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ORIGINAL ARTICLE

Direct actions of dapagliflozin and interactions with LCZ696 and spironolactone on cardiac fibroblasts of patients with heart failure and reduced ejection fraction

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Abstract

Aims Inhibitors of SGLT2 (SGLT2i) have shown a positive impact in patients with chronic heart failure and reduced ejection fraction (HFrEF). Nonetheless, the direct effects of SGLT2i on cardiac cells and how their association with main drugs used for HFrEF affect the behaviour and signalling pathways of myocardial fibroblasts are still unknown. We aimed to determine the effects of dapagliflozin alone and in combination with sacubitril/valsartan (LCZ696) or spironolactone on the function of myocardial fibroblasts of patients with heart failure and reduced ejection fraction (HFrEF).

Methods and results Myocardial fibroblasts isolated from HFrEF patients (n = 5) were treated with dapagliflozin alone (1 nM–1 μM) or combined with LCZ696 (100 nM) or spironolactone (100 nM). The migratory rate was determined by wound-healing scratch assay. Expression of heart failure (HF) markers and signalling pathways activation were analysed with multiplexed protein array. Commercially available cardiac fibroblasts from healthy donors were used as Control (n = 4). Fibroblasts from HFrEF show higher migratory rate compared with control (P = 0.0036), and increased expression of HF markers [fold-change (Log2): COL1A1–1.3; IL-1b–1.9; IL-6–1.7; FN1–2.9 ($P < 0.05$)]. Dapagliflozin slowed the migration rate of HFrEF fibroblasts in a dose-dependent manner and markedly decreased the expression of IL-1β, IL-6, MMP3, MMP9, GAL3, and FN1. SGLT2i had no effect on control fibroblasts. These effects were associated with decreased phosphorylation of AKT/GSK3 and PYK2 kinases and the signal transducer and activator of transcription (STAT). A combination of dapagliflozin + LCZ696 further decreased fibroblast migration, although it did not have a significant effect on the regulation of signalling pathways and the expression of biomarkers induced by SGLT2 inhibition alone. In contrast, the combination of dapagliflozin + spironolactone did not change the migration rate of fibroblast but significantly altered SGLT2i responses on MMP9, GAL3, and IL-1b expression, in association with increased phosphorylation of the kinases AKT/GSK3 and ERK1/2.

Conclusions SGLT2i, LCZ696, and spironolactone modulate the function of isolated myocardial fibroblasts from HFrEF patients through the activation of different signalling pathways. The combination of SGLT2i + LCZ696 shows an additive effect on migration, while spironolactone modifies the signalling pathways activated by SGLT2i and its beneficial effects of biomarkers of heart failure.

Keywords Systolic heart failure; Sodium-glucose cotransporter-2 inhibitors; Dapagliflozin; Sacubitril-valsartan; Spironolactone; Pharmacology

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Introduction

Heart failure with reduced ejection fraction (HFrEF) is a worldwide leading cause of morbidity and mortality with a high impact on healthcare system resources and cost.[1] There have been extraordinary advances in pharmacological and device therapy in the last decades to treat acute and chronic HFrEF.[2] In the pharmacological scenario, the development of the sodium-glucose cotransporter 2 Inhibitors (SGLT2i) substantially advances the treatment options for patients with HFrEF.[3–6] Currently, SGLT2i has been defined as the fourth pillar of HFrEF treatment besides beta-blockers, angiotensin receptor-neprilysin inhibitors (ARNI), and mineralocorticoid receptor antagonists (MRA).[1]

Myocardial fibrosis contributes to left ventricular dysfunction, leading to heart failure (HF).[7] Myocardial interstitial fibrosis (MIF) is defined by the diffuse, disproportionate accumulation of collagen in the myocardial interstitium contributing to left ventricular dysfunction and predisposing patients to develop HFrEF. In recent years, magnetic resonance mapping techniques have been widely used for diagnosing interstitial fibrosis.[8] However, the objective of targeting MIF to improve the treatment of HF is still scarcely performed. Few studies at the preclinical level have mainly evaluated the use of aldosterone antagonists in MIF.[9]

