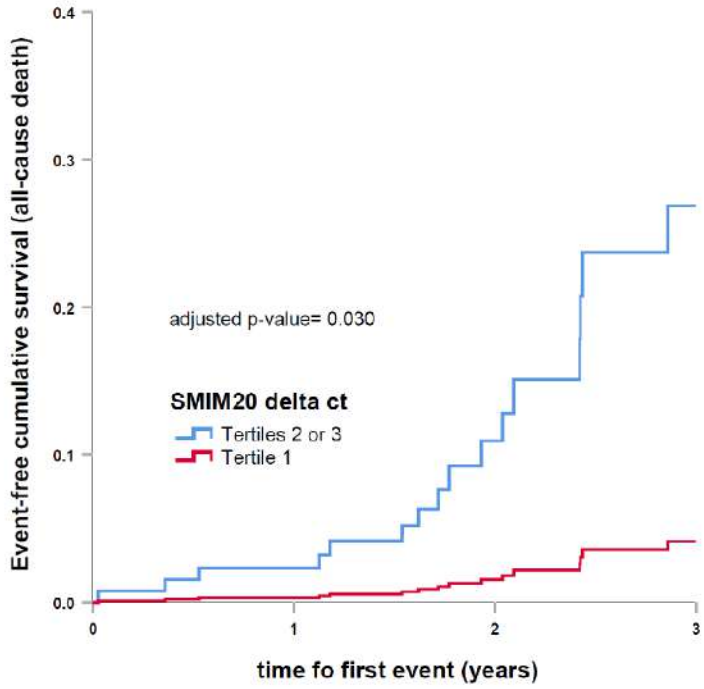
Panel A: *FTMT* mid-range expression (tertile 2 of ∆ct) compared to high expression (lower tertile of ∆ct –tertile 1) or low expression (higher ∆ct –tertile 3) of *FTMT*.



Panel B: SIRT7 mid-range expression (tertile 2 of ∆ct) or low expression (higher ∆ct – tertile 3) compared to high expression (lower tertile of ∆ct –tertile 1) of SIRT7.



Panel C: SMIM20 mid-range expression (tertile 2 of ∆ct) or low expression (higher ∆ct – tertile 3) compared to high expression (lower tertile of ∆ct –tertile 1) of SMIM20.



Discussion

Our study provides a comprehensive analysis into the mechanisms involved in the crosstalk between HF, iron homeostasis, metabolism, and cardiovascular homeostasis. In the first part of our research, we outline the pivotal role of HF and its characteristic neurohormonal activation in the myocardial iron homeostasis, by utilizing two different experimental models: an isoproterenol-induced HF mice model and stimulated H9c2 cardiac cells stimulated with Nor and AT2, to serve as proxy for the neurohormonal activation present in HF. Our data confirm that HF itself directly leads to significant disruptions in the genetic expression of iron regulating elements within the myocardium, culminating in iron depletion and mitochondrial damage and dysfunction. Remarkably, we found that these alterations within the cardiac cells, are predominantly influenced by the main components of HF neurohormonal activation(168). In the second part of our research, we further defined the crosstalk between these main stakeholders of iron regulation and HF axis, within a real-world cohort of HF patients. Certainly, the whole-transcriptome analysis of blood samples indicates that ID in HF induces alterations in key biological pathways, encompassing iron metabolism, mitochondrial function, oxidative stress, cardiovascular function, and systemic inflammation. Moreover, the in-depth evaluation of the trends in genetic expression of the key regulators of these pathways revealed that these trends were associated with HF readmissions and all-cause death(169).

The present findings are particularly significant for several reasons. HF is a syndrome characterized by an intricate interaction between multiple biological pathways that arise from different organs and systems. Iron is essential in various physiological processes, including oxygen transport and energy metabolism; therefore, its dysregulation can have profound implications within the whole body(114,119,120). They also indicate that HF and ID simultaneously contribute to worsening myocardial function and overall cardiovascular health, and the aberrations in metabolic processes seem to play a crucial role in this relationship.

Neurohormonal Activation is The Bridge Between Iron Deficiency and Heart Failure

The use of isoproterenol to induce a HF phenotype in mice and the application of AT2 and Nor in H9c2 cardiac cells provide a robust model to study the effects of neurohormonal stress on the heart. We validated the neurohormonal activation model by assessing cardiac hypertrophy markers such as B-type natriuretic peptide (BNP) and Myosin Heavy Chain 7 (Myh7). Our in vitro model induced both mRNA and protein expression levels for these markers. Nonetheless, combined stimuli did not yield a synergistic effect, suggesting that individual stimuli already maximized the response.

We found changes in the transcription of genes responsible for iron regulation within the myocardium and mitochondria and were able to connect these changes with the presence of ID, impaired energy production and increased oxidative stress within the cardiac cells and the myocardium. Neurohormonal activation is a central mechanism of disease progression in patients with HF. Despite it is a compensatory response to diminished cardiac output, its chronic activation leads to deleterious effects on cardiovascular system including maladaptive remodeling, myocardial fibrosis, systolic and diastolic dysfunction, impaired microvascular function and glomerular (29,170). In this regard, elevated levels of catecholamines and AT2 are markers of this neurohormonal stress and have been associated with more severe disease states and worse clinical outcomes, and pharmacological interventions targeting these pathways, such as beta-blockers, angiotensin converting enzyme inhibitors, angiotensin receptor blockers and mineralocorticoid receptor antagonists, are foundational in HF treatment(1,2). Patients with more severe HF and with worse outcomes have a higher degree of neurohormonal stress and patients tolerating higher doses of HF specific pharmacological treatments tackling these neurohormonal axis tend to present better outcomes. Our study suggests it also contributes to alterations in iron metabolism. The absence of a synergistic effect in cells stimulated with both AT2 and Nor might indeed indicate both a ceiling effect in the response of cardiomyocytes to neurohormonal stress and a particular effect for each neurohormonal towards each specific pathway.

The primary pathway through which neurohormonal activation modulates cardiac iron homeostasis is by regulating the key components of iron uptake. Intracellular iron balance is critically dependent on IRPs with our models showing reduced mRNA expression of IRP1 and IRP2. This downregulation aligns with observations in the myocardium of HF patients, who exhibit decreased IRP activity, mitochondrial dysfunction and compromised myocardial contractility (130). Concurrently, our models demonstrated a diminished expression of Tfr1, which is indispensable mediator for iron uptake within cardiomyocytes. The significance of Tfr1 in both iron regulation and HF pathophysiology is supported by clinical and experimental data. There is an inverse relationship between iron bioavailability and TFR1 levels, and elevated serum levels of sTFR1 levels have been consistently linked to greater HF severity (139,171,141). Maeder et al. observed that cardiomyocytes stimulated with aldosterone or Nor treatment exhibited reduced levels of Tfr1 (172). In line with previous research by Xu et al., our data reaffirms the importance of TFR1-mediated iron uptake for cardiomyocyte health. Xu et al.'s Tfr1 knockout mouse model demonstrated that Tfr1 deficiency precipitates ID within cardiomyocytes, leading to mitochondrial dysfunction and a consequent decline in energy production, a HF phenotype and death (155). Finally, we noted a minor downregulation of divalent metal DMT1, which is associated with the uptake of non-transferrin bound iron, following neurohormonal stimulation, but this change was not observed in the hearts of mice induced with isoproterenol. Kobak et al. have suggested that DMT1 could potentially compensate for iron import in scenarios where TFR1 mediated iron uptake is compromised(173).

All these findings parallel with our observations, where neurohormonal activation and isoproterenol-induced models similarly exhibit these changes in iron uptake regulatory pathways. Overall, these insights suggest that the IRPs-Tfr1 axis might function as a defensive mechanism against HF by ensuring an uninterrupted supply of iron. It also underscores the profound interaction between iron homeostasis and mitochondrial function, and the need for maintaining an adequate iron supply to reduce disease progression.

Despite our novel findings, ID in patients with HF has been classically linked with factors such as insufficient dietary intake, inadequate absorption, gastrointestinal losses, and chronic inflammation. In this regard, hepcidin plays a pivotal role in systemic iron regulation and it is intimately linked with all these factors. The hepcidin axis mainly affects iron export from cells through the internalization and degradation of FPN and as a consequence, inhibiting iron export (126). However, despite patients with HF are exposed to a chronic inflammatory state, hepcidin levels in patients with HF have been mostly found downregulated, suggesting that in HF, its regulation might be more influenced by iron stores rather than inflammation (174).

