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TESI DI DOTTORATO

EFFECTS OF SODIUM-GLUCOSE COTRANSPORTER 2 INHIBITORS ON IRON METABOLISM IN A CONTEMPORARY COHORT OF PATIENTS AFFECTED BY CHRONIC HEART FAILURE WITH REDUCED EJECTION FRACTION

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ABSTRACT

Background. Sodium/glucose cotransporter 2 inhibitors (SGLT2i) have modified heart failure's therapy and prognosis. Their contribution to cardiac reverse remodeling and the merging effects on iron metabolism could be the substrate of the reduction in cardiovascular morbidity and mortality. Aim of our study was to prospectively analyse biochemical, clinical and echocardiographic parameters at 6 months follow-up in comparison to baseline in a cohort of 100 consecutive patients with heart failure with reduced ejection fraction (HFrEF) treated with SGLT2i, and to identify independent predictors of cardiac reverse remodeling at follow-up.

Methods. We enrolled 100 patients with symptomatic HFrEF, already on other optimal medical therapy, who initiated SGLT2i from November 2021 to May 2022. We performed a comprehensive evaluation at baseline and at follow-up at 6 months including medical history, physical examination, electrocardiogram, blood sampling and echocardiography.

Results. Eighty-seven patients continued to take SGLT2i for the entire 6 months follow-up while 13 had to stop for adverse effects or other clinical events. Mean age was 65 years and the cause of heart failure was ischemic in 50% of cases. At follow-up we observed a minor non clinically significative decrease of systolic blood pressure (112±14 mmHg vs 115 ± 15 mmHg, p=0.05), a significant decrease of renal filtration (eGFR 64 ± 24 ml/min/1.73m2 vs 69± 23 ml/min/1.73m2, p=0.005) and sharp decline of NT-proBNP (684 pg/mL vs 838 pg/mL, p=0.002). Patients at follow-up had higher values of hemoglobin (14.8 ± 1.5 g/dL vs 14.2 ±1.6 g/dL, p=0.006) and red blood cells (5.0 ± 0.8 x 10^12/L vs 4.7 ±0.6 x 10^12/L, p<0.001) while iron and ferritin levels declined significantly (respectively 80 μg/dL vs 92 μg/dL, p=0.002 and 160 μg/L vs 194 μg/L, p=0.01). Hepcidin values dropped (4.0 nmol/L vs 6.7 nmol/L, p=0.003) with a consistent increase of erythroferrone serum concentration (0.99 ng/mL vs 0.39 ng/mL, p<0.001). Considering echocardiographic parameters a decrease of end diastolic volume of the left ventricle (LV) (185 ± 67 ml vs 196 ± 61 mL, p=0.01) with a consistent increase of LV ejection fraction (36 ± 16% vs 33 ± 10%, p=0.05) were observed with a reduction of restrictive pattern and (5.7% vs 8.1%, p=0.039) and moderate to severe mitral regurgitation (20% vs 25% of cases, p<0.001). Patients had a better function of left atrium (PALS 17.8 ± 8.8% vs 15.3 ± 9.6, p=0.007) and right ventricle (RV free wall strain 18±5.2% vs 15.4 ± 4.3%, p=0.001). Basal levels of hepcidin was as the only independent predictor of LV reverse remodeling at multivariable analysis. Clinical outcome was not assessable due to the small number of events.

Conclusion. SGLT2i shown to strongly impact on HFrEF cardiac remodeling and the ability to modify iron pathway and possibly inflammation could be an additional strength of their action.

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BACKGROUND

Heart failure with reduced ejection fraction (HFrEF) remains a major cause of morbidity and mortality worldwide. However, improvements in pharmacotherapy during the last three decades significantly delay clinical progression and prolong disease-free survival. Combination therapy with an angiotensin-converting enzyme inhibitor (ACEi), an angiotensin receptor blocker (ARB) or an angiotensin receptor-neprilysin inhibitor (ARNI), a beta blocker (BB) and a mineralocorticoid receptor antagonist (MRA), has been the corner stone of guideline-recommended therapy for patients with HFrEF for several years [1]. Most recent guidelines added sodium/glucose cotransporter 2 inhibitors (SGLT2i) to the standard regimen, as first line therapy in patients with HFrEF. The last 2021 European Society of Cardiology (ESC) guidelines on heart failure (HF) recommend dapagliflozin or empagliflozin in addition to optimal medical therapy (OMT) (namely an ACEi/ARNI, a beta-blocker and an MRA) as essential drugs for patients with HFrEF regardless of the presence of diabetes mellitus (class I – level A). Of note, prognosis in HFrEF is further impacted by comorbidities and iron deficiency plays a key role in event free survival and quality of life. Intravenous iron supplementation with ferric carboxymaltose is recommended by current guidelines in symptomatic patients recently hospitalized for HF with documented iron deficiency (ID) [2,3, 4, 5,6].

Sodium/glucose cotransporter 2 inhibitors in heart failure

Sodium/glucose cotransporter 2 inhibitors have been initially developed as drugs to treat type 2 diabetes mellitus (T2DM): they reduce blood glucose by increasing urinary glucose excretion[7]. Glycosuria occurs when the renal threshold of glucose is reached, approximately with a blood glucose of 160-180 mg/dl. At this level, the proximal tubule in kidney glomerulus cannot reabsorb all the filtered glucose, resulting in glycosuria. Sodium/glucose cotransporter

2 (SGLT2) is expressed in the proximal tubule and is capable of reabsorption of nearly 90 percent of the filtered glucose load. Its inhibition promotes renal excretion of glucose and thereby modestly lower blood glucose levels and glycated hemoglobin A1C (HbA1C) in patients affected by T2DM. SGLT2i do not usually provoke hypoglycemia in non-diabetic patients, since their ability in lowering blood glucose is directly proportional to plasmatic glucose levels. Moreover, SGLT2i showed to be effective also in improving weight loss (together with advice in lifestyle and diet) and in lowering systolic blood pressure[8].

Initially developed as anti-diabetic drugs, SGLT2i were later demonstrated to significant improve cardiorenal outcomes in diabetic patients[9], [10]. This reduction in cardiovascular (CV) mortality is particularly important since it is estimated that 65% of people with diabetes will die as a result of cardiovascular complication[11],[12], [13].

Subsequent studies showed that SGLT2i also prompt significant cardiorenal benefits in patients affected by heart failure regardless of the presence of diabetes[14], [15], [16], [17].

The use of SGLT2i has been associated with a significant reduction in the rates of hospitalization for heart failure and cardiovascular death, consistently across the spectrum of left ventricular ejection fraction (LVEF) and regardless of the presence of type 2 diabetes mellitus[15],[16],[17].

The DAPA-HF Trial, published in 2019, demonstrated that dapagliflozin compared to placebo lead to a 26% reduction of composite end-point of worsening heart failure or death from cardiovascular causes was lower among patients with symptomatic, and these findings were similar regardless of the presence or absence of type 2 diabetes [14].

Subsequently, the EMPEROR-Reduced Trial showed in 2020 that the composite of cardiovascular death or hospitalization for heart failure was reduced by 25% among patients with symptomatic HFrEF treated with empagliflozin than in those with placebo, with or without diabetes. Although there was not a significant reduction in cardiovascular mortality (one of the

two components of the composite endpoint), a recent meta-analysis of the DAPA-HF and EMPEROR-Reduced Trial found no heterogeneity in cardiovascular mortality[18].

The last 2021 European Society of Cardiology (ESC) guidelines on heart failure recommend dapagliflozin or empagliflozin in addition to optimal medical therapy (namely an ACEi/ARNI, a beta-blocker and a MRA) as cornerstone therapies for patients with HFrEF regardless of the presence of diabetes mellitus (class I – level A).

In 2020 the DAPA-CKD Trial focused on patients with an estimated glomerular filtration rate (eGFR) of 25 to 75 ml per minute per 1.73 m^2 of body-surface area (BSA) demonstrated that the risk of a composite of sustained decline in eGFR of at least 50%, end-stage kidney disease or death from renal or cardiovascular causes was significantly lower in patients treated with dapagliflozin than in patient treated with placebo, regardless of the presence of type 2 diabetes[19]. Of note, a small reduction in eGFR following initiation of SGLT2i should be expected and should not lead to premature discontinuation of the drug. Indeed, these changes are generally transient, occur despite improvement in patients' outcomes and these drugs slower worsening of renal function in the long term. An increase in serum creatinine of < 50% above baseline, as long as serum creatinine is < 3 mg/dl, or a decrease in eGFR < 10% from baseline, as long as it is > 25 ml/min/1.73m^2, can be considered acceptable.

Moreover, caution is needed since SGLT2i could increase the risk of fungal genito-urinary tract infections and therefore patients should be monitored and properly made aware of this possible complication. In EMPEROR-Reduced trial empagliflozin showed to be associated with urinary tract infections (4.9% of patients) and with genital tract infections (1.7% of patients).

Mechanisms of action of SGLT2i in heart failure

The filtered load of glucose induces osmotic diuresis and natriuresis, a process already called "smart osmotic diuresis" resulting in improved mobilization of interstitial fluid and also improving intrarenal hemodynamics[20] .

However, it is now considered unlikely as the driving mechanism of SGLT2i beneficial effect, since an increase in diuresis has been noted only transiently after the beginning[21].

Moreover, there are no data showing that the beneficial effects of these drugs in patients with HFrEF are related to reduced glucose levels.

Several mechanisms have been proposed to describe SGLT2i benefits, among which there are: autophagy restoration, alteration in ion homeostasis, improvements in myocardial metabolism and energetics, and improvements in endothelial function that promote vasodilation and reduce afterload [22],[23], [24],[25], [26], [27].