Currently, two randomized clinical trials evaluating the effect of SGLT2i on myocardial fibrosis employing cardiac magnetic resonance have suggested that SGLT2 inhibition reduces myocardial fibrosis and extracellular matrix.[10,11] In a diabetic mice preclinical model, SGLT2 inhibition ameliorates myocardial fibrosis partly through inhibition of collagen formation and deposition.[12] Dapagliflozin at 300 nM reduced LPS-induced activation of inflammation in mouse cardiofibroblasts, an effect that was mediated through Dapa-induced AMPK phosphorylation and reduced NHE-1 expression.[13,14] Furthermore, previous studies have shown that ARNI and MRAS may be associated with a reduction in circulating myocardial fibrosis biomarkers.[15,16] Nevertheless, the effects of these drugs on the relevant cell populations, especially cardiac fibroblasts, have not been studied entirely.

The association of SGLT2i, ARNI, and MRAS have been evaluated in the DAPA-HF and EMPEROR -Reduced trial.[17–19] In both trials, the association of SGLT2i and ARNI has not modified the clinical benefit of SGLT2 inhibition. Moreover, in a comparative analysis of three randomized controlled trials, a comprehensive disease-modifying pharmacological therapies (SGLT2i, ARNI, and MRAS) schedule was associated with a halt or delay in clinical progression and extended survival in patients with HFrEF.[20] Of note, the biological interplay at a molecular level of the mechanisms of action of these drugs on myocardial fibrosis has not been studied in detail.

Expansion of cardiac fibroblasts and their acquisition of an inflammatory and matrix-synthetic phenotype are prominent features in the pathophysiology of MIF.[21,22] These highly reactive cells are provided with membrane receptors for several neurohumoral factors, making them critical pharmacological targets in cardiac remodelling and failure. In the present study, we aimed to determine the effects of pharmacological therapy with SGLT2i alone and in combination with ARNI or MRAS on the function and signalling pathway activation of myocardial fibroblasts of patients with HFrEF. Changes in functional and signalling phenotypes of HFrEF fibroblasts were determined compared with fibroblasts from healthy donors and following pharmacological treatments.

Methods

Patient inclusion and cardiac fibroblast isolation

Cardiac fibroblasts were obtained in patients with advanced ischemic heart disease undergoing heart transplantation with a preceding clinical history of uncontrolled HF. Immediately after the cardiotomy, a myocardial fragment (~2 g) was obtained from the left ventricular tissue in regions without visually recognizable scar tissue. Samples were immersed in culture media (DMEM-F12 + 10% FBS + 1% penicillin–streptomycin + 1% amphotericin B) and processed subsequently. The extraction is carried out according to the mechanical dispersion method described by Nagaraju *et al.*[23] Briefly, the pieces of the myocardium are digested in 20 μm filtered Hank's balanced salts w/o calcium, phenol red, or magnesium (Biowest) with collagenase-A (Roche) in an orbital shaker at 37°C. Finally, the cells are centrifuged at 1900 g for 10 min, 70 μm filtered, and suspended in the culture media. Control cells were obtained from four different batches of commercial primary human cardiac fibroblasts isolated from the ventricles of healthy human heart tissues [PromoCell® (Heidelberg, Germany) and Cell Applications Inc. (San Diego, CA, USA)]. The studies were carried out under the approval of the Ethics Committee for Clinical Research (CEIC) of the HCPB (CEIC#HCB/2015/0639), following the principles of the Declaration of Helsinki. Patients serving as myofibroblast donors signed an informed consent form.