Our experiments showed that cardiac cells treated solely with AT2 experienced an increase in Hamp expression, which is consistent with a cellular response to conserve iron, by the internalization and degradation of FPN. In contrast, Hamp mRNA levels were decreased under conditions involving isoproterenol and Nor, or the combination of AT2 and Nor. This means that, under sympathetic activation, the overall cellular response appears to involve the inhibition of hepcidin production, leading to an increase in iron loss because of the persistence of FPN at the cellular membrane. Additionally, our experimental models revealed that, Fpn1 mRNA levels decreased in cardiac cells with AT2 and Nor independent stimulation, but no change was noted when both stimuli were applied together. In contrast, mice treated with isoproterenol demonstrated diminished Fpn1 mRNA and protein levels compared to controls. Furthermore, the FPN protein levels remained unaltered under various neurohormonal stimuli. These findings highlight the complexity of cardiac cells iron regulation and suggest the presence of potential secondary effectors regulating the HAMP and FPN synthesis and degradation *in vivo.*

Previous experimental models have evidenced that the absence of hepcidin leads to severe cardiac dysfunction (143,175) and the deletion of the cardiac-specific isoform of FPN leads to myocardial iron accumulation. On the other hand, the knockout of systemic Fpn1 gene induces widespread iron overload, akin to the presentation observed in hemochromatosis, but does not impact the cardiomyocyte iron regulation (176). This implies that the regulation of myocardial iron is autonomously managed in an autocrine manner, with Hamp serving as the primary driver inhibiting the FPN function through its

degradation aiming to prevent cardiac iron depletion. In our experiments, we observed that the HF characteristic neurohormonal activation behaved in opposing ways. The renin-angiotensin-aldosterone axis represented by AT2 promoted the preservation of iron stores within cardiac cells, whereas sympathetic activation promoted iron release. This aligns with our previous research where we underscored the importance of sympathetic activation in the distribution of iron within tissues in patients with HF(133). To the best of our knowledge, this is the first time that sympathetic activation, and myocardial iron regulation and energetics are so intimately linked. Additionally, this suggests that beta-blocking agents, known for their predominant beneficial effects on myocardial function and remodeling, may play a partial role in inhibiting iron release from cardiac cells (177). Nonetheless, recent research by Lakhal-Littleton et al. adds a layer of complexity, proposing that furin, a cellular endoprotease, contributes to the cellular response to ID by promoting the conversion of prohepcidin to active hepcidin (175). It is thus, a limitation, that in our experiments, we did not test the behavior of furin in any of the experimental conditions. Finally, our study found that the levels of the cytoplasmic iron storage protein, FTN remained unchanged across all experimental conditions. This is likely due to the potential influence of factors beyond those investigated in our experiments, encompassing both iron regulatory elements and the explored HF components.

Mitochondrial Dysfunction links Iron Deficiency and Neurohormonal Activation in Heart Failure

Iron-deficient environments within cardiomyocytes were shown to negatively impact the mitochondrial membrane potential, energy production and lead to an increased production of ROS. Iron is essential for various mitochondrial enzymes involved in oxidative phosphorylation, antioxidative defense, and oxygen transport and regulates mitochondrial biogenesis. Low levels of intracellular iron lead to impaired mitochondrial respiration, compromising the energy production process and impacting cardiomyocyte function (147).

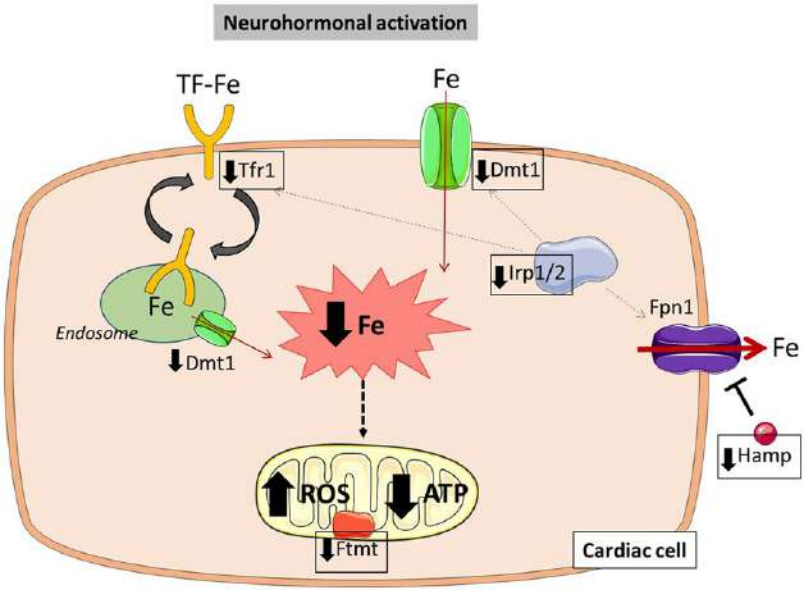
The mitochondrial membrane potential plays a critical role in preserving mitochondrial structural integrity, facilitating ion transport and intramitochondrial biological pathways. Metrics such as ROS generation and ATP production serve as indicative measures of mitochondrial function and efficiency. The integrity of mitochondria and the organelle biological processes are pivotal in regulating intracellular metabolic pathways, protein synthesis and degradation, and initiating apoptosis processes. These considerations highlight the intricate connection between mitochondrial homeostasis and cellular processes crucial for maintaining overall cellular health and functionality.

Mitoferrins are fundamental in mediating the transfer of iron from the cytosol to the mitochondria, essential for regulating intracellular iron dynamics. In our experiments, Mfrn2 expression was found to be decreased in the heart tissue of the isoproterenol-treated mice. This finding is crucial given that Paradkar et al. have previously demonstrated that reductions in Mfrn1 and Mfrn2 are associated with decreased mitochondrial iron content (178). Therefore, it would suggest that in in-vivo conditions, here might be a connection between iron transport across the mitochondrial membrane and its dysfunction. However, our cell-based experiments revealed no changes in Mfrn1 and Mfrn2 mRNA or protein levels under any type of neurohormonal stimulation, indicating that their regulation might be influenced by additional factors beyond neurohormonal stimuli.

In fact, in our study we found decreased expression of Ftmt across all experimental models and conditions. This observation is noteworthy as Ftmt expression is independent

of cellular iron levels, suggesting a potential influence of neurohormonal activation on its synthesis (179). This is crucial, given FTMT's dual role in iron storage and meticulous regulation of oxidative processes, particularly significant in cells with high mitochondrial demands like cardiomyocytes, where it contributes to cellular defense against oxidation and other stressing conditions. (180,181).

Figure 8. **Changes in iron metabolism due to neurohormonal activation in cardiac cells**: the net outcome of stimulation by AT2 and Nor is a modification in key elements of iron regulation. This leads to decreased iron intake, increased iron loss, impairment of mitochondrial structure and function, and a reduction in ROS scavenging capacity by diminishing levels of antioxidant elements like FTMT.



Mitochondrial function and ATP production are essential for maintaining the coupling between calcium handling by the sarcoplasmic reticulum and myocardial contraction and relaxation. Efficient ATP supply by mitochondria is necessary for the ATP-dependent calcium pumps, like sarco(endo)plasmic reticulum calcium-ATPases (SERCA), which facilitate the uptake of calcium into the sarcoplasmic reticulum during diastole, and for the function of the myosin ATPase during the contractile cycle. Therefore, disruption in mitochondrial ATP production can lead to impaired calcium cycling and consequent cardiac dysfunction(182) (183). Moreover, mitochondria are a significant source of ROS, which are pivotal in contributing to myocardial remodeling and HF progression(146). Therefore, considering FTMT's main function as an antioxidant and its relationship with

both iron and mitochondrial function, its downregulation suggests that neurohormonal activation may increase the susceptibility of cardiomyocytes to oxidative stress, a scenario that aligns with other research indicating reduced levels of antioxidant enzymes in the hearts of HF patients.