SGLT2i has also been postulated to reduce myocardial fibrosis and to alter adipokines and cytokines production [28],[29]. They have also been shown to reduce the burden of atrial fibrillation by mechanism that may induce electrical and structural atrial remodeling[26],[27], [30], [31], [32]. Of course, also the beneficial effects of SGLT2i on renal function may contribute to improved outcomes in HF patients. In particular, they showed to be able to reactivate macula densa function at glomerular level and thus to reduce renin-angiotensin-aldosterone system activation. All these mechanisms may influence myocardial remodeling, which could be the substrate of the reduction in cardiovascular morbidity and mortality; the evidence of a slow and gradual decline of natriuretic peptides in HF patients after SGLT2 inhibition may confirm this hypothesis [33], [34].

Therefore, the increasing interest in assessing atrial and ventricular remodeling has recently resulted in studies exploring these hypotheses via echocardiography or magnetic resonance imaging (MRI)[35], [36].

Cardiac reverse remodeling

Most studies were performed on patients with T2DM, with only few studies enrolling people with HF irrespective of the presence of T2DM. A recent review and metanalysis focused on the changes in cardiac functional properties after the begin of SGLT2i [37]. Moreover, the majority of studies used echocardiography as the imaging method employed for the cardiac measurements. According to the results of the reported meta-analysis, the use of SGLT2i was associated with an improvement in LVEF and in left ventricular global longitudinal strain (LV-GLS). Regarding volumes, left ventricular end-diastolic volume (LV-EDV) did not show significant reduction at follow-up after the initiation of SGLT2i, while a significant reduction in left ventricular end-systolic volume (LV-ESV) and in left atrial volume index (LAVi) was noted. Finally, an improvement in the ratio of mitral peak velocity of early filling (E') to early diastolic mitral annular velocity (e') (E/e' ratio) was also present following SGLT2 inhibition, and this could account for the beneficial effects seen in the difficult-to-treat category of HFpEF patients.

In HFrEF patients also right ventricular function may be adversely affected in addition to left ventricular (LV) functions. Only few studies analysed the impact of this new category of HF drugs on right ventricle suggesting an improvement in the RV systolic function after few months of therapy [20], [21], [38].

Moreover, it is well known that secondary mitral regurgitation (MR) in HFrEF is associated with a poor prognosis[39], [40]. In fact, left ventricular dilation leads to dilation of the mitral annulus and reduced coaptation of the mitral valve leaflets causing secondary MR. Pivotal studies demonstrated that neurohormonal modulation prompts cardiac remodeling with reduction in left ventricular size and volume, leading to reduction of MR severity[41]. The PRIME-study demonstrated that sacubitril/valsartan was able to reduce secondary functional MR to a greater extent than did valsartan alone.

After the introduction of SGLT2i as a crucial therapy in HFrEF, a recent study underlined the early and substantial reverse remodeling effects of SGLT2i, even if data on MR were not reported[42]. Moreover, another recently published study on a rat model demonstrated that dapagliflozin was able to suppress cardiac fibrosis and to improve hemodynamics in an MR-induced HF rat model, including partial restoration of LV-EF and end-systolic pressure volume relationship[43]. An ongoing trial is evaluating the hypothesis that a SGLT2i, ertugliflozin, could be effective in improving MR in patients with functional MR secondary to LV dysfunction and dilation *(ClinicalTrials.gov Identifier: NCT04231331*). Nevertheless, at the moment strong evidence that SGLT2i are able to reduce MR severity, due to their ability to reduce LV size and volume, is still lacking.

Iron metabolism and heart failure

General concepts of iron metabolism

Iron is a micronutrient essential for a wide range of metabolic processes in all living organisms[44] including cellular energy metabolism, anaerobic respiration, synthesis of hemoglobin and nucleotides. Moreover, iron is involved in many other processes in the body such as oxidative metabolism and cellular immune response[45]. However, free iron is highly reactive and may be toxic because of its capability to generate oxygen-derived free radicals (Reactive Oxygen Species-ROS) that can cause severe damages to biological molecules. Systemic iron homeostasis is highly conservative and, in the adult, the total amount of iron in the body is about 3-4 g. Under physiological conditions, red blood cells (RBCs) contain the largest proportion of body iron (~ 2 g), and the 20-25 mg of iron needed for daily production of new RBCs derive almost totally from continuous recycling of aged erythrocytes by splenic macrophages[46]. Every day, 1-2 mg/d of iron is absorbed from the diet and the same amount is lost through skin and mucosal exfoliation, plus menses in fertile women. It should be noted that

the organism does not have an active iron excretion mechanism, thus the control of the duodenal absorption plays a vital role in iron homeostasis[47]

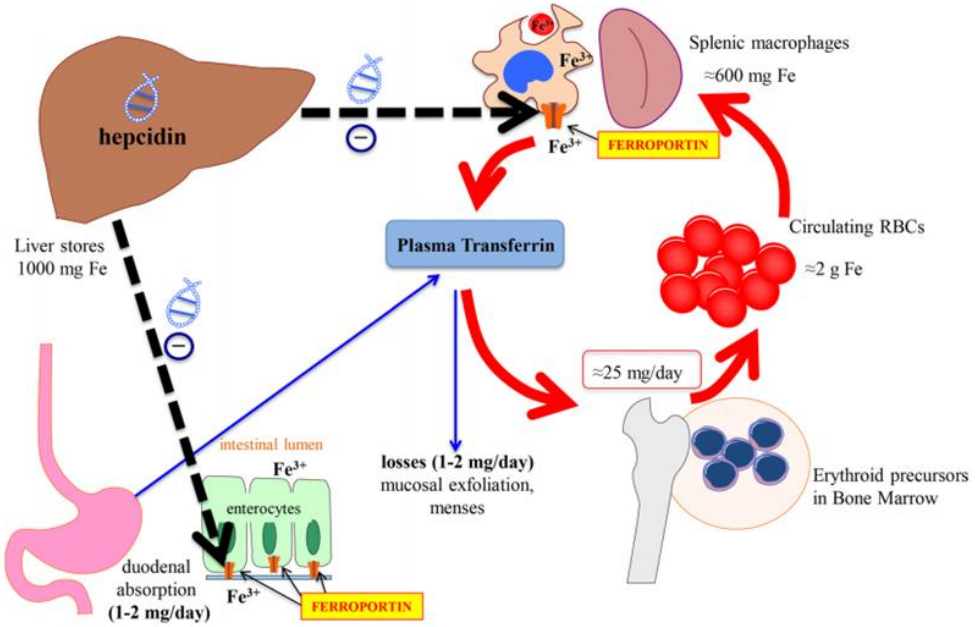

Figure 1: *Systemic Iron Homeostasis (adapted from Girelli D, 2018* [48]*)*

Hepcidin is a hormone synthesized in the liver and its main role is to regulate the plasma pool of iron. Hepcidin is sometimes called the "master iron regulator" because it controls how much iron is available for cells via different mechanism. It acts by controlling iron absorption from the diet through enterocytes, iron recycling from macrophages involved in the destruction of senescent RBCs, and iron mobilization from the deposits. Hepcidin negatively regulate the iron homeostasis by binding to ferroportin, the channel responsible for the efflux of iron from enterocytes, macrophages and hepatocytes into the plasma. After binding to ferroportin, hepcidin causes its destruction by endocytosis into the cell's lysosomes [49]. Iron depletion suppresses hepcidin, leading to increased activity of iron exporter ferroportin releasing stored iron, enhanced iron recycling by macrophages, and up-regulation of either iron uptake from enteric lumen or its export to circulation [50],[51]. Other conditions that regulate hepcidin expression are chronic infections and inflammatory diseases. Indeed, hepcidin levels turned to be hundred-

times higher in patients suffering from inflammatory conditions than in healthy individuals[52]. Interleukin-1β (IL-1β), IL-6 and IL-22 increase hepcidin expression leading to macrophage iron sequestration and hypoferremia that reduces erythropoietic response leading to anemia[53], [54]. Erythroferrone (ERFE) is a hormone synthesized by erythroblasts in the bone marrow, which suppresses hepcidin by trapping its stimulator BMP6. ERFE is member of the C1q/TNF-related protein (CTRP) family. In iron deficiency anemia, hypoxia strongly induces EPO synthesis in the kidney and erythroferrone released from EPO-stimulated erythroid precursors further down-regulate hepcidin[55].

Iron trafficking is a dynamic process, using transferrin to transport iron between sites of absorption, recycling, and storage to sites of iron utilization. Transferrin-iron is taken up by binding transferrin receptor 1 (TfR1) ubiquitously expressed on all cells. The highest concentration of TfR1 is found on erythroid precursors, hemoglobin producing cells with the highest iron requirements in the body. However, its ligand diferric transferrin is very low if ID is present and, when unbound, TFR1 is cleaved by a local protease. Even if its function remains unclear, the soluble form of Transferrin Receptor 1, usually referred to as soluble Transferrin Receptor (sTfR) is typically upregulated in ID [56].

Iron deficiency and anemia in heart failure

Iron deficiency (ID) and anemia are common conditions and their prevalence increases with aging[57], however ID remains often underdiagnosed and undertreated[58]. According with current epidemiological data, near one third of the world population is affected by anemia. Increased iron needs, unbalanced iron supply, low iron intake, reduced absorption and chronic loss are the most common causes and are often present in combination. According to the World Health Organization criteria, anemia is defined as a haemoglobin concentration <12 g/dL in women

and 13 g/dL in men. Iron deficiency induces striking modification of cell and systemic iron homeostasis, especially affecting the hepcidin/ferroportin axis[44].

Heart failure is a complex syndrome and nowadays accepted with a substantial inflammatory component. Accepted definition of ID in patients with HF is either a serum ferritin concentration <100 ng/mL or 100-299 ng/mL with transferrin saturation (TSAT) <20%[2]. ID, with or without anemia, is present in up to 55% of patients with chronic heart failure[59]. In addition to inflammation, it may be caused by several mechanisms including increased loss and reduced intake or absorption (i.e. malnutrition, gut congestion). Patients with HFrEF and ID, independently from anemia, have reduced functional capacity, poor quality of life, risk of hospitalization, increased all cause and cardiovascular mortality and increased costs [60]. Anemia is also related to unfavourable outcomes, and correcting anemia with erythropoiesis stimulating agents did not showed to improve prognosis. Furthermore blood transfusions are associated with increased mortality in patients with HF[61]. Large randomized clinical trials showed that iron supplementation with i.v. ferric carboxymaltose or derisomaltose is safe and improves symptoms, exercise capacity, and QOL of patients with HFrEF and iron deficiency [62], [63], [64], [65], [66], while oral iron therapy is not an effective alternative[67]. Thus current guidelines recommend to regularly screen patients with HFrEF for anaemia and iron deficiency with full blood count, serum ferritin concentration, and TSAT and to infuse ferric carboxymaltose in recently hospitalized patients when iron deficiency is found (Class IIa – level of evidence B)[2] (***Figure 2***).