Cell culture and treatments

Cardiac fibroblasts were plated onto 0.2% gelatin-coated culture dishes and studied before cell confluence between passages 5 and 7. Cells were maintained in culture in DMEM-F12 supplemented with 10% (v/v) fetal bovine serum (FBS) and growth factors (Thermo Fisher Scientific, Waltham, MA, USA). One hour before treatments, the culture medium was changed to low-serum (1%) media, and incubation proceeded overnight. Both control and HFrEF fibroblasts were then treated for 48 h with an increasing dose (1 nM to 1 μM) of SGLT2i (Dapagliflozin - #S1548, Selleckchem, Houston, TX,

USA). For drug association protocols, cells were treated with a combination of 100 nM SGLT2i and 100 nM LCZ696 (#S7678, Selleckchem, Houston, TX, USA) or 100 nM SGLT2i and 100 nM spironolactone (#S4054, Selleckchem, Houston, TX, USA). These concentrations represent the concentration that promoted the submaximal effect on fibroblast migration in preliminary studies and is close to the active drug concentration found in patients.[24] All drugs were dissolved in the organic solvent dimethyl sulfoxide (DMSO), and untreated cells received the same final concentration of 0.1% DMSO (v/v) as a vehicle. The composition of amino acids, inorganic salts, and other metabolic components (such as glucose, pyruvate, total protein, and lipid) present in the fibroblast culture media during treatments are shown in detail in *Table S1*.

Migration rate

A standardized scratch assay determined the migration rate of cardiac fibroblasts.[25] Fibroblasts were grown in 12-well plate and treated with increasing concentrations of SGLT2i alone or in combination with LCZ696 or spironolactone as described above. Monolayers were manually scraped with a 100 μL pipette tip, gently washed twice with PBS to remove non-adherent cells, and incubated in media supplemented with 1% FBS to reduce proliferation. Images of the scratched area were taken immediately after (T0) and at 6, 12, 24, 36, and 48 h after injury using the 5× objective of an inverted microscope (Axiovert 200 Zeiss, Oberkochen, Germany) and ZEN software. The scratched area was measured using NIH ImageJ software with MRI Wound Healing Tool, and the percentage of the invaded area was determined as [% Healing = 100·(Area T0 − Area TX)/Area T0; where Area T0 = initial area (T = 0) and Area TX = area at each time (X) h after injury.

Analysis of heart failure biomarkers

Following 48 h of treatments, simultaneous assessment of cellular concentrations of nine biomarkers of heart failure: atrial natriuretic polypeptide (ANP); fibronectin (FN1); galectin 3 (GAL3); collagen type I-alpha-1 (COL1A1); matrix metalloproteinase-2 (MMP-2), -3 (MMP-3), and -9 (MMP-9); interleukins (IL) IL-6 and IL-1β, was performed using commercially available multiplex bead-based immunoassay following the manufacturer's instructions (R&D Systems, Inc. Minneapolis, MN, USA). Briefly, cells were lysed in RIPA buffer (Thermo Fisher Scientific, Waltham, MA, USA) and Halt™ protease and phosphatase inhibitors (Thermo Fisher Scientific, Waltham, MA, USA). Protein concentration was measured using the BCA protein assay (Thermo Fisher Scientific, Waltham, MA, USA). Equal volumes of cell lysates (25 μL per well) or standards (25 μL per well) were incubated with pre-mixed bead sets in pre-wetted 96-well microtiter plates at 4°C overnight. After recommended washing, the fluorescent detection antibody mixture was added and incubated for 1 h, followed by incubation with streptavidin-phycoerythrin for an additional 30 min at room temperature. The concentration of each biomarker was determined using a Luminex™ 200™ Instrument (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA), and data were reported as mean fluorescent intensities normalized by the amount of protein (mg) determined by the BCA test. The expression of procollagen I C-terminal propeptide (PICP), and N-terminal procollagen III propeptide (PIIINP) was determined in parallel using commercially available ELISA kits (Elabscience Biotechnology Inc., Houston, TX, USA).