Metabolic Aberrances and Reduced Redox Capacity links systemic Iron Deficiency with Heart Failure

The contemporary perspective on HF is shifting from a strictly hemodynamic framework to a more integrative approach wherein the complex interactions between different organs and systems including metabolism and microvascular function are emerging as key components(95). One of the key recent advancements in HF is the possibility to identify distinct HF patterns by integrating clinical and biological data, genetic profiling, advanced imaging, and proteomics with advanced machine learning techniques. To date, diverse HF phenotypes have been proposed, categorizing patients based on unique clinical features according to the presence of cardiovascular risk factors, the etiology of the cardiomyopathy, the extent of multiorgan involvement, and their correlation with clinical features like symptom burden, treatment efficacy, tolerance and the overall prognosis (74,75,73).

ID is emerging as an essential component to further understand HF pathophysiology considering its role in energetics and its peripheral cardiovascular and myocardial effects (184). In both of our experimental models, we disclosed some of the main biological pathways linking HF neurohormonal activation - as the main driver of HF effects - and ID at a myocardial level, but the interplay between ID and HF within the cardiovascular system and its potential role in disease progression was not completely disclosed. Consequently, we decided to investigate the genetic expression patterns affected by ID, analyzing the transcriptome in blood samples of patients with HF. Because, in spite of the compelling evidence, the pathophysiological consequences of ID in HF have often been mostly related to anemia, we included a cohort of patients with only ID and normal hemoglobin levels. This comprehensive analysis of mRNA levels allowed us to better understand the trends and interconnections among key regulatory networks in HF that influence iron homeostasis, cardiovascular function, and systemic metabolism.

Our transcriptome analysis identified significant alterations in critical biological pathways regulating iron metabolism, mitochondrial function, intracellular energy production, oxidative stress response, apoptosis, calcium ion signaling, platelet aggregation, and transforming growth factor signaling pathways. Interestingly, ID has already been

associated with various physiological processes, including inflammation, metabolic regulation, and tissue remodeling (185). A recent study has demonstrated that the experimental elevation of central venous pressure as indicative of systemic congestion, produced the elevation of plasma markers of oxidative stress and inflammation (186). In addition, as previously discussed, metabolism is intimately connected to cardiovascular disease and previous research has consistently underscored the presence of metabolic disturbances across the HF spectrum. Indeed, hyperglycemia, dyslipidemia, and obesity are known to induce endothelial dysfunction, oxidative stress, and inflammation, affecting the delivery of nutrients and oxygen and promoting tissue fibrosis (90,187) (188). Hence, the insights from our research propose a direct connection between the iron levels, inflammation, oxidative stress and cardiovascular function, indicating that metabolic processes might be central nexus among these conditions. Consequently, we selected 22 genes for in-depth analysis based on their statistical significance and relevance to cardiovascular function, metabolism, and intracellular homeostasis. Gene ontology analyses confirmed that the functions of the selected genes are aligned with crucial pathophysiological pathways identified in our whole-transcriptome analysis, involving in cardiovascular homeostasis, systemic inflammation and oxidative stress resilience. Notably, among these genes, we observed significant variations in the mRNA expression of six genes coding for the mitochondrial ferritin (MTFT), sirtuin 7 (SIRT7), the small integral membrane protein 20 (SMIM20), adrenomedullin (ADM), and the endothelin converting enzyme 1 (ECE1), relative to the presence of systemic ID.

To the best of our knowledge, this indicates that there is a strong link between iron-related metabolic processes and microvascular function, thereby confirming our initial hypothesis. In particular, the gene expression of TFR1 was notably higher in patients with ID, underscoring the main role of this gene in iron regulation at a tissular level in HF. In fact, recent research has indicated that the serum levels of the soluble TFR1 correlate with adverse HF outcomes, and worse functional capacity and quality of life, even in the presence of normal serum iron levels (141,171). However, in our study, specific trends in TFR1 gene expressions did not correlate with clinical outcomes. Among the same lines, the mRNA levels ECE1 and ADM were lower in patients with ID, indicating a close connection between ID, microvascular function and HF. However, we failed to identify a

specific genetic expression trend that correlated with adverse outcomes. ADM is secreted by endothelial cells in response to congestion, whereas ECE1 is present in the endothelium, the cardiomyocytes, in the liver and at the nervous tissue, participating in the processing of ET1, promoting vasoconstriction and myocardial hypertrophy within the cardiovascular system (189,190). Both ADM and ET1 high levels correlate with congestion and adverse outcomes in HF patients, yet whether these elevated levels are a response to disease worsening or an active participant in disease progression remains unclear. In fact, based on our study results and considering the roles of ADM and ECE1 in regulating vascular tone and maintaining endothelial barrier function, it suggests a scenario where the absence of ADM and ECE1 in an ID environment might contribute to disease progression, but elevated levels of both might indicate worsening HF. In fact, the abovementioned study on the experimental induction of venous congestion showed an increase in the levels of ET1, vascular adhesion molecule 1, cluster of differentiation 146 antigen, and cytokines such as tumor necrosis factor α and interleukin 6(186). Considering our results, it would be interesting to evaluate the behavior of iron homeostasis and the markers we evaluated in our studies, before and after this experiment. Independently, these findings signal a straightforward path to further understand the interconnection between ID, congestion, and microvascular function in patients with HF, that deserve further investigation.

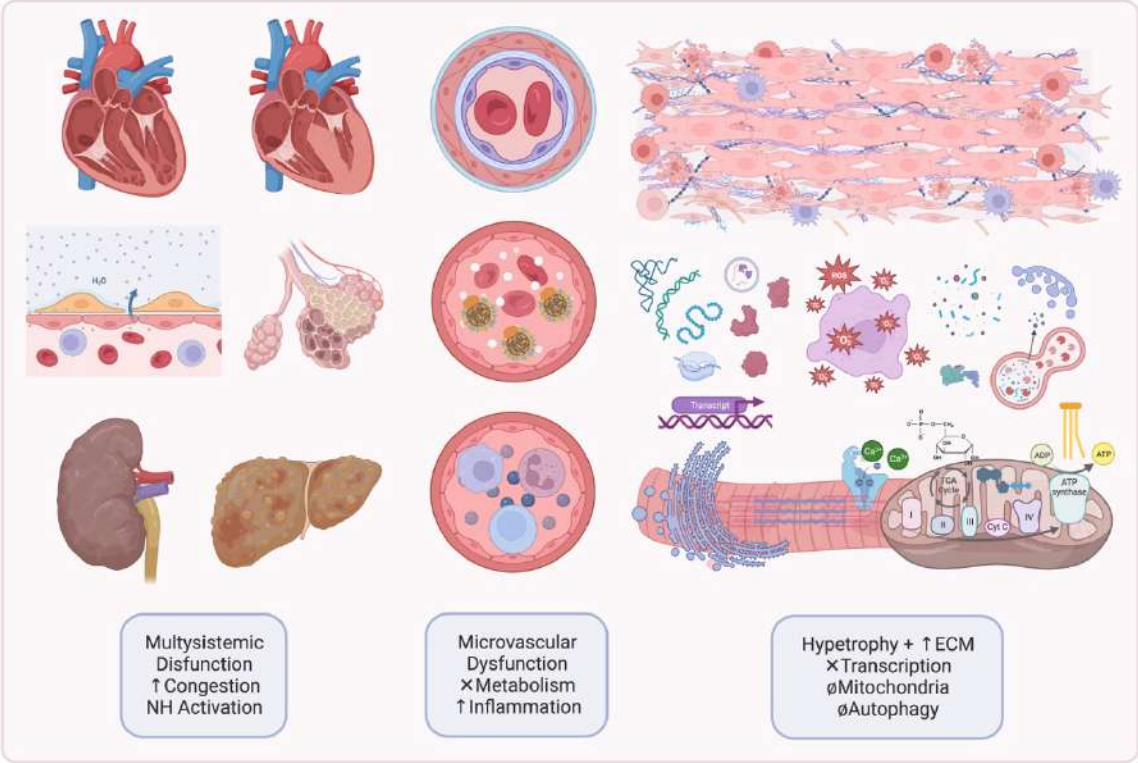
Furthermore, we found low levels of FTMT, SIRT7 and SMIM20 gene expression in the blood from patients with ID, when compared to those without ID. More precisely, we identified that an intermediate change in the gene expression of the FTMT and either intermediate or low change in the gene expression of SIRT7 or SMIM20 were correlated with the composite study outcomes and all-cause death. Sirtuins are a family of NAD+-dependent deacetylases that play a crucial role in the regulation of various cellular processes, such as aging, inflammation, adipogenesis, shifting cellular metabolism from glycolysis to fatty acid oxidation, mitochondrial biogenesis, autophagy and apoptosis (191). Moreover, they have also shown to exert protective effects by enhancing endothelial nitric oxide synthase activity and regulating the production of vasoconstrictors like ET1, thus impacting vascular tone and reactivity and promoting angiogenesis (192). In HF, sirtuins regulate energy production, detoxify oxidative stress, handle intracellular