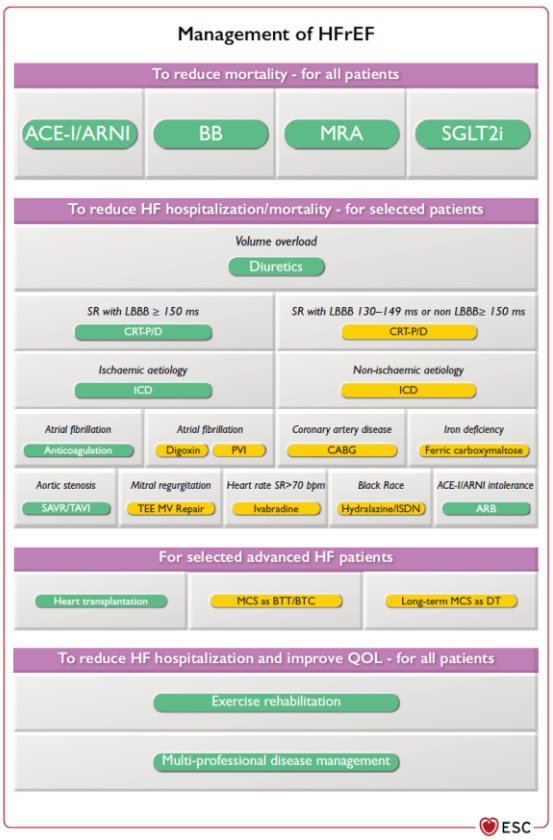

Figure 2. Strategic phenotypic overview of the management of heart failure with reduced ejection fraction. *Adapted from 2021 ESC Guidelines for the diagnosis and treatment of acute and chronic heart failure*[2].

Effects of Sodium/glucose cotransporter 2 inhibitors on iron metabolism

Iron metabolism and type 2 diabetes (DM2) are strictly correlated since iron affects glucose metabolism and glucose metabolism impinges on several iron metabolism pathways. ID may impair glucose metabolism predisposing to incident DM2 and diabetic patients with ID have worse glycaemic control [68]. Previous studies demonstrated that subjects with impaired glucose tolerance or type 2 diabetes showed significantly increased levels of sTfR and higher serum ferritin and insulin sensitivity is negatively associated with sTfR serum levels[69]. Furthermore it has been shown that treatment with SGLT2i in diabetics patients leads to increased haematocrit due to hemoconcentration and enhanced erythropoiesis as indicated by a rise in plasma

erythropoietin (EPO) and reticulocytes in patients treated with these drugs [70]. Some authors proposed the improved renal cortical oxygenation as mechanism leading to increased EPO levels [71]. Heyman et all [72] conversely proposed that intensified hypoxia at the renal cortical medullary junction induced by SGLT2i causes enhanced EPO transcription. Thus SGLT2i improving tubulointerstitial hypoxia which is the common final pathway of end-stage renal disease represent a new class of diuretics with renoprotective effects [73].

Other proposed mechanisms of increased haematopoiesis linked to SGLT2i therapy are enhancement of iron absorption due to reduced intestinal interstitial oedema[74], reduced systemic inflammation leading to increase of transferrin receptor function[75], improved serum ferritin concentration[76], increased haematopoiesis in bone marrow via reduction of macrophage infiltration and fat metabolism [77].

More recently Ghani et al [78] evaluated a cohort of 52 obese type 2 diabetes patients randomized to dapagliflozin or placebo reporting a significant increase in haemoglobin concentration and haematocrit in SGLT2i patients but also significantly reduced circulating hepcidin and ferritin concentrations while causing a significant increase in levels of the hepcidin inhibitor, erythroferrone, and a transient increase in erythropoietin. Additionally, patients treated with dapagliflozin had increased plasma transferrin levels and expression of transferrin receptors 1 and 2 and a decrease in hypoxia-induced factor-1α in mononuclear cells, while there was no change in the expression of the iron cellular transporter, ferroportin. Another study evaluated the effects of empagliflozin on 42 proteins plasma levels of subjects across the spectrum of glucose tolerance[79] showed 34% of ferritin concentration reduction after 4 weeks of therapy.

Empagliflozin treatment was also associated with an early increase in plasma erythropoietin levels accompanied by an increase in hematocrit and reduced ferritin and RBC hemoglobin concentration in patients with type 2 diabetes mellitus and coronary artery disease[80]. Considering the setting of heart failure few evidence are available. Recent sub-analysis from

DAPA_HF trial confirmed the efficacy of dapagliflozin irrespective from ID. Transferrin saturation, ferritin, and hepcidin were reduced and total iron-binding capacity and soluble transferrin receptor increased with dapagliflozin compared with placebo[81]. Furthermore Andresen et al[82] showed serum-erythropoietin increased in patients with HFrEF treated with empagliflozin compared to placebo from baseline to 12 weeks, plasma-hepcidin decreased, and mean corpuscular volume increased, while no changes were observed for mean corpuscular haemoglobin concentration or plasma-iron.

AIMS

The main aim of our study was to evaluate iron metabolism in a contemporary cohort of patient with HFrEF referring to heart failure clinic of the Cardiology Unity at the University of Verona already on optimal medical therapy (OMT) who started treatment with SGLT2i.

In details the objectives of the study were:

- To evaluate clinical biochemical parameters (haemoglobin, haematocrit, iron status, hepcidin, erythroferrone, sTfR, etc.) in order to find out SGLT2i effects on iron metabolism parameters
- To compare echocardiographic data at baseline and at 6 months follow up after SGLT2i treatment looking for any improvement of cardiac volumes or function and valve disease
- To compare and analyse the correlations between the biochemical and clinical/ echocardiographic parameters collected in order to gain insights into the mechanisms involved in SGLT2i action in patients with HFrEF

METHODS

Eligibility criteria

For this prospective single-centre study, all the patients who attended our outpatient clinic for heart failure and who initiated SGLT2i from November 2021 to May 2022 were enrolled. Inclusion criteria were: age ≥18 years, symptomatic HFrEF with left ventricular ejection fraction of ≤40% and optimal medical therapy (OMT) for HFrEF, starting therapy with SGLT2i (empagliflozin or dapagliflozin).

OMT for HFrEF is defined as the combination of an angiotensin converting enzyme inhibitor or angiotensin receptor blocker or sacubitril/valsartan + beta-blocker + mineralocorticoid receptor antagonist, each drug at the maximum tolerated dose for the single patient.

Exclusion criteria were: type 1 diabetes mellitus, renal impairment defined as an estimated glomerular filtration rate < 20 ml/min/1.73m^2, pregnancy, prior heart transplant or left ventricular assist device, end-stage hypertrophic or arrhythmogenic cardiomyopathy, cardiac amyloid, Anderson-Fabry disease.

This prospective single- centre study was performed within a framework of the observational *Heart Failure Verona Registry of the University of Verona – CESC number 1959*. Written informed consents were obtained from each subject after a detailed explanation of the studies. For analyses, all data were anonymized. The study conformed to the Declaration of Helsinki and to local regulations for prospective studies and data handling.

Study protocol

We performed a comprehensive evaluation at baseline including medical history, physical examination, electrocardiogram (ECG), blood sampling and echocardiography.

Blood tests included blood count, hemoglobin (Hb), hematocrit (Hct), iron, ferritin, transferrin saturation, serum creatinine, eGFR (CKD-EPI formula), potassium (K), N-terminal pro-brain natriuretic peptide (NT-proBNP), hepcidin, erythroferrone (ERFE) and sTfR.

Serum Hepcidin Assay

Hepcidin-25 was measured by LC-MS/MS after minor modifications of a method included in an international harmonisation study[83]. Hepcidin-25 standards (native and the isotopic-labelled internal standard [Asp-Thr-His-[13C9,15N]Phe-Pro-Ile-Cys-Ile-[13C9,15N]Phe-Cys-Cys-[15N]Gly-Cys-Cys-His-Arg-Ser-Lys-Cys-Gly-Met-Cys-Cys-Lys-Thr] (Mr2810.2)) were purchased from Peptide International (Louis-ville, KY, USA); a volume of 80 µL was used for each serum and standards. Blank serum (deprived from hepcidin by charcoal treatment) was used as reference. Samples were treated by solid-phase extraction using Oasis hydrophilic-lipophilic balanced reversed-phase (HLB) cartridges (Waters, Milan, Italy). HPLC was performed using an X-Terra MS C182.5µm (Waters), and detection was obtained using a Triple Quad LC-MS/MS (Agilent Technologies,Santa Clara, CA, USA). Normal ranges (according to sex and age) were available as obtained in a previous population study [84]. Hepcidin concentration is expressed as nmol/L.

Serum ERFE Assay

Erythroferrone analysis was performed using the Human Erythroferrone IETM ELISA kit (Intrinsic Lifesciences-The BioIron CompanyTM, USA), which is a double monoclonal antibody sandwich ELISA assay. The measurements were obtained according to manufacturer's instructions. In brief, after the incubation of serum samples in the assay plate pre-coated with mouse anti-human erythroferrone monoclonal antibody, a second monoclonal anti-erythroferrone antibody conjugated to horseradish peroxidase was added. The

quantification of the enzyme conjugate bound to the immobilized ERFE was done by the addition of substrate, then after stopping the reaction with an acid solution, the plate was read at 450 nm in a PowerWaveTM XS Microplate Reader (BioTek Instruments). The standard curve was prepared with Human erythroferrone standard concentrations ranging from 10 ng/ml to 0.16 ng/ml (8 points). Samples, standards and controls were assessed in duplicates and diluted (1:10 dilution). Absorbance values were used for data interpolation and reduction by GraphPad Prism 8 software (San Diego, CA, USA). Concentrations were expressed in ng/ml. Samples presenting a CV > 14% in repeated measurements were re-assayed. The LOD of Human Erythroferrone ELISA kit is 0.16 ng/mL. Normal range obtained with same methodology in healthy individuals was reported in a recent publication and the authors indicated that the median concentration of ERFE was 0.51 ng/mL (IQR: 0.12–1.25), with men having higher levels than women (0.67 vs 0.32 ng/mL)[85].