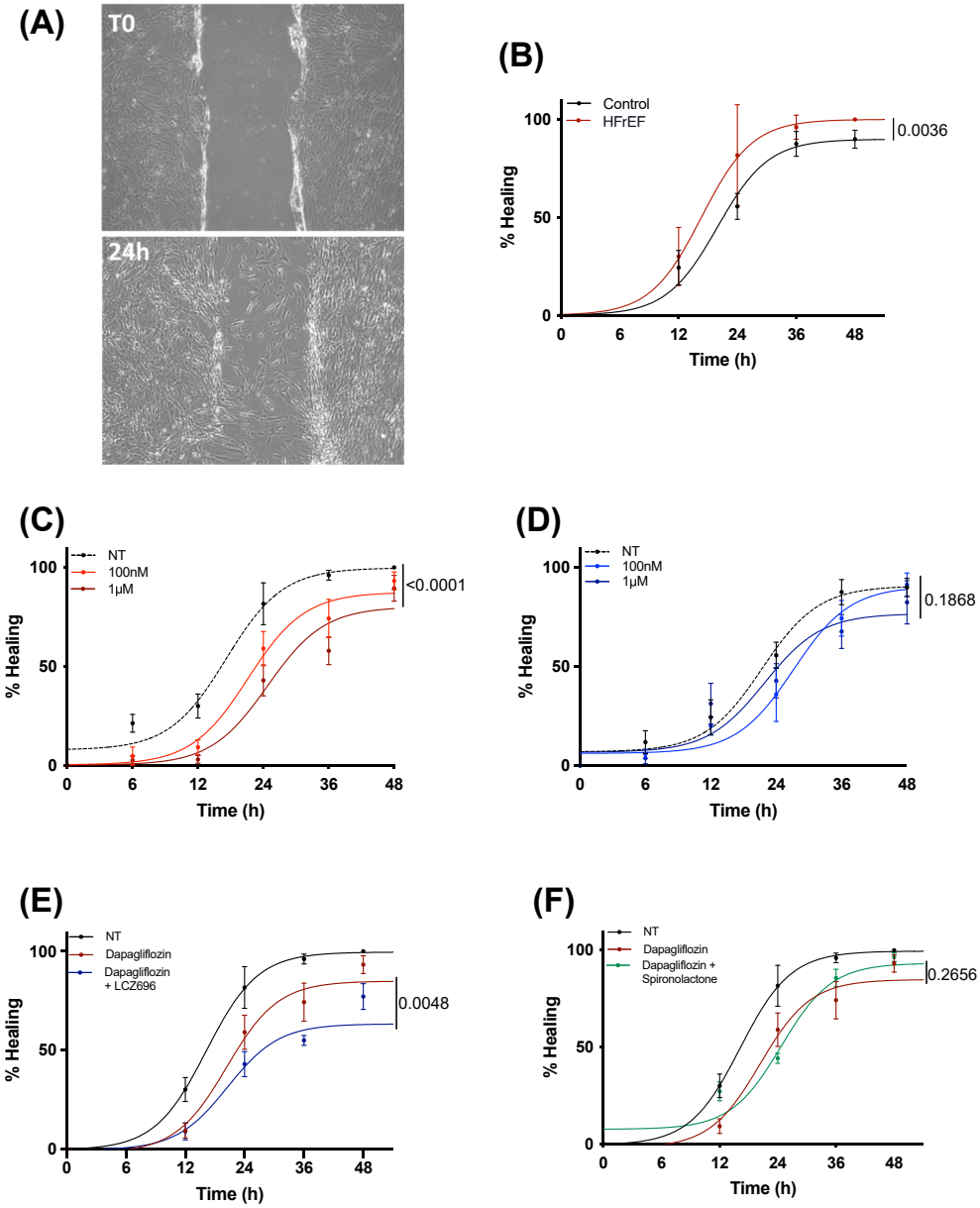
Multiplex intracellular phosphorylation analysis