Ca2+, and play a crucial role in preserving cardiac function, promoting angiogenesis and suppressing fibrosis in response to pressure overload (188,193–195). Thus, the decrease in SIRT7 levels suggests an increased susceptibility to oxidation, implying a diminished capacity to mitigate oxidative damage. On the other hand, both SMIM20 and FTMT have key roles in mitochondrial function, albeit in distinct competences: SMIM20, is a small integral membrane protein localized predominantly in mitochondria that has been implicated in cellular metabolism and bioenergetics, contributing to the regulation of the cytochrome c oxidase complex. For instance, the cleavage of SMIM20 produces phoenixin a neuropeptide implicated in a variety of physiological processes, including stress response and substrate use in mitochondrial metabolism. While SMIM20 operates within the mitochondrial membrane, phoenixin functions in an endocrine manner within the rest of the cell. Considering the biological roles of sirtuins and SMIM20, the associations between a higher increase in SIRT7 and SMIM20 gene expressions may indicate a compensatory pathway attempting to mitigate the progression of HF provoked by metabolic inefficiency and oxidative stress. Conversely, the observed low levels of gene expression might suggest that in the presence of ID, this compensatory pathway could become insufficient, yet it's important to note that our study did not measure the overall levels of these proteins. FTMT, on the other hand, is a specialized form of ferritin localized in mitochondria that maintains iron homeostasis and functions as an antioxidant playing a pivotal role in iron regulation and DNA repair. FTMT plays a crucial role in modulating this balance, and previous studies have underscored the critical importance of maintaining mitochondrial iron homeostasis (179,180). The decrease in FTMT levels in the presence of ID implies an elevated vulnerability to oxidative stress and a potential decrease in the capacity to store iron within the mitochondria(179–181). The relationship between lower and higher changes in FTMT gene expression in blood is challenging to understand; however, it underscores the importance of finely regulating this protein in response to stress conditions such as in HF. Consequently, similar to previously mentioned points, it suggests that either a minimal change in FTMT expression due to reduced production capacity (as seen in ID) or excessive gene expression due to persistent damage correlate with disease progression. Indeed, the observed trends in FTMT gene expression and its association with adverse outcomes, highlight the intimate

relationship between HF pathophysiology and metabolism, with ID playing a central role in promoting this adverse biological pathway.

The biological relevance of these proteins and the observed trends also emphasize the role of mitochondrial iron homeostasis in HF. This dual focus—on bioenergetics through SIRT7, SMIM20 and iron regulation through FTMT—provides a more comprehensive understanding of the molecular pathways that may be targeted for HF management. An inadequate mitochondrial function exacerbates the cardiomyocyte inability to meet the metabolic demand, worsening cardiac function and exacerbating deleterious processes affecting diverse organs within the cardiovascular system that culminate in worse clinical outcomes. Hence, our results underscore the significance of the connexon between metabolic adaptation and cardiovascular protection.

Figure 9. An integrative model of HF from the myocardial function to microvascular disease, inflammation and altered metabolism. Microvascular function plays a pivotal role in the regulation of critical cellular processes such as aging, inflammation, metabolism, and the adaptive response to stress conditions. This vital function is primarily controlled by endothelial cells, which are central to microvascular biology by controlling angiogenesis and regulating vascular tone. Consequently, metabolic abnormalities trigger a complex sequence of events that occur at a systemic, myocardial, cellular, and biological levels. These intricate mechanisms involve processes such as oxidative stress, altered transcriptional activity, and impaired cellular maintenance, ultimately leading to multiorgan damage, inflammation and interstitial tissular fibrosis.



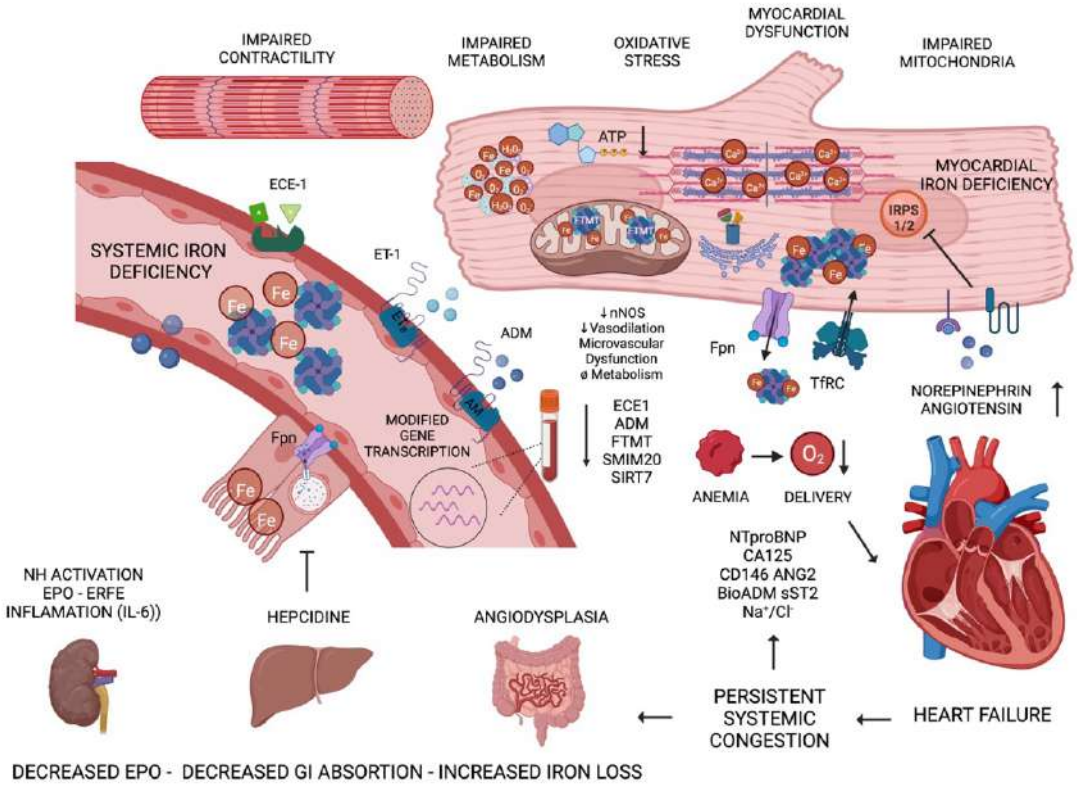
The interconnections within the cardiovascular system explain the disease continuum in patients with HF, wherein metabolism stands as one of the key components of disease progression. Therapeutic interventions influencing all these metabolic pathways, constitute a promising therapeutic opportunity for diseases affecting microvascular function and systemic metabolism. In line with our research, previous studies have shown that ID alters whole-body energy metabolism towards a glycolytic profile and despite the evidence supporting treatments focused on mitochondria in HF is scarce, new therapeutic agents like sodium glucose transporter inhibitors (SGLT2i) have shown their effectiveness across HF phenotypes by tackling both systemic and myocardial metabolism (196)(197). In fact, the SGLT2i expert its cardioprotective effects via growth differentiation factor 15, suppressing ROS generation and boosting myocardial ATP levels through enhanced autophagy and sirtuin-involved signaling pathways (194)(198). What's more, treatment with SGLT2 inhibitors is linked to alterations in iron-regulating elements and increased erythropoiesis, further suggesting a close association between fluid congestion,

metabolic dysregulation, and iron homeostasis(199). Recent research has highlighted the potential for glucagon-like peptide agonists to improve outcomes in patients with HF especially in those with preserved LVEF, wherein metabolic comorbidities, such as obesity and dyslipidemia(200). However, there is currently no data on the effect of these compounds on iron homeostasis, and uncertainty remains in its effect throughout the HF spectrum(201).