Serum sTfR Assay

Soluble Transferrin Receptor was measured using the DRG sTfR ELISA kit (DRG Instruments GmbH) and performed according to manufacturers' instructions. This ELISA assay is a sandwich ELISA, where the microtiter wells were coated with a monoclonal (mouse) antibody directed towards a unique antigenic site of the sTfR molecule. An aliquot of patients' serum containing endogenous sTfR were incubated in the coated well with enzyme conjugate, which is an anti-sTfR antibody conjugated with horseradish peroxidase. After adding the substrate solution, the intensity of color developed was proportional to the concentration of the sTfR in patients' samples. The standard curve was prepared in six points with sTfR standard concentrations ranging from 160 ng/ml to 10 ng/ml. The concentration of sTfR was obtained from the mean absorbance of the standard curve. Samples, standards and controls were assessed in duplicates and diluted (1:51 dilution). Absorbance values were used for data interpolation

and reduction by GraphPad Prism 8 software (San Diego, CA, USA). Concentrations were expressed in ng/ml. The normal expected values reported by DRG sTfR ELISA's datasheet is 480-3090 ng/ml (min.-max.) and a mean of 1430 ng/ml, observed in a group of 176 healthy human subjects. The absorbance was read at 450 nm on the PowerWaveTM XS Microplate Reader (BioTek Instruments). Samples presenting a CV > 14% in repeated measurements were re-assayed.

Standard echocardiographic examination

All patients underwent a baseline standard transthoracic and Doppler echocardiographic examination using commercially available echocardiography systems (Philips EPIQ 7C, GE Vivid T8). All examinations were conducted by experienced sonographers.

Classic echocardiographic parameters were evaluated. Simpson's biplane method was used to assess LV diastolic and systolic volumes, LV-EF and left atrial volume in apical 4-chamber and 2-chamber views. Diastolic function was determined by measuring the ratio of mitral peak velocity of early filling (E) to late diastolic filling velocity (A) (E/A ratio), E/E' ratio, deceleration time, left atrial volume and maximum velocity of tricuspidalic regurgitation. Right chambers were examined with tricuspidalic annular plane systolic excursion (TAPSE) and systolic pulmonary arterial pressure (PAPs). Valve function was assessed with color-Doppler, continuous-wave Doppler and pulsed wave Doppler in all the appropriate projections.

Standard echocardiographic measurements were obtained in accordance with the current guidelines of the European Association of Cardiovascular Imaging / American Society of Echocardiography [86], [87].

Images were saved digitally and subsequently analysed offline using the TomTec software by two independent expert operators (GU, JP) blinded to the data. Discordance was resolved by

consensus. Cardiac reverse remodeling was defined as an improvement of LV-EF of at least 5% or reduction in LV-EDV of at least 10% from baseline or both criteria [88], [89] .

Strain analysis

Speckle-tracking strain analysis was performed for each patient with the aid of a dedicated software (AUTOSTRAIN, TOMTEC-ARENA, TOMTEC Imaging Systems GmbH, Unterschleissheim, Germany).

Apical four, two and long-axis views and right ventricle focused view file images were uploaded into a computer for subsequent offline strain analysis. Speckle tracking analysis was calculated applying an automated contouring detection algorithm, with manual adjustments where necessary. If the offline images had a poor acoustic window, strain analysis was not performed.

We assessed left ventricular global longitudinal strain, right ventricular (RV) free-wall strain and peak left atrial longitudinal strain (PALS), in accordance with current guidelines [90], [91], [92].

Statistical analysis

Statistical analyses were performed using the SPSS (Statistical Package for the Social Sciences, Chicago, Illinois) software Release 26. Variables were tested for normality via Shapiro-Wilk test. Continuous variables are expressed as mean ± standard deviation (SD) or median and interquartile range (IQR) in case of non-normal distribution; binary variables are expressed as counts and percentages. Student t test was used for normally distributed variables, Wilcoxon test was used for variables that were not normally distributed. Frequencies are expressed ad percentage. Comparisons were performed using the Student t-test for continuous variables and the Chi-Squared ($\chi 2$) test for categorical variables. A p-value <0.05 was considered statistically significant. Difference between the study groups were analyzed using one-way ANOVA (for

normally distributed variables) or Kruskal-Wallis test (in case of non-normal distribution). Bonferroni correction was used for post-hoc analysis.

Univariate and multivariable logistic regression analyses was additionally performed to assess the independent association with left ventricular reverse remodeling.

RESULTS

Study population

For this prospective study we enrolled 100 consecutive patients with HFrEF, who initiated SGLT2i from November 2021 to May 2022. Six months follow-up was completed at the end of November 2022.

The characteristics of the study population are illustrated in **Table 1**. Male gender represented the majority (88%) and mean age was 65.6±11.9 years. The cause of heart failure was ischemic in a half of the cases and non-ischemic in the other 50% of patients, and the median of disease duration was 10 ± 9 years. The proportion of patients affected by type 2 diabetes mellitus was 20%. Around one third of patients (36%) had iron deficiency at baseline. Seventy-eight per cent of patients had implantable cardiac defibrillator (ICD) and 34% had cardiac resynchronization therapy-defibrillator (CRT-D). Among our 100 patients, 85 began therapy with dapagliflozin, 15 with empagliflozin. This disproportion is due to the different approval date in our country of the two molecules. Regarding concomitant therapies for heart failure, 98% of patients assumed beta-blockers and 94% an inhibitor of renin-angiotensin system (respectively 69% of patients in therapy with ARNI, 20% with ACEi and 5% with ARB). Seventy-three percent of patients were on MRA therapy (see table 1 for other population characteristics). Eighty-seven patients completed the 6 months follow-up, while 13 patients precociously interrupted SGLT2i for the following reasons: genito-urinary infections (6 patients, 6%), general discomfort (1 patients, 1%), arterial hypotension (1 patient, 1%), palpitation (1 patient, 1%), heart transplant (1 patients, 1%) and left ventricular assist device implantation (1 patient, 1%), worsening of chronic kidney disease (1 patient, 1%), end stage cancer (1 patient, 1%).

Table 1. Characteristics of the study population.

HFrEF POPULATION	
n=100	
Males, n (%)	88 (88%)
Age, years	65.6 ± 11.9
Etiology, n (%)	
Ischemic heart disease	50 (50%)
Non-ischemic heart disease	50 (50%)
Disease history, years	10 ± 9
Arterial hypertension, n (%)	53 (53%)
Diabetes mellitus type 2, n (%)	20 (20%)
Iron Deficiency	
ferritin < 100 mcg/L	27 (27%)
ferritin <299 mcg/L with TSAT <20%	36 (36%)
BMI, kg/m2	26.6 ± 4.8
ICD, n (%)	78 (78%)
CRT, n (%)	34 (34%)
Drug, n (%)	
Dapagliflozin	85 (85 %)
Empagliflozin	15 (15%)
Concomitant drugs, n (%)	
ACEi	20 (20%)
ARB	5 (5%)
ARNI	69 (69%)
Beta blockers	98 (98%)
MRA	73 (73%)
Ivabradin	8 (8%)

ACEi: angiotensin converting enzyme inhibitor; ARB: angiotensin receptor blocker; ARNI: angiotensin receptor-neprilysin inhibitor; TSAT: transferrin saturation; BMI: body mass index; CRT: cardiac resynchronization therapy; HFrEF: heart failure with reduced ejection fraction; ICD: implantable cardiac defibrillator; MRA: mineralocorticoid receptor antagonist.

Clinical events at follow-up

During the 6 months follow-up 4 patients were admitted for acute heart failure (4.6% of population) and 3 patients had in hospital admission for non cardiac causes (assuming infective issues). This small number of clinical events is probably due to the small sample size and the short term follow-up.

Clinical and biochemical data at baseline and at follow-up

We excluded from the analysis 13 patients who stopped the therapy before the 6 months follow-up. Thus considering the 87 patients who continued to take SGLT2i for the entire 6 months follow-up we found a minor, clinically non significative change in blood pressure (112±14 mmHg vs 115 ± 15 mmHg, *p=0.05*) and any difference in NYHA functional class as expected from literature. Furthermore, patients in our population had same rate of atrial fibrillation at baseline and at 6 months follow-up and comparable levels of serum potassium. We found a significant decrease of renal filtration (eGFR 64 ± 24 ml/min/1.73m2 vs 69± 23 ml/min/1.73m2, *p=0.005*), a difference of 5 ml consistent with what reported in previous studies[93]. Interestingly patients had a sharp decline in NT-proBNP values (838 pg/mL vs 684 pg/mL, *p=0.002*) with no difference in diuretic dosages. Considering blood count, patients showed significantly higher values of haemoglobin (14.8 ± 1.5 g/dL vs 14.2 ±1.6 g/dL, *p=0.006*) and red blood cells (5.0 ± 0.8 x 10^12/L vs 4.7 ±0.6 x 10^12/L, *p<0.001*) at 6 month follow-up but not difference in haematocrit values nor MCV, MCHC, RDW, white blood cells or platelets suggesting a mechanism different from hemoconcentration.