The degree of phosphorylation of multiple kinases was determined in each untreated sample (NT) and cells treated with SGLT2i alone or combined with LCZ696 or spironolactone with a membrane-based Proteome Phospho-Kinase Array (R&D Systems, Inc. Minneapolis, MN, USA). After treatments, cells were solubilized in lysis buffer (1×10^7cells/mL) provided with the kit supplemented with Halt™ protease and phosphatase inhibitors (Thermo Fisher Scientific, Waltham, MA, USA). Cell lysates were incubated with array membranes O.N. at 4°C on a rocking platform shaker. Following recommended washes and incubation with detection antibody plus streptavidin-HRP, the chemiluminescent signal in each membrane was visualized by the LAS4000 imaging system (GE Healthcare). Densitometric analyses of phosphorylation signals were performed using ImageJ software and normalized by the signal of reference spot in each membrane.

Data analysis and statistics

Data normality was assessed using the Kolmogorov–Smirnov test. According to their distribution, continuous variables were expressed as mean ± standard deviation (SD) or median with interquartile range (IQR). A nonlinear model was employed for migration assay to analyse the time-course of migrator rate, and factorial ANOVA determined differences of data sets (curves). The area under the curve (AUC) was calculated and expressed as arbitrary units from each migration curve. The degree of change (Log2) in the expression of heart failure biomarkers or the phosphorylation of kinases were analysed by multiple *t*-tests with false discovery rate (FDR) correction using the method of Benjamini and Hochberg. Brown Forsythe and Welch ANOVA performed a comparison of means across independent variables (treatments), followed by Dunnett's T3 post hoc analysis multiple comparisons. Data were analysed and plotted using GraphPad Prism version 8.4 (GraphPad Software, San Diego, CA, USA). Significance was considered when $P < 0.05$.

Figure 1 Migration rate of cardiac fibroblasts: role of heart failure and SGLT2 inhibition. Representative image of migratory rate at 24 h following scratch (A) and time course of mean migratory rate (% healing) in cardiac fibroblasts of healthy controls and patients with heart failure with reduced ejection fraction (HFrEF) (B). Migration rate was analysed in cells of HFrEF (C, $n = 5$) or control (D, $n = 4$) groups treated with vehicle and increasing concentrations (100 nM and 1 μM) of SGLT2 inhibitor (SGLT2i). The effects of SGLT2i interaction with other frequently used drugs in heart failure patients, LCZ696 (E, $n = 5$), and spironolactone (F, $n = 5$), on the migration rate of cardiac fibroblasts, were determined in cells isolated from HFrEF patients. The extra sum-of-squares F determined differences in the fit of time-course curves. P values and comparisons are expressed next to each time-course curve. Significance is considered when $P < 0.05$.