In conclusion, our findings suggest a multifaceted mechanism by which ID exacerbates myocardial dysfunction. It also suggests that neurohormonal activation in HF could exacerbate disease progression through intracellular iron depletion. Our findings indicate that neurohormonal activation may render cardiac cells more susceptible to oxidative stress and mitochondrial dysfunction. The myocardium is highly sensitive to changes in ion concentrations, given its crucial role in contractility and impulse propagation. Within the myocardium, altered expression of genes related to iron regulation could compromise the hemodynamic function, leading to decreased cardiac output and worsening the disease course (Figure 9). Thus, the treatment with intravenous iron therapy offers a potential to optimize these biological processes, targeting microvascular function and systemic metabolism, and providing an opportunity to positively impact in disease progression (202).

Figure 10. Novel insights into the intricate links between ID and HF reveal that ID arise from diverse biological processes and concurrent comorbidities yet neurohormonal activation contributes to both systemic and intramyocardial ID. HF patients exhibit systemic congestion and compromised oxygen and nutrient delivery, fostering tissue hypoxia. This hypoxic state is exacerbated by the presence of ID intrinsic to the HF system. Intracellular iron perturbations ultimately impact mitochondrial function, compromising ATP production and calcium intracellular fluxes, thereby impairing contraction and relaxation at the sarcomere. In HF patients, ECE1, ADM, FTMT, SMIM20, and SIRT7 play pivotal roles in connecting ID, intracellular metabolism, and microvascular function, culminating in exacerbated cardiac dysfunction and heightened tissue hypoxia. As HF advances, persistent congestion and inflammation may trigger increased hepcidin secretion, diminishing iron absorption. Furthermore, limited tissue iron availability reduces hemoglobin production, intensifying the hypoxic response. Prolonged hypoxia and congestion

contribute to the development of gastrointestinal angiodysplasia, potentially leading to gastrointestinal bleeding, anemia, and further iron loss.



Limitations and Future Perspectives

Despite the comprehensive and detailed studies performed in the present work, we acknowledge that it has some limitations that need to be acknowledged. First, our research was limited to one in vivo HF model using isoprenaline and did not include other models like aortic coarctation or metabolic HF models. Similarly, in our cellular models, we only considered AT2 and Noradrenaline to mimic the HF neurohormonal environment. Future studies should consider the broader systemic metabolic alterations in HF beyond ID and evaluate the impact of pharmacological treatments and iron administration on the observed abnormalities.

Moreover, in our analyses of the influence of systemic ID in systemic metabolism, we only analyzed mRNA in whole blood. We have to acknowledge that integrating transcriptomics with proteomics and metabolomics may had provided a more comprehensive disease understanding. Moreover, applying machine learning to these data could help in obtaining more insights into the regulation of microvascular function and metabolic pathways, and the discovery of new biomarkers and therapeutic strategies for vascular and myocardial health, that could potentially enable for an earlier intervention and alter the disease trajectory.

Finally, neither in *in-vivo* nor in real-world data we stratified HF severity beyond dose control of AT2 and Nor in the in-vitro model and thus, we did not explore the relationship between disease severity and iron regulation *per se*. Likewise, while we analyzed HF through endocrine and paracrine lenses, we did not account for the impact of hemodynamics on iron regulation, despite potential links between hemodynamic profiles and iron homeostasis.

In order to address these additional inquiries, our research team is conducting further experiments to investigate the impact of various HF pharmacological treatments on the iron regulation elements identified in our study. Moreover, as part of these research future steps, I dedicated 12 months to the Advanced HF Unit at Montefiore Medical Center, affiliated with Albert Einstein University in New York City, USA. This tenure was a strategic move to delve deeper into these questions, aligning with a research team that has a well-established background in the field.

As mentioned earlier, ID has long been associated with the occurrence of anemia. While ID is acknowledged as an independent prognostic factor, some authors have emphasized the primacy of anemia over ID in the prognosis of HF patients. However, it is important to note that despite anemia and ID have many common biological pathways in HF, they originate from distinct sources and biological dysregulations, contributing to their unique profiles. Certainly, comprehending the intricate biological interconnections between both identities, namely anemia and ID holds the key to a deeper understanding of their significance in the progression of the disease and offers insights into potential treatment approaches. Previous research by Patel et al. has drawn attention to arteriovenous malformations (AVMs) in advanced HF patients (203), as AVMs are known to be associated with bleeding events, especially in those with durable ventricular assist devices(204).

From an integrative perspective, neurohormonal activation contributes to both systemic and intramyocardial ID in patients with chronic HF. The systemic congestion and compromised oxygen and nutrient delivery in HF foster tissue hypoxia, which is aggravated by the intrinsic presence of ID. Intracellular alterations in iron levels ultimately affect mitochondrial function, compromising ATP production and calcium intracellular fluxes, leading to impaired contraction and relaxation at the sarcomere. In HF patients, ECE1, ADM, FTMT, SMIM20, and SIRT7 play crucial roles in connecting ID, intracellular metabolism, and microvascular function, resulting in exacerbated cardiac dysfunction and increased tissue hypoxia. Despite not being the primary cause of ID, as HF advances persistent congestion and progressive decreased oxygen and nutrient delivery leads to inflammation, potentially stimulating increased hepcidin secretion and reducing iron absorption and delivery from stores. This sequential progression perspective proposes that chronic HF initiates the presence of ID triggering metabolic disturbances that impact intracellular metabolism. This, in turn, induces a hypoxic state and inflammation, ultimately contributing to the development of anemia, amplifying the hypoxic response. Prolonged hypoxia and congestion contribute to the development of mucosal angiodysplasia, potentially leading to gastrointestinal bleeding, persistence of anemia, and further iron loss. Viewing anemia as a progressive stage of ID and HF rather than

independent factors might help explain certain research findings that prioritize anemia as a predictor of adverse outcomes (205,206).

During my stay in the US, we compared the iron levels between patients with and without AVMs, revealing a tendency for those with AVMs to have lower iron and TSAT, but the statistical results were borderline due to low sample size. Previous research by Vukelic et al. emphasized the role of HIFα in AVM development and the relationship between angiopoietin 2 (Ang2) and vascular endothelial growth factor (VEGF) levels in the presence of AVMs(207). However, we could not find a definitive correlation between Ang2 and iron levels, but similar to AVM presence, we observed a trend indicating lower iron levels and TSAT, specially in those patients with increased central venous pressure, as indicative of systemic congestion.

Hemodynamics traditionally serve as the gold standard for evaluating patients with HF. However, in recent years, non-invasive echocardiographic measurements have provided clinicians with alternatives to the invasiveness of pulmonary catheterization for both evaluation and optimization of patients. Chronic HF is characterized by elevated filling pressures, as indicated by the capillary wedge pressure (reflecting left ventricular elevated filling pressures) and the right atrium or central venous pressure (indicating right ventricular elevated filling pressures). While increased left ventricular filling pressures are closely associated with exercise limitation and pulmonary hypertension, systemic congestion in chronic HF is predominantly linked to multiorgan dysfunction. This aspect has gained significant attention in recent years due to its correlation with HF decompensations and dysfunction in organs such as the kidneys and liver, both closely linked with increased mortality.

Following this thread, we assessed 417 patients with chronic advanced HF, categorizing iron status and hemodynamics based on WHO anemia definitions and current guidelines for ID. Anemia was defined as WHO (hemoglobin <12g/dL in women and <13g/dL in men) and was present in 51% of patients, with 66.5% showing TSAT<20%. Low TSAT occurred with and without anemia but was less common when TSAT>20% (Figure 10). Notably, anemia with low TSAT was associated with younger age, higher bilirubin, lower BMI, and pulmonary vascular resistance (Table 2). Moreover, low TSAT without anemia correlated

with higher biventricular filling pressures and slightly lower hemoglobin. There was a trend towards higher NTproBNP (N-terminal pro b-type natriuretic peptide) levels when TSAT <20% independent from the presence of anemia, but this was statistically non-significant. Overall, anemia was correlated with the need for a higher cardiac output poorer renal function and higher need for inotropic support while low TSAT was more related with elevated filling pressures, particularly high central venous pressure.

Figure 11. Prevalence of anemia and ID in the cohort of advanced HF patients. The cohort was divided relative to the presence of anemia and TSAT 20%. The distribution of the cohort was different according to the anemia and ID subtypes (p<0.001). The most common phenotype was anemia and TSAT <20%. There was a clear uncoupling between anemia and ID: ID was present in almost 22% of patients even in absence of anemia and 12% of patients presented anemia without ID.