In the iron-related biochemical assessment, seven patients were excluded since they received i.v. iron-therapy during the follow up period, a strong confounder in iron assessment. After 6 months of SGLT2i therapy, in our population iron and ferritin levels declined significantly (respectively 80 μg/dL vs 92 μg/dL, *p=0.002* and 160 μg/L vs 194 μg/L, *p=0.01*) with no

difference in transferrin saturation. Furthermore, hepcidin values have dropped (4.0 nmol/L vs 6.7 nmol/L, *p=0.003*) with a consistent increase of erythroferrone serum concentration (0.99 ng/mL vs 0.39 ng/mL, *p<0.001*). No difference was observed for sTfR at baseline and at follow-up. Correlations of these parameters at baseline (T0) and follow-up (T1) are shown in ***Figure 3***.

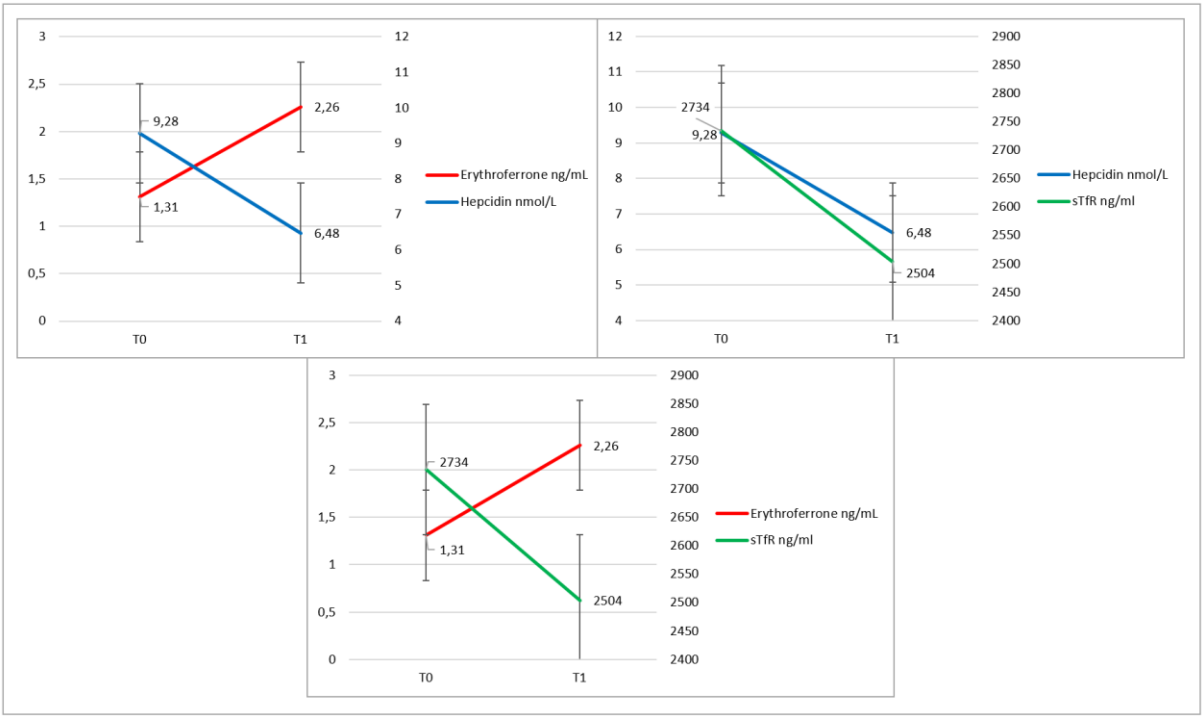

Figure 3: Trend over time of hepcidin, erythroferrone and sTfR. *Results are reported as mean and standard deviation. T0= basal; T1=6 months follow-up.*

If we exclude 3 cases of in hospital admission for non cardiac causes (assuming infective issues) the iron-related data remained the same. For complete data see **Table 2**. Six patients performed cardiopulmonary test at baseline and at follow-up. In this exiguous subgroup no difference was found for the main prognostic parameters (VE/VCO2: minute ventilation/carbon dioxide production slope; VO2: peak oxygen consumption; %VO2: percent achieved of predicted peak VO2).

Table 2. Clinical and biochemical data at baseline and at follow-up.

	BASELINE	**FOLLOW-UP**	**p-value**
	n=87	n=87	
Systolic arterial pressure, mmHg	115 ± 15	112±14	**0.05**
Diastolic arterial pressure, mmHg	72 ±12	70 12	0.20
NYHA functional class, n (%)			
I - II	73	73	NS
III - IV	13	14	NS
I	18	22	
II	56	51	
III	12	13	
IV	1	1	
Atrial fibrillation, n (%)	48 (48%)	48 (48%)	NS
Blood test			
Hemoglobin, g/dL	14.2 ±1.6	14.8 ± 1.5	**0.006**
Hematocrit, %	44.4 ±5.3	45.3 ± 6.3	0.30
RBC, 10^12/L	4.7 ±0.6	5.0 ± 0.8	**<0.001**
MCV, fL	93.6 ±7.4	93.8 ± 7.8	0.30
MCHC, g/L	319 ±34	313 ± 56	0.388
RDW, %	14.1± 1.7	14.4 ± 2.9	0.22
WBC, 10^9/L	7.2 ±2.0	7.2 ± 2.0	0.868
PLT, 10^9/L	210 ±75	205±66	0.29
eGFR, ml/min/1.73m2	69± 23	64 ± 24	**0.005**
Potassium, mmol/L	4.2 ± 0.3	4.1 ± 0.7	0.25
NT-proBNP, pg/mL	838 (72 - 1604)	684 (65 - 303)	**0.002**
Iron, µg/dL	92 (67 - 116)	80 (61 – 98)	**0.002***
Ferritin, µg/L	194 (131 - 326)	160 (108 - 212)	**0.01 ***
Transferrin saturation, %	27.5 (20.5 – 34.5)	26 (20.5 – 31.5)	0.31 *
Hepcidin nmol/L	6.7 (0.5 - 13)	4.0 (0.6 –7.3)	**0.003 ***
Erythroferrone ng/mL	0.39 (0.1- 1.6)	0.99 (0.1- 2.1)	**<0.001 ***
sTfR ng/ml	2283 (1122 - 3444)	2127 (1450 - 2805)	0.127 *
Diuretic therapy			

Furosemide dose, mg	25 (0 - 50)	12,5 (0-50)	0,276

* For these iron-related biochemical parameters 7 patients were excluded since they received i.v. iron-therapy during the follow up period

NYHA: New York Heart Association; RBC: red blood cells; MCV mean corpuscular volume; MCHC: Mean Corpuscular Haemoglobin Concentration; RDW: Red blood cell Distribution Width; WBC: white blood cells; PLT: platelets; eGFR: estimated glomerular filtration rate; NT-proBNP: N-terminal pro-brain natriuretic peptide; sTfR: Soluble Transferrin Receptor. Normal ranges for hepcidin (nmol/L) Age Hepc-25 (CI 95%), 60-69 years: 7.62(6.20-9.36) ; 70-79 years: 6.55 (5.10-8.41). Normal range for ERFE: 0.51 ng/mL (IQR: 0.12–1.25), Normal range for sTfR: 480-3090 ng/ml.

Echocardiographic data at baseline and at follow-up

All the patients performed a baseline and a 6 months follow-up rest echocardiography. A decrease of end diastolic volume of the left ventricle (185 ± 67 ml vs 196 ± 61 mL, *p=0.01*) with a consistent increase of left ventricle ejection fraction (36 ± 16% vs 33 ± 10%, *p=0.05*) were observed. Moreover, the restrictive pattern, well established as a bad prognostic feature in heart failure, was present in a significant lower percentage of patients at follow-up as compared to baseline (5.7% vs 8.1%, *p=0.039*). Finally mitral regurgitation at follow-up had a significantly lower grade than at baseline (moderate or severe in 20% vs 25% of cases, *p<0.001*). No differences in terms of atrial dimensions or TAPSE while pulmonary artery systolic pressure was significant lower at follow-up (sPAP 31 ± 11 mmHg vs 35 ± 13 mmHg, *p=0.01*)

Considering second level techniques as speckle tracking echocardiography, our patients at follow-up had a better function of left atrium (PALS 17.8 ± 8.8% vs 15.3 ± 9.6, *p=0.007*) and right ventricle (RV free wall strain 18±5.2% vs 15.4 ± 4.3%, *p=0.001*). Other echocardiographic data could be found in **Table 3**.

Considering echocardiographic threshold for ICD implantation, at baseline 23 patients (27% of the population) had LVEF higher than 35% while at follow up 32 patients (36%) (p<0.001). Thus, 8 patients showed a significant LVEF improvement leading to a theoretical escape from

ICD implantation. Furthermore 16 patients during follow-up had an improving trend of LVEF (+ 5%).

Table 3. Echocardiographic data at baseline and at follow-up.

	BASELINE	**FOLLOW-UP**	**p-value**
	N=87	N=87	
EDV, ml	196 ± 61; 194 (151-234)	185 ± 67; 173 (1140-227)	**0.01**
EDVi, ml/m^2	101 ± 30; 101 (78-11)	95 ± 34; 92 (6 -116)	0.07
LVEF, %	33 ± 10; 32 (27-36)	36 ± 16; 34 (28-38)	**0.05**
LAVi, ml/m^2	55 ± 21; 53 (42-62)	52 ± 26; 47 (37-57)	0.258
TAPSE, mm	18 ± 4; 18 (16-20)	18 ± 5; 18 (16-20)	0.26
MR≥2, n (%)	22 (25%)	18 (20%)	**<0.001**
PAPs, mmHg	35 ± 13; 31 (27-42)	31 ± 11; 29 (25-35)	**0.01**
Restrictive pattern	7 (8.1%)	5 (5.7%)	**0.039**
Speckle tracking echocardiography			
LV GLS (%)	9.5 ± 3.2; 9.5 (7.8-11.4)	10.0 ± 4.3; 9.9 (7.8 -12.0)	0.336
RV free wall strain (%)	15.4 ± 4.3; 15,7 (11.8-19.8)	18 ± 5.2; 18.3 (15.2-21.0)	**0.001**
LA PALS (%)	15.3 ± 9.6; 13.3 (10-20.7)	17.8 ± 8.8; 14.9 (9.1 -23.9)	**0.007**

EDV: end-diastolic volume; EDVi: end-diastolic volume index; LA PALS: peak left atrial longitudinal strain; LAVi: left atrial volume index; LVEF: left ventricular ejection fraction; LV GLS: left ventricular global longitudinal strain; MR≥2: mitral regurgitation at least moderate; PAPs: pulmonary artery systolic pressure; TAPSE: tricuspid annular plane systolic excursion.