Results

Migratory profile of fibroblasts: Role of heart failure and treatments

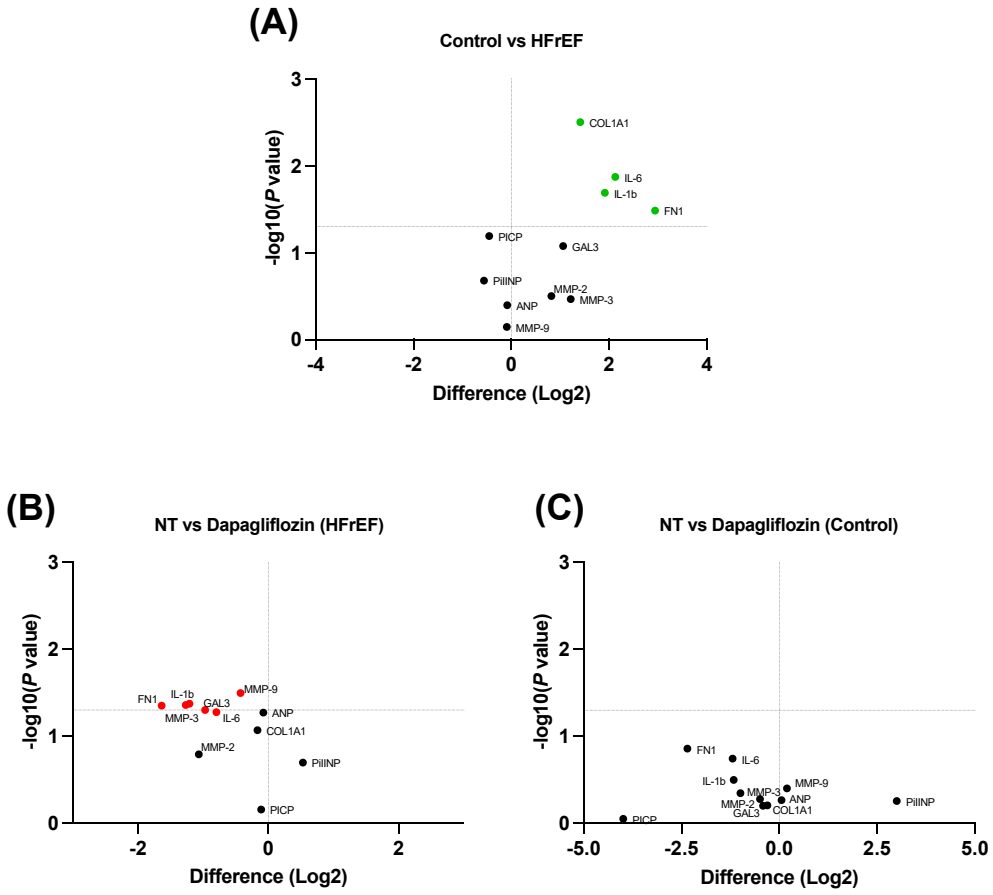
Fibroblasts were obtained from patients with HFrEF ($n = 5$) and healthy donors ($n = 4$). In this study, we observed that cardiac fibroblasts isolated from hearts of patients with HFrEF have a higher migration rate than those isolated from hearts of healthy donors (*Figure 1A* and *1B*), as evidenced by the increase in the Area Under de Curve (AUC: HFrEF = 333 ± 25.7 vs. Control = 275 ± 30.8; $P = 0.012$). When fibroblasts of HFrEF patients were treated with increasing concentrations of dapagliflozin, there was a decrease in cell migration in a

dose-dependent manner peaking at concentration 1 μM (*Figure 1C, Figure S1*). Conversely, SGLT2i did not affect cell migration in fibroblasts of the control group (*Figure 1D*, *Figure S2*). Before determining the effects of the association of SGLT2i with other frequently used drugs in heart failure patients [sacubitril/valsartan (LCZ696) and spironolactone], we tested the effects of those drugs alone on fibroblast. All drugs used individually induced similar inhibition patterns on fibroblast migration in HFrEF to those observed in the dapagliflozin experiment (*Figure S3*). When we combine cells treatments, that is, 100 nM SLGT2i + 100 nM LCZ696 or 100 nM SGLT2i + 100 nM spironolactone, we observed that the combination of SLGT2i + LCZ696 amplified the inhibitory effect of SLGT2i alone on fibroblast migration (*Figure 1E*). The association of SLGT2i with spironolactone did not modify the inhibitory response of SLGT2i alone (*Figure 1F*).