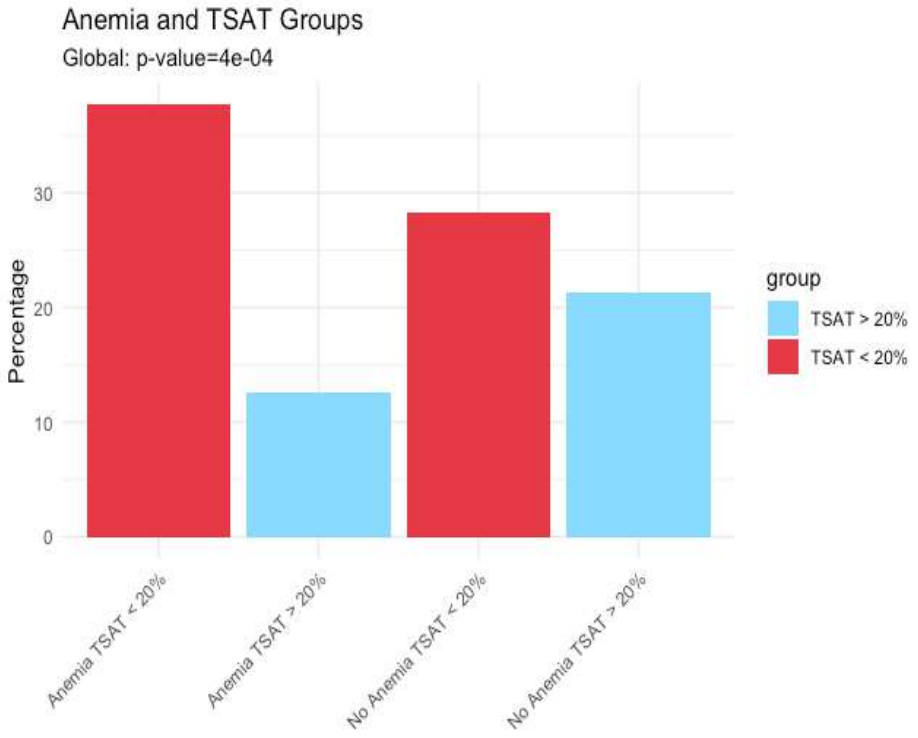


Table 2: Clinical characteristics summaries and between group comparisons according to the anemia status and the transferrin saturation index.

	Anemia TSAT<20 %	Anemia TSAT>20%	p-value	No Anemia TSAT<20%	No Anemia TSAT>20%	p-value
	N = 151	N = 49		N = 112	N = 83	
Baseline Characteristics						
Age-yr.	56.5 ± 11	59.6 ± 9	0.046	56.0 ± 11	57.6 ± 11	0.308
BMI (kg/m2)	26.3 ± 5.6	27.6 ± 5.4	0.014	29.7 ± 6.6	28.6 ± 5.8	0.197
Male sex - no. (%)	109 (74.2)	37 (72.6)	0.968	74 (67.3)	57 (67.8)	1
NYHA III-IV – no.(%)	139 (94.5)	44 (86.3)	0.078	101 (91.8)	75 (89.3)	0.601
IHD	100 (68.0)	36 (70.5)	0.869	60 (54.6)	49 (58.3)	0.703
Diabetes	43 (29.3)	12 (23.5)	0.545	31 (28.1)	18 (21.4)	0.363
CKD	40 (27.2)	17 (23.6)	0.514	28 (25.5)	21 (25.0)	1
COPD	34 (16.3)	9 (17.3)	1	24 (21.8)	21 (25.0)	0.727
Serum markers – SD						
Creatinine mg/dl	1.5 (1.0-1.9)	1.4 (1.0-1.8)	0.766	1.3 (1.0-1.6)	1.3 (1.0-1.6)	0.850
eGFR mL/min/1.73m2	75 (44-92)	68 (44-85)	0.212	81.1 (58-96)	67 (47-88)	0.005
Sodium mEq/L	138 (134-140)	139 (136-141)	0.085	139 (137-141)	139 (136-141)	0.975
Albumin g/dL	3.8 (3.4-4.1)	3.9 (3.5-4.3)	0.237	4.1 (3.7-4.3)	4.2 (3.8-4.5)	0.184
Bilirubin mg/dL	0.8 (0.5-1.5)	0.6 (0.5-1.2)	0.048	0.9 (0.6-1.4)	0.9 (0.6-1.4)	0.648
BNP pg/ml (N=301)	1547 (784-2598)	847 (541-1304)	0.03*	850 (359-2101)	666 (216-1941)	0.602*
Hematology values – SD						
Hemoglobin g/dl	11.1 ± 1.24	11.1 ± 1.3	0.818	13.9 ± 1.2	14.2 ± 1.2	0.02
Platelets k/uL - IQR	222 (177-270)	207 (179-240)	0.231	221 (183-258)	212(177-258)	0.712
Ferritin ng/ml	128 (63.-296)	184 (105-375)	0.045*	116 (72-191)	175 (109-322)	<0.001*
TSAT %	0.12 (0.09-0.15)	0.24 (0.22-0.28)	<0.001*	0.14 (0.11-0.16)	0.26 (0.22-0.31)	<0.001*

Hemodynamics – IQR						
MAP (mmHg)	81.2 (74-88)	81 (71-92)	0.750	84 (76-91)	83 (74-88)	0.171
HR (beats/min)	89 (73-100)	82 (75-96)	0.301	80 (69-100)	80 (70-90)	0.680
RAP mmHg	13 (9-17)	8 (5-15)	<0.001	13 (7-20)	8 (5-14)	<0.001
CWP mmHg	25 (21-30)	20 (15-26)	<0.001	24 (18-30)	22 (15-25)	0.013
CI l/m^2	1.9 (1.6-2.2)	2.0 (1.72-2.26)	0.250	1.70 (1.41-1.97)	1.72 (1.40-2.16)	0.355
SVRI (dynes s m2/cm5)	2880 (2335-3534)	2829 (2363-3372)	0.743	3334 (2844-4140)	3380 (2793-4116)	0.950
PVR (WU)	3.0 (2.0-4.0)	2.4 (1.5-3-3)	0.049	2.9 (1.78-4.12)	2.62 (1.79-4.22)	0.530
HF Treatment %						
ACEI/ARB	44 (28.9)	14 (28.0)	0.755	30 (27.3)	20 (24.1)	0.847
BB	115 (75.7)	40 (80.0)	0.760	89 (80.0)	68 (81.9)	0.727
ARNI	36 (23.7)	13 (26.0)	1	47 (42.0)	42 (50.6)	0.264
MRA	64 (42.1)	21 (42.0)	0.928	53 (47.7)	44 (53.0)	0.468
SGLT2 inh.	3 (1.9)	1 (1.9)	1	4 (3.6)	1 (1.2)	0.391
HDZ/NTG	42 (27.6)	12 (24.0)	0.646	27 (24.5)	15 (18.0)	0.616
Loop Diuretics	148 (97.4)	49 (98.0)	0.805	110 (99.1)	79 (95.2)	0.103
Distal	26 (17.7)	7 (13.7)	0.815	17 (15.5)	7 (8.3)	0.314
Digoxin	27 (17.7)	6 (12.0)	0.907	26 (23.6)	15 (18.0)	0.553
Amiodarone	48 (31.5)	16 (32.0)	0.924	38 (34.5)	32 (38.6)	0.553
Inotropes	100 (65.8)	30 (60.0)	0.725	56 (50.5)	38 (45.7)	0.636
VKA	26 (17.7)	12 (23.5)	0.553	23 (20.9)	21 (25.0)	0.616
DOAC	41 (27.9)	14 (27.5)	1	37 (33.6)	18 (21.4)	0.088
ASA	86 (58.4)	28 (54.9)	0.792	56 (50.9)	47 (53.9)	0.580
Oral Iron	37 (24.3)	10 (20.0)	0.838	7 (6.4)	11 (13.2)	0.170
AICD	38 (25.0)	15 (30.0)	0.594	23 (20.7)	20 (24.1)	0.515

* Estimates for log_BNP, log_ferritin, log_iron and log_tsat.

Data are expressed as means ± SD (standard deviation) when normally distributed or as medians with interquartile range when non-normally distributed. Between group comparison were tested using Student's t-test for normal variables, or U Wilcoxon test, in cases where the assumptions of normality or homogeneity of variances were not met. Inter-group differences were tested one-way analysis of variance with covariates (ANCOVA) or Kruskal-Wallis test where the assumptions of normality or homogeneity of variances were not met. Between group and intergroup comparisons for categorical variables were analyzed using Chi-square tests or Fisher's exact tests, as deemed appropriate for the specific variable type.