Predictors of left ventricular reverse remodeling

Subsequently we divided our population in two groups based on the presence of cardiac reverse remodeling, namely improvement of LV-EF of at least 5% or reduction in LV-EDV of at least 10% from baseline or both criteria [88], [89].

In our population 27 patients (31%) among 87 had left ventricular reverse remodeling.

Patients who developed cardiac reverse remodeling at follow-up underwent less frequently to ICD implantation (56% vs 85%, *p=0.006*). No difference was found between ischemic and non ischemic patients and none echocardiography parameter differed from the two groups. Considering blood tests only hepcidin showed a significant difference, being statistically higher at baseline in patients who developed cardiac remodeling at follow-up (12.3 nmol/L vs 4.6 nmol/L, *p=0.01*). A basal hepcidin value of 12.3 nmol/L is above the normal range for patients similar to our population[84]. Complete data are shown in **Table 4**.

Table 4. Differences between patients without and with left ventricular reverse remodeling.

	No reverse remodeling (60)	**Reverse remodeling (27)**	**p-value**
Males, n (%)	54 (90 %)	21 (78 %)	0.07
Age, years	64 ± 12	67 ± 11	0.38
Non-ischemic etiology, n (%)	26 (43%)	16 (59%)	0.09
Disease history, years	11 ± 9	9 ± 10	0.72
Arterial hypertension, n (%)	30 (50 %)	18 (67 %)	0.10
Diabetes mellitus type 2, n (%)	12 (20 %)	6 (22 %)	0.755
ICD, n (%)	51 (85%)	15 (56%)	**0.006**
CRT, n (%)	17 (28 %)	7 (26 %)	0.97
Dapagliflozin, n (%)	54 (90%)	21 (78%)	0.50
ID definition 1	16 (27%)	8 (29%)	0.8
ID definition 2	22 (37%)	9 (33%)	0.7
ID definition 3	18 (30%)	10 (37%)	0.6
Systolic arterial pressure, mmHg	113 ± 12	120 ± 20	0.06
Diastolic arterial pressure, mmHg	72 ± 9	72 ± 17	0.85
Hemoglobin, g/dL	14.4 ± 1.5	14.1 ± 1.9	0.42
eGFR, ml/min	69 ± 22	68 ± 22	0.89
Potassium, mmol/L	4.2 ± 0.5	4.3 ± 0.6	0.13
NT-proBNP, pg/mL	929 (59 - 799)	868 (118 - 1618)	0.37
Iron, μg/dL	88 (63 - 113)	93 (66 - 120)	0.76

Ferritin, μg/L	186 (40 - 332)	201 (55 - 256)	0.81
Transferrin saturation, %	24 (16 - 32)	27 (20 - 34)	0.76
Hepcidin nmol/L	4.6 (1 – 8.2)	12.3 (4.8 – 19.8)	**0.01**
Erythroferrone ng/ml	0.54 (0.04 – 2.05)	0.32 (0.04 – 1.73)	0.17
sTfR ng/ml	2137 (2044 - 3922)	2454 (1271 - 3638)	0.76
VE/VCO2	34.8 ± 6.3	34.2 ± 5.1	0.74
EDV, ml	194 ± 65	185 ± 48	0.8
EDVi, ml/m2	100 ± 30	97 ± 28	0.86
LVEF, %	32 ± 7	33 ± 9	0.65
LAVi, ml/m2	55 ± 17	55 ± 21	0.85
TAPSE, mm	18 ± 4	17 ± 5	0.48
PAPs, mmHg	22 ± 19	26 ± 20	0.87
MR≥2, n (%)	12 (20 %)	9 (15 %)	0.15
LV GLS, %	9.4 ± 2.5	8.3 ± 3.8	0.34
RV free wall, %	16 ± 5	15 ± 4	0.67
LA PALS, %	16 ± 9	13 ± 10	0.37

CRT: cardiac resynchronization therapy; EDV: end-diastolic volume; EDVi: end-diastolic volume index; eGFR: estimated glomerular filtration rate; ICD: implantable cardiac defibrillator; LA PALS: peak left atrial longitudinal strain; LAVi: left atrial volume index; LVEF: left ventricular ejection fraction; LV GLS: left ventricular global longitudinal strain; MR≥2: mitral regurgitation at least moderate; NT-proBNP: N-terminal pro-brain natriuretic peptide; PAPs: pulmonary artery systolic pressure; TAPSE: tricuspid annular plane systolic excursion; VE/VCO2: minute ventilation/carbon dioxide production slope; ID definition 1: ferritin level below 100 ng/ml irrespective of transferrin saturation (TSAT); ID definition 2: ferritin between 100–300 ng/ml if TSAT is <20%; ID definition 3 sTfR >3000 ng/ml.

We also performed the same statistical analysis excluding the seven patients who received i.v. iron-therapy during the follow up period and similar results were confirmed. ICD presence and hepcidin values at baseline were the only parameters that differ between patients with and without left ventricular reverse remodeling (see **Table 5**).

Table 5. Differences between patients without and with left ventricular reverse remodeling excluding patients who underwent iron i.v.

	No reverse remodeling (57)	**Reverse remodeling (23)**	**p-value**
Males, n (%)	52 (91%)	17 (74 %)	0.19
Age, years	64 ± 12	67 ± 10	0.20
Non-ischemic etiology, n (%)	26 (46 %)	15 (65 %)	0.09
Disease history, years	11 ± 9	10 ± 10	0.63
Arterial hypertension, n (%)	29 (51 %)	15 (65 %)	0.18
Diabetes mellitus type 2, n (%)	11 (19 %)	5 (22 %)	0.51
ICD, n (%)	49 (86 %)	12 (52 %)	**0.002**
CRT, n (%)	16 (28 %)	6 (26 %)	0.59
Dapagliflozin, n (%)	52 (91%)	20 (87 %)	0.32
Systolic arterial pressure, mmHg	114 ± 13	120 ± 19	0.08
Diastolic arterial pressure, mmHg	72 ± 9	72 ± 16	0.98
Hemoglobin, g/dL	14.04 ± 1.6	14.0 ± 2.0	0.29
eGFR, mL/min	69 ± 22	68 ± 22	0.89
Potassium, mmol/L	4.2 ± 0.5	4.3 ± 0.6	0.13
NT-proBNP, pg/mL	872 (796 - 2389)	811 (68 - 1555)	0.94
Iron, μg/dL	89 (65 - 114)	102 (77 - 1127)	0.95
Ferritin, μg/L	201 (70 - 332)	210 (70 - 350)	0.94
Transferrin saturation, %	25 (19 - 32)	27.5 (18 - 31)	0.89
Hepcidin, nmol/L	6.0 (1.0 – 11.0)	12.3 (6.1 – 18.1)	**0.048**
Erythroferrone, ng/mL	0.54 (0.04 – 1.78)	0.31 (0.04 – 1.66)	0.24
sTfR, ng/mL	2137 (1285 - 2990)	2255 (1062 - 1448)	0.94
VE/VCO2	35,5 ± 6.4	33,6 ± 4.0	0.68
EDV, mL	194 ± 65	189 ± 50	0.75
EDVi, mL/m2	100 ± 29	99 ± 29	0.93
LVEF, %	33 ± 6	35 ± 7	0.42
LAVi, mL/m2	55 ± 22	54 ± 18	0.88
TAPSE, mm	19 ± 3	17 ± 5	0.33
PAPs, mmHg	23 ± 20	23 ± 17	0.94
MR≥2, n (%)	11 (19 %)	7 (30 %)	0.29

LV GLS, %	9.6 ± 2.5	8.5 ± 4.0	0.50
RV free wall, %	16.2 ± 5.3	15.3 ± 4.3	0.72
LA PALS, %	16 ± 9	14 ± 10	0.54

CRT: cardiac resynchronization therapy; EDV: end-diastolic volume; EDVi: end-diastolic volume index; eGFR: estimated glomerular filtration rate; ICD: implantable cardiac defibrillator; LA PALS: peak left atrial longitudinal strain; LAVi: left atrial volume index; LVEF: left ventricular ejection fraction; LV GLS: left ventricular global longitudinal strain; MR≥2: mitral regurgitation at least moderate; NT-proBNP: N-terminal pro-brain natriuretic peptide; PAPs: pulmonary artery systolic pressure; TAPSE: tricuspid annular plane systolic excursion.

We analyzed cardiac reverse remodeling in terms of LVEF improvement and LVEDV reduction separately for the group of patients with ischemic and with non-ischemic etiology of heart failure but any significant difference was found. These results are probably to be attributed to the low number of patients who remodel in the two groups (13 patients in the ischemic group vs 13 patients in the non ischemic group, p=0.8).

Furthermore, we analysed the prevalence of metabolic disease considering BMI, presence of diabetes mellitus and/or dyslipidaemia between patients without and with left ventricular reverse remodelling. Again, no significant difference was found.

Iron deficiency prevalence considering three different definitions (definition 1: ferritin level below 100 ng/ml irrespective of transferrin saturation (TSAT); definition 2: ferritin between 100–300 ng/ml if TSAT is <20%; definition 3: sTfR >3000 ng/ml) did not differ between the remodelling and not remodelling group (see **Table 4**). However patients without ID according to definition 1 and 2 had higher LVEF at baseline than patients with ID. This was not true with the third definition (see **Table 6**). Moreover no difference of basal left ventricular end diastolic volume was found between patients with or without ID considering all the 3 definitions (p always > 0.1).

Table 6. Basal LVEF in patients with or without ID.