Abbreviations: AICD: Automatic implantable cardiac defibrillator. ARB: angiotensin receptor blockers. ACEI: angiotensin converter enzyme inhibitors. ASA: Acetylsalicylic acid. BMI: Body mass index. BNP: Brain natriuretic peptide. CI: Cardiac Index. CKD: Chronic kidney disease. COPD: Chronic obstructive pulmonary disease. CVA: Cerebral Vascular Accident. CWP: capillary wedge pressure. DOAC: Direct anticoagulants. eGFR: estimated glomerular filtration rate. HDZ/NTG: Hydralazine and Nitrates. HR: Heart rate. IHD: Ischemic heart disease. MAP: Mean arterial pressure. MCV: Mean corpuscular volume. MCHC: Mean corpuscular hemoglobin concentration. NYHA: New York Heart Association functional class. PVR: pulmonary vascular resistance. SGLT2: sodium glucose transporter 2 inhibitors. SVRI: indexed systemic vascular resistance TSAT: Transferrin saturation index. VKA: vitamin K antagonists. WU: Wood Units.

Additionally, we categorized patients into four groups based on ferritin levels and transferrin saturation (TSAT): Global ID (Ferritin < 100 ng/mL), Delivery-ID (TSAT < 20%), Storage-ID (Ferritin < 100, TSAT > 20%), and No-ID (Ferritin > 100, TSAT > 20%). The detailed analysis of the ID phenotype revealed Delivery ID in 39.1%, Global-ID in 25.2%, and Storage-ID in 7% of the cohort. An absence of ID was less common in anemic patients, and abnormal iron delivery tended to be more prevalent in this group, emphasizing the crucial role of iron availability rather than overall iron body content.

The prevalence of anemia and ID phenotype in the advanced HF patient cohort displayed distinct patterns based on the presence of anemia ($p < 0.001$) (Figure1). The distribution of ID phenotypes indicated that abnormal iron delivery (Ferritin > 100 and TSAT < 20%) was the most common type in both anemic and non-anemic patients (Panel A and B). Although there were no significant statistical differences in the percentage of patients with abnormal iron delivery between anemic and non-anemic groups ($p = 0.058$), patients without any type of ID were more common in those without anemia.

Interestingly, from a clinical perspective, patients with global and iron delivery deficiency required more inotropes and presented higher filling pressures in the hemodynamic study, suggesting a higher degree of systemic congestion (Table 3). Overall, global ID or iron deliver deficiency (both with TSAT <20%) behaved similarly and indicated more severe HF, whereas iron storage deficiency showed characteristics of a more stable clinical scenario, as patients without any type of ID.

Figure 12. Prevalence of anemia and ID phenotypes in a cohort of advanced HF patients. The distribution of ID subtypes was different according to the presence of anemia (p<0.001). Panel A) shows the distribution of ID phenotypes in anemic patients. The most common type in anemic and non-anemic patients was abnormal iron delivery (Ferritin >100 and TSAT <20%). There were no significant statistical differences in the percentage of patients with abnormal iron delivery between anemic and non-anemic (p=0.058). Patients without any type of ID were more common in patients without anemia (p<0.05).

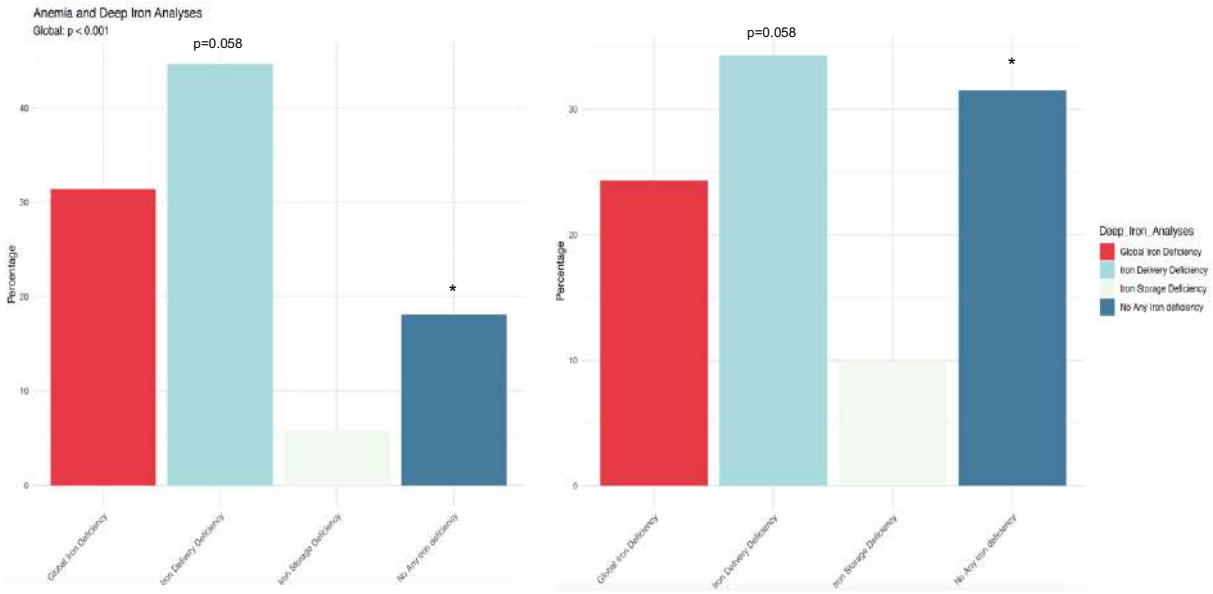


Table 3: Clinical characteristics summaries and between group comparisons according to in-depth iron status analyses.

	Global Deficiency	Delivery Deficiency	Storage Deficiency	No Deficiency	p-value
	N = 106	N = 149	N = 29	N = 89	<0.001
Baseline Characteristics					
Age-yr.	57 ± 10	56 ± 11	58 ± 12	58± 10	0.736
Male sex - no. (%)	66 (65.05)	110 (75.34)	17 (58.62)	67 (74.16)	0.462
NYHA Class III-IV– no. %	97 (91)	142 (95.3)	27 (93.1)	77 (86.5)	0.391
BMI (kg/m2)	27.31 ± 6.28	27.98 ± 6.44	28.83 ± 5.67	28.35 ± 5.83	0.727
Comorbidities no. (%)					
IHD	70 (67.96)	88 (60.27)	18 (62.07)	57 (62.64)	0.594
COPD	23 (22.33)	23 (15.75)	8 (27.59)	20 (21.98)	0.443

Diabetes	28 (27.18)	45 (30.82)	7 (24.14)	20 (21.98)	0.401
CKD	26 (25.24)	41 (28.08)	7 (24.14)	25 (27.47)	0.979
Serum markers - SD					
Creatinine mg/dl	1.2 (1-1.6)	1.5 (1.1-2)	1.3 (1-1.5)	1.3 (1-1.65)	0.005
eGFR mL/min/1.73m 2	77.65 (53-95.5)	76.75 (47.25-92.55)	76.7 (50-87.6)	68 (46-87.25)	0.138
Sodium mEq/L	139 (136.5-141.5)	138 (134-140)	139 (136-142)	139 (136-141)	0.047
Total bilirrubin mg/dL	0.8 (0.5-1.5)	0.9 (0.6-1.4)	0.7 (0.5-1.3)	0.8 (0.55-1.3)	0.086
Albumin g/dL	4 (3.7-4.3)	3.9 (3.43-4.2)	4 (3.6-4.4)	4.1 (3.7-4.4)	0.044
BNP pg/ml (N=299)	1978 (878-3525)	2271 (988.5-4476)	1276.5 (822.25-1854)	1899 (896-3693.25)	0.084*
Hematology values - SD					
Hemoglobin g/dl	12.13 ± 1.71	12.30 ± 1.87	13.03 ± 1.41	12.96 ± 2.15	0.001
Hematocrit %	38.59 ± 5.32	38.41 ± 5.71	40.47 ± 3.72	40.06 ± 6.31	0.005
Platelets k/uL - IQR	221 (174-268.5)	223 (182.25-257.75)	232 (181-274)	207 (179-254)	0.021
Iron values - IQR					
Ferritin ng/ml	56.1 (40.65-80.5)	210 (138.23-385.88)	74.2 (60-87)	234 (155-390.4)	<0.001*
Iron ug/dl	44 (33-56)	41 (35-51.5)	92 (80-100)	82 (69-96.5)	<0.001*
Transferrin mg/dl	312 (279-348)	254 (223-287)	269 (241-319)	243 (212-281)	<0.001
TSAT %	0.12 (0.08-0.16)	0.14 (0.11-0.16)	0.26 (0.23-0.3)	0.26 (0.24-0.31)	<0.001*
Hemodynamics – IQR					
MAP (mmHg)	84 (75.06-90.09)	81.51 (75.57-88.44)	78.54 (73.59-88.11)	81.84 (73.67-89.18)	0.714
Heart Rate (bpn)	82 (69-99.5)	87.5 (72-100)	77 (67.5-85.5)	80 (70-94)	0.149
RAP mmHg	12 (7-17)	14 (8-19)	8 (5-13)	8 (5-15)	0.142
CWP mmHg	25 (18-28)	25 (20-32)	19 (14-23.5)	22 (15-26)	0.070
Cardiac Index l/m^2	1.88 (1.5-2.13)	1.76 (1.44-2.06)	1.83 (1.54-2.3)	1.9 (1.55-2.19)	0.313
SVRI (dynes s m2/cm5)	2915.25 (2571.50-3534.72)	3062.92 (2587.56-3969.23)	2836.12 (2509.99-3916.58)	3140.4 (2541.47-3630.95)	0.885
PVR (WU)	3.01 (1.96-4.12)	2.75 (1.91-4.1)	2.11 (1.51-3.84)	2.5 (1.84-3.46)	0.302
HF Treatment %					

ACEI/ARB	27 (26.21359223)	39 (26.71)	8 (27.59)	21 (23.08)	0.924
BB	79 (76.70)	111 (76.03)	22 (75.86)	75 (82.42)	0.151
ARNI	36 (34.95)	43 (29.45)	12 (41.38)	39 (42.86)	0.025
MRA	50 (48.54)	57 (39.04)	13 (44.83)	44 (48.35)	0.088
SGLT2 inh.	3 (2.91)	4 (2.74)	1 (3.45)	0 (0)	0.081
HDZ/NTG	23 (22.33)	42 (28.77)	1 (3.45)	20 (21.98)	0.010
Loop Diuretics	103 (97.2)	146 (98.7)	29 (100)	84 (94.4)	0.05
Metolazone	14 (13.59)	28 (19.18)	4 (13.79)	9 (9.89)	0.064
Digoxin	25 (24.27)	25 (17.12)	7 (24.14)	16 (17.58)	0.099
Amiodarone	26 (25.24)	55 (37.67)	5 (17.24)	37 (40.66)	0.008
Inotropes	50 (48.54)	97 (66.44)	12 (41.38)	46 (50.55)	0.002
VKA	15 (14.56)	33 (22.6)	6 (20.69)	23 (25.27)	0.068
DOAC	40 (38.83)	36 (24.66)	7 (24.14)	23 (25.27)	0.012
ASA	53 (51.46)	86 (58.9)	13 (44.83)	51 (56.04)	0.087
Oral Iron	19 (18.45)	23 (15.75)	4 (13.79)	13 (14.29)	0.173
AICD	16 (15.53)	38 (26.03)	9 (31.03)	22 (24.18)	0.275

* Estimates for log_BNP, log_ferritin, log_iron and log_tsat.

Data are expressed as means ± SD (standard deviation) when normally distributed or as medians with interquartile range when non-normally distributed. Overall group differences were tested one-way analysis of variance with covariates (ANCOVA) or Kruskal-Wallis test where the assumptions of normality or homogeneity of variances were not met. Chi-square tests or Fisher's exact tests, as deemed appropriate for the specific variable type.

Abbreviations: AICD: Automatic implantable cardiac defibrillator. ARB: angiotensin receptor blockers. ACEI: angiotensin converter enzyme inhibitors. ASA: Acetylsalicylic acid. BMI: Body mass index. BNP: Brain natriuretic peptide. BUN: blood urea nitrogen. CKD: Chronic kidney disease. COPD: Chronic obstructive pulmonary disease. CWP: capillary wedge pressure. DOAC: Direct anticoagulants. eGFR: estimated glomerular filtration rate. HDZ/NTG: Hydralazine and Nitrates. IHD: Ischemic heart disease. MAP: Mean arterial pressure. MCV: Mean corpuscular volume. MCHC: Mean corpuscular hemoglobin concentration. NYHA: New York Heart Association functional class. PVR: pulmonary vascular resistance. SGLT2: sodium glucose transporter 2 inhibitors. SVRI: indexed systemic vascular resistance TSAT: Transferrin saturation index. VKA: vitamin K antagonists. WU: Wood Units.

In addition, we analyzed patients with normal ferritin levels (ferritin 300ug/L) but TSAT <20%: these patients displayed signs of more advanced disease and incorporated plasma markers of worse multiorgan function. In fact, we were able to determine that a central venous pressure of 12 or higher marked the beginning of a trend for increased ferritin levels. Therefore, this reinforces the idea that ferritin is not a marker of iron disposal in patients with HF and rather a marker of disease severity. Moreover, relying on ferritin as a marker to determine the need for iron supplementation may overlook patients with tissue ID, potentially depriving them of a beneficial treatment. On the other hand, anemia was related with a higher output state and a higher use of inotropes, suggesting a worse

native cardiac performance and a higher need of cardiac output to reach the tissular metabolic demands.

Overall, the data presented in this last section of the thesis help to establish a connection between the hemodynamic features of HF and the iron axis. Our data reveal a strong association between ID and high cardiac filling pressures, as a marker of congestion. This underscores the concept of ID and anemia only as a continuum of disease initiated by HF congestion, progressing through ID gradual, pump failure, hypoxic tissual state, multiorgan involvement accompanied by inflammation, and iron blockage that ultimately might culminate in the development of anemia. It also provides insights into recent research on ID and underscores the significance of TSAT as a dependable indicator of ID in HF, irrespective of ferritin levels. This association could help us understand the more noticeable benefits of iron supplementation in patients with signs of congestion and the capacity of iron replacement to prevent the incidence of decompensations(106). Furthermore, this perspective may help to partially understand the negative results observed in the recent HEART-FID study, where most patients had a TSAT greater than 20%, indicating an absence of relevant ID in the context of HF, according to our data (136). Ultimately, given that anemia and ID can have diverse origins and that HF patients may have a range of comorbidities affecting overall morbidity and mortality, treating patients with iron supplementation without addressing its specific origin and the associated comorbidities could lead to suboptimal outcomes. Our research also emphasizes the critical role of the crosstalk between the heart and the broader spectrum of organs within the cardiovascular system, as individual endocrine entities essential for maintaining systemic homeostasis. We have previously highlighted the close correlation between congestion and neurohormonal activation (particularly SNS). Considered our earlier reports exposed in this thesis, ID, oxidative stress, and mitochondrial function emerge stronger as pivotal components at the intersection of cardiac failure, cardiovascular function, and multiorgan damage.

We advocate for sustained research to deepen our understanding of how these various factors interact across clinical scenarios and how experimental interventions in these elements can lead to either the improvement or worsening of disease processes within

these metabolic pathways. By improving our knowledge, we will not only to optimize HF management but will also expand to a myriad of other cardiovascular conditions where aberrant metabolism is a key contributor.

Conclusions

1. Our findings highlight that ID might not merely a comorbidity but an integral component of the HF syndrome and takes a central role in the interaction between HF and myocardial metabolism.
2. HF induces changes in the expression of iron-related genes within the heart that provoke myocardial iron depletion in experimental models.
3. Neurohormonal activation is crucial to understand intracellular iron depletion and making cardiac cells more metabolically inefficient and vulnerable to oxidative stress.
4. ID might also play a critical role in the interplay between the heart and the broader cardiovascular system, resulting in changes in biological pathways related to systemic metabolism, oxidative stress, inflammation, cellular proliferation, autophagy, and senescence.
5. These metabolic changes happen irrespective of anemia and are represented by distinct genetic expression patterns in Sirtuin7, Small integral membrane protein 20, and Mitochondrial ferritin that were associated with adverse clinical outcomes.
6. Our findings underscore the importance of metabolism and antioxidant capacity in understanding HF pathophysiology and the central role of iron in this intricate regulation.

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