	No ID	**ID**	**p-value**
Basal LVEF – ID definition 1	33 ± 5%	28 ±6%	**p=0.001**
Basal LVEF – ID definition 2	33 ± 5%	29 ±6%	**p=0.009**
Basal LVEF – ID definition 3	32 ± 5%	31 ± 6%	p=0.5

Univariate and multivariable analysis

At univariate analysis performed considering all the parameters that differed significantly between patients without and with left ventricular reverse remodelling (see **Table 4**) systolic blood pressure, not having an ICD and basal levels of hepcidin were the only parameters significantly correlated to LV remodelling at follow-up (see **Table 7**)

Table 7. Univariate analysis of predictors of left ventricular reverse remodeling.

	OR	95% CI	p-value
Female	1.02	0.97-1.07	0.9
Etiology*	1.86	0.51-6.79	0.3
No-ICD	**4.81**	**1.6-14.6**	**0.006**
Systolic blood pressure	**1.04**	**1.01-1.07**	**0.03**
Hepcidin	**1.07**	**1.01-1.13**	**0.01**
Delta Hepcidin (%)^	1.02	0.97-1.07	0.46
Delta ERFE (%)^	0.96	0.80-1.15	0.6
Delta sTfR (%)^	0.99	0.99-1.00	0.37

* ischemic vs non ischemic, ^ even testing absolute value delta p always > 0.1

Finally, we performed a multivariable analysis with variables chosen for biological plausibility in order to identify independent predictor of left ventricular reverse remodeling (see **Table 8**). In our R^2 multivariable analysis, basal level of hepcidin was as the only independent predictor of left ventricular reverse remodeling. Event forcing all the significant variables in the model, hepcidin remains significantly associated with LV remodeling.

Table 8. Multivariable analysis of independent predictors of left ventricular reverse remodeling.

Model R2= 0.196 **P = 0.019**	Odd Ratio	P value	95 C.I. Inferior	95 C.I. Superior
Age	*1.04*	0.111	0.99	1.09
LVEF	*1.02*	0.328	0.97	1.07
Systolic blood pressure	*1.03*	0.071	1,00	1.07
Hepcidin	**1.08**	**0.023**	**1.01**	**1.15**

The higher was hepcidin at baseline, the higher is the probability for the patient to have a reduction of left ventricle volumes and an increase of LVEF. Patient with low levels of hepcidin have a 20% of probability to remodel and this percentage grows linearly with the increase of hepcidin levels. If basal hepcidin is higher than 25 nmol/L patients in our population have 50% of probability to remodel as shown in ***Figure 4***.

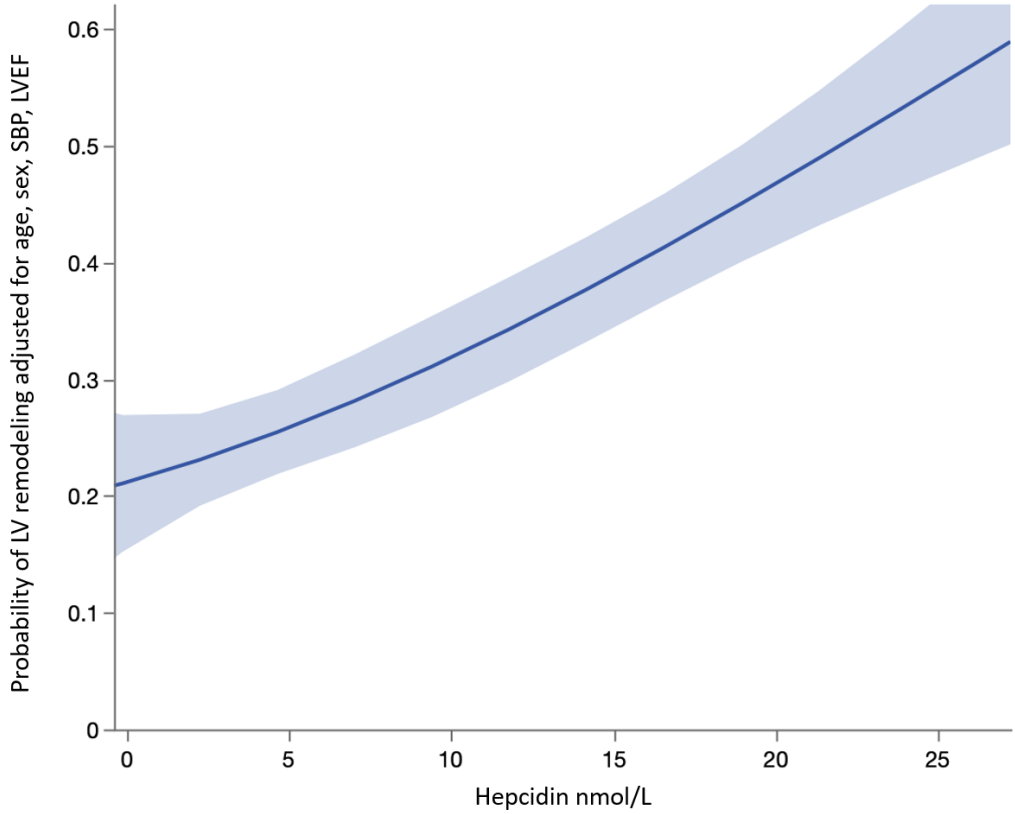

Figure 4. **Correlation between basal levels of hepcidin and probability of LV remodeling after SGLT2i therapy adjusted for age, sex, systolic blood pressure (SBP) and LVEF.**

Finally, as shown in ***Figure 5***, the correlation high basal-hepcidin on LV remodeling remains consistent across clinically relevant subgroups of patients even if this correlation is stronger in older patients, males, with higher LVEF and an ICD, lower eGFR, non relevant MR and lower ferritin.

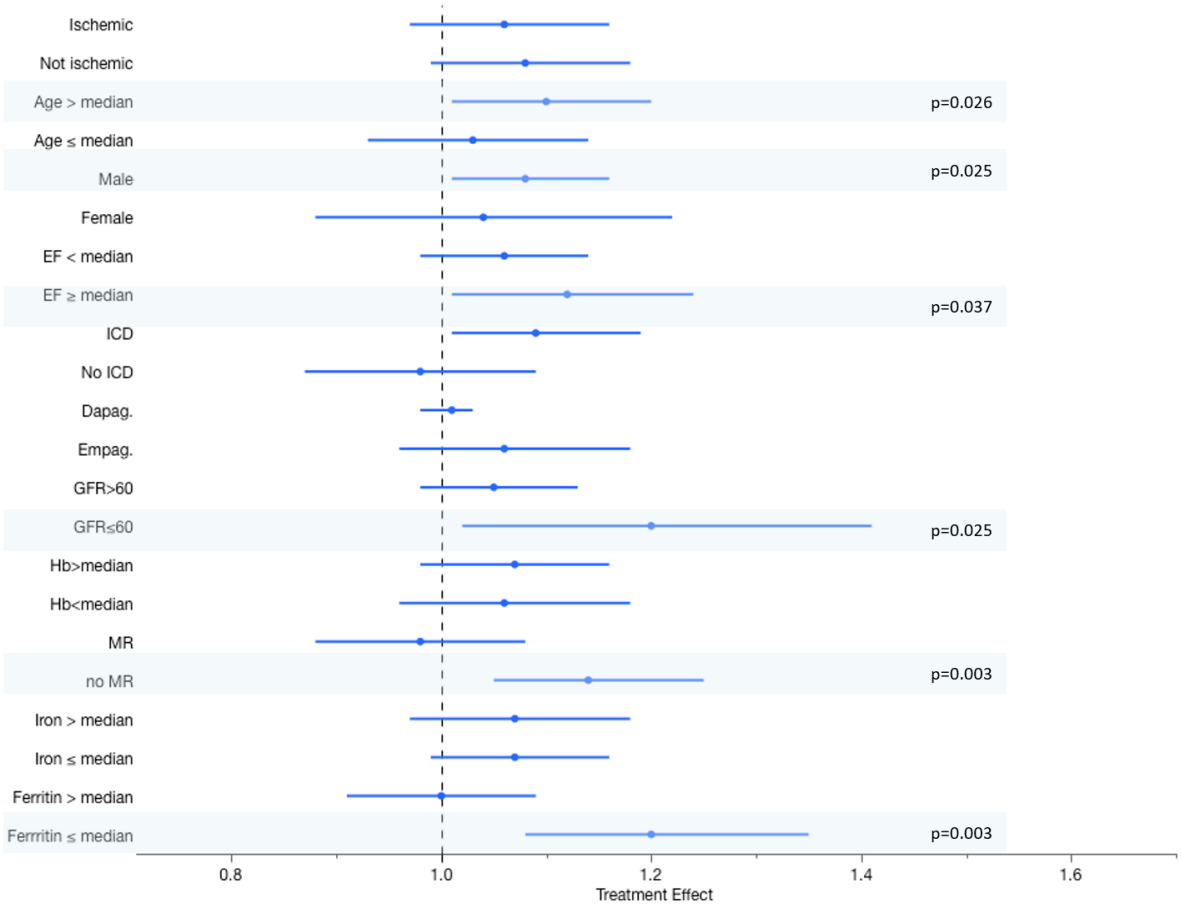

***Figure 5*. Correlation of basal hepcidin levels and LV remodeling outcome according to clinically relevant subgroups of patients.**

A visual summary of the main results of our stud is provided in ***Figure 6***.

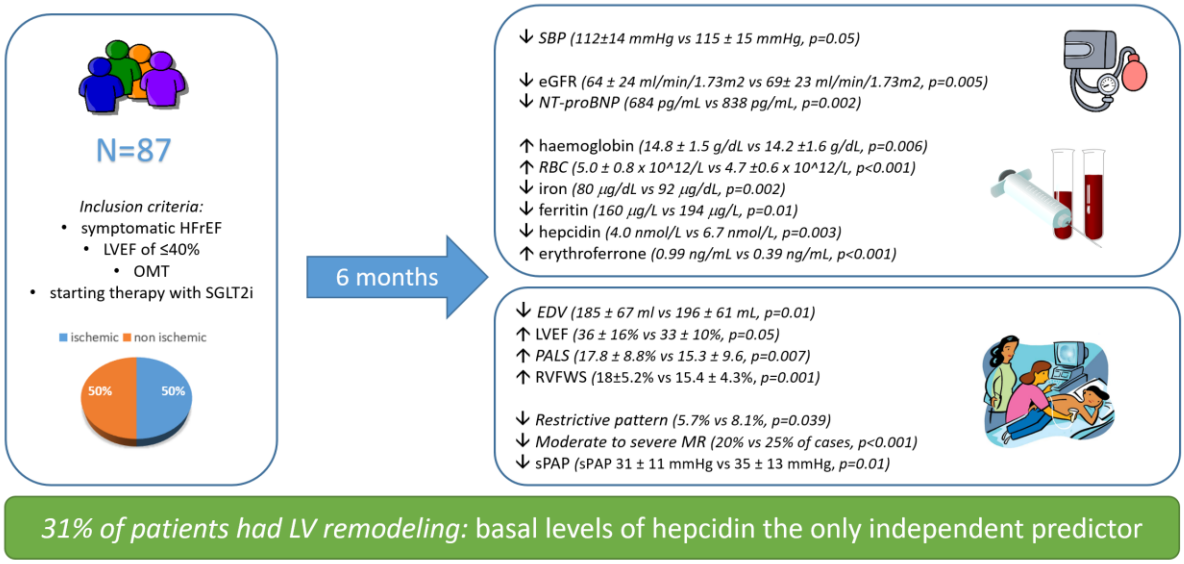

Figure 6. Visual abstract of the study. *HFrEF: heart failure with reduced ejection fraction; LVEF: left ventricular ejection fraction; OMT: optimal medical therapy; SBP: systolic blood pressure; RBC: red blood cells; EDV: end diastolic volume; PALS: peak left atrial longitudinal strain; RVFWS: right ventricle free wall strain; sPAP: systolic artery systolic pressure; LV remodeling: improvement of LV-EF of at least 5% or reduction in LV-EDV of at least 10% from baseline or both criteria*

DISCUSSION

The prevalence of heart failure is increasing due to aging, increased cardiovascular risk factors in the overall population and reduction of mortality after acute myocardial infarction[94]. Nowadays, heart failure is the leading cause of hospitalization of patients over 65 years [95] and its mortality remains high, even if stepwise improvements have been made in pharmacotherapy in the last three decades, significantly delaying clinical progression and prolonging disease-free survival. Sodium-glucose cotransporter 2 inhibitors have further improved heart failure's therapy, being the newest class of drugs able to show significant benefit in terms of cardiovascular morbidity and mortality. On the backbone of beta blockers, ARNI/ACEi/ARB and MRA, SGLT2i configure as the fourth pillar of pharmacological management of heart failure with reduced ejection fraction. Heart failure prognosis is strongly influenced by comorbidities and iron deficiency is one of the most common. Iron metabolism and heart failure physiopathology are strictly connected and drugs recommended for heart failure may impact also on iron setting.

In our prospective study we firstly analysed the trend over time of iron parameters after introduction of SGL2i on top of optimal medical therapy for HFrEF.

According to literature our patients showed after 6 months a significant increase of hemoglobin levels with no difference of hematocrit but with a significant rise of RBC count.

Even if we do not have data about EPO levels, these results suggest a possible increase of erythropoiesis induced by SGLT2i as previously reported[96] instead of a consequence of hemoconcentration. Ferritin and iron levels decreases over time and these data are consistent with stored iron consumption for erythropoiesis. Furthermore hepcidin significantly decrease after 6 months of therapy as previously reported [78]. As previously suggested [97], SGLT2i reduce hepcidin and ferritin and upregulate transferrin receptor protein 1 raising cytosolic Fe2+ available to mitochondria resulting in higher production of heme in erythroid precursors and

ATP in cardiomyocytes. Hepcidin and ferritin decrease showed in our population supports the hypothesis of an anti-inflammation role of SGLT2i in HFrEF patients already described by literature[98], [99].

Of note, in our population erythroferrone levels significantly increase and this evidence is new[82]. This data can explain the decline of hepcidin levels since erythroferrone suppresses hepcidin synthesis in the liver, thus hepcidin and erythroferrone trends in our population are consistent.

We also observed a significant reduction in NT-proBNP concentration (from 838 to 684 pg/ml, *p=0.002*). This finding supports the hypothesis of left ventricular reverse remodeling mediated by SGLT2 inhibitors, which leads eventually to a reduction in NT-proBNP production, as already reported in literature[33], [34].

Thus, we analyzed echocardiographic data and we found a significant reduction in LV-EDV and an increase in LV-EF. The change of both these parameters corroborates the real improvement of LV function. These findings are in line with what already reported in literature, even if a recent metanalysis showed that LV-EDV did not decrease significantly at follow-up after the initiation of SGLT2i[37].

Of note, there are very few studies analyzing the impact of this new category of HF drugs on right ventricular function and none analyzed their effect on right ventricular strain and on left atrial strain. In our cohort right ventricular function measured by means of TAPSE did not vary at follow-up but RV free wall strain significantly improved. Similarly, LAVi did not vary but peak left atrial longitudinal strain significantly improved at follow-up. In both cases, strain improvement could be the precursor of a more evident improvement in right ventricular function and of a reduction in left atrial volume.

Moreover, the percentage of patients with mitral regurgitation at least moderate was significantly lower at follow-up (from 25% to 20%, *p<0.001*). It is well known that secondary mitral regurgitation in HFrEF is associated with a poor prognosis[39], [40]. In fact, left ventricular dilation leads to enlargement of the mitral annulus and reduced coaptation of the mitral valve leaflets causing secondary MR. Even if our study is performed on a relatively small population, it gives one of the first evidences in literature that SLGT2i could reduce significantly relevant mitral regurgitation, thanks to their ability to reduce LV size and volume, acting in a synergistic way with other HF drugs such as sacubitril/valsartan.

Finally, by means of a univariate analysis, we found as predictors of left ventricular reverse remodeling not having an implanted ICD, a higher systolic blood pressure and higher hepcidin levels at baseline. The first two parameters are clinically reasonable since these patients probably have a less severe disease with a better cardiac output. We thus imply that patients who did not need ICD were more prone to improvement. This, in our opinion, is coherent with current clinical practice and summarizes multiple clues detected by the clinicians (for example, LVEF trend, no late gadolinium enhacement at CMR, no arrhythmias recorded…) that guided the clinical choice. We did not aim to test the effectiveness of CRT in improving LV function, which is quite well established in RCTs and goes beyond the scope of the study (particularly considering that only 31 patients had CRT).

The most interesting result is the correlation of high levels of basal hepcidin with positive LV remodeling at follow-up. This correlation is so strong that at multivariable analysis basal hepcidin is the only independent predictor of outcome. In particular, our study showed that the higher is hepcidin at baseline the higher is the possibility for the patient to reduce LV volume and improve LVEF after 6 months of therapy. The most convincing explanation from our point of view is the anti-inflammatory and anti-fibrotic effects of SGLT2i already reported not only in cardiovascular setting [98], [100], [101], [102]. Hepcidin is directly induced by the cytokine IL-6 and

as such is among the acute phase proteins secreted by the liver as part of the inflammatory response[103]. Hepcidin is also expressed in hypoxic conditions[104].

Including LVEF in the multivariate model would be unnecessary in the absence of interaction between LVEF at baseline and the likelihood of LV reverse remodeling. However, it is quite established that the baseline LVEF can affect LV remodeling (also supported by the trend between baseline LVEF and "delta LVEF" in our study p=0.04). We, therefore, prefer to include the LVEF in the multivariable model. Even testing the model without LVEF and with CRT results are stable.

Considering the correlation of basal hepcidin levels and LV remodeling according to clinically relevant subgroups of patients in our population, the most evident association is for older male patients with an ICD and worse eGFR. These conditions are known to be associated with systemic inflammation and hypoxia so our data could support the hypothesis that this is the setting in which SGLT2i perform better. However even in patient with higher LVEF at baseline, no severe MR and lower ferritin baseline hepcidin levels strongly correlates with LV remodeling at follow-up and these data are of uncertain interpretation.

Finally we found a significant, even if small (5 ml min/1.73m^2), decrease in renal function at 6 months follow-up. A small reduction in eGFR following initiation of SGLT2i is expected in all patients during the first weeks of treatment[105]. These changes are generally transient and occur even if these drugs slower worsening of renal function in the long term[19]. The reduction that we have seen in eGFR at follow-up is lower than 10% of baseline eGFR. A possible explanation is that this decrease belongs to the initial decline in renal function, which later may stabilize and may also remain preserved. It would be interesting to see at a longer time, such at 12 months-follow-up, the evolution of the renal function of our patients.

Of note our population was on real optimal medical therapy at baseline with a percentage of RAASI, β-blockers and MRA noticeably higher than expected on real population [106], [107] so further advantages of new drugs could be difficult to highlight.

LIMITS OF THE STUDY AND FUTURE PROSPECTIVES

The main limits of our study is that is not randomized and conducted in a small population of patients. We knowledge that the selected primary endpoint is based on echocardiographic measurements that hold intrinsic inter and intra-observer variability; however, the association between Hepcidin and LV reverse remodeling permitted when different LVEF cutoffs were adopted ($p<0.01$ for both 8% and 10% LVEF change). Furthermore, while inter and intra-observer variability is relatively high in all-comer patients, it is remarkably reduced in the presence of a core laboratory and board-certified operators as in our institution[108]. We also completed a short follow-up and results have to be confirmed and re-evaluated after a longer period of observation. Furthermore, in order to confirm our "inflammation hypothesis" we do not have other inflammatory markers to correlate with our outcome as well as no data about EPO are available to corroborate the "erythropoiesis hypothesis".

CONCLUSIONS

Heart failure with reduced ejection fraction in an endemic disease with poor prognosis and complex physiopathology. Iron metabolism is strictly affected by the disease and its therapy. New drugs as SGLT2i shown to strongly impact on HFrEF outcome and the ability to modify inflammation and iron pathway could be an additional strength of their action.

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