Expression profile of biomarkers of heart failure

At basal state in cell culture, fibroblasts from HFrEF patients displayed an increased expression of components of extracellular matrix (COL1A1 and FN1) and pro-inflammatory cytokines (IL-6 and IL-1β) when compared with healthy control cells (*Figure 2A*). The treatment of fibroblasts from HFrEF patients with 100 nM of SGLT2 markedly reduced the expression of the extracellular matrix components (MMP-3, MMP-9, and FN1), the beta-galactoside-binding protein Galectin-3

Figure 2 Expression of biomarkers of heart failure in cardiac fibroblasts. Volcano plots show the values of fold change (Log2, *x*-axis) by the FDR-adjusted *P*-value (−Log10 of *P*-value, *y*-axis) in the concentration of the heart failure biomarkers atrial natriuretic polypeptide (ANP); fibronectin (FN1); galectin 3 (GAL3); collagen type I-alpha-1 (COL1A1); matrix metalloproteinase-2 (MMP-2), -3 (MMP-3), and -9 (MMP-9); interleukins (IL) IL-6 and IL-1β; procollagen I C-terminal propeptide (PICP) and N-terminal procollagen III propeptide (PIIINP). Highlighted are biomarkers that were at least 1.5-fold down-regulated (red) or up-regulated (green), with $P < 0.05$. Changes in heart failure biomarkers were analysed in untreated fibroblasts from patients with HFrEF ($n = 5$) relative to the levels found in untreated cells of healthy controls ($n = 4$) (A). The effects of SGLT2 inhibition in the expression of heart failure biomarkers were determined in cells from HFrEF (B, $n = 5$) or healthy controls (C, $n = 4$) treated with 100 nM of SGLT2i expressed as fold change relative to their respective untreated cells. Differences were analysed by multiple *t*-tests with false discovery rate (FDR) correction using the method of Benjamini and Hochberg.



(GAL3); and the inflammatory cytokines IL-1β and IL-6 (*Figure 2B*). There was no significant change in protein expression in control cells after treatment with SGLT2i (*Figure 2C*). The association of SGLT2i with LZC696 did not significantly modify the responses observed with treatment with SGLT2i alone, although a slightly more pronounced decrease in IL-6 expression was seen in the SGLT2i + LZC696 group (*Figure 3*). In contrast, there was a partial loss in the benefits of SGLT2 inhibition when dapagliflozin was associated with spironolactone. This association abolished the down-regulation of GAL3, MMP9, and IL-1β induced by the treatment with SGLT2i alone (*Figure 3*).

Phosphorylation profile induced by SGLT2i and drug associations

A phosphoprotein array determined the potential signalling pathways regulated by the inhibition of SGLT2i. From 35 kinases and associated proteins analysed, SGLT2i decreased the degree of phosphorylation of 6 proteins (*Figure 4A*). SGLT2i treatment reduced the phosphorylation of the protein kinase AKT at both Threonine-308 and Serine-473 sites (*Figure 4B*) along with a decreased phosphorylation of its downstream glycogen synthase kinase 3 (GSK3) (*Figure 4C*). Moreover, SGLT2i decreased the phosphorylation of protein tyrosine kinase 2 (PYK2) (*Figure 4D*) and the downstream transcription factor signal transducer and activator of transcription 3 (STAT3) (*Figure 4E*). The combination of SGLT2i with LCS696 did not modify the response of SGLT2i on protein phosphorylation in AKT/GSK3 and PYK2/STAT3 cascades (*Figure 4B–D*). On the other hand, the association of SGLT2i with spironolactone abolished the response of SGLT2i alone on AKT/GSK3 phosphorylation cascade (*Figure 4B* and *Figure 4C*) and, besides, increased the degree of phosphorylation of components of the mitogen-activated protein kinase cascade, MEK1/2 (*Figure 4F*) and ERK1/2 (*Figure 4G*).

Discussion

This study shows that (1) dapagliflozin, a SGLT2i has a direct effect on cardiac fibroblast function, decreasing the migratory rate of dysfunctional cells of patients with HFrEF and the secretion of pro-inflammatory cytokines and significant structural components of extracellular matrix (ECM) turnover; (2) these effects are associated with a decrease of phosphorylation of key regulators of cardiac remodelling, including the AKT/GSK3 cascade and the STAT3; (3) the combination of dapagliflozin + LCZ696 shows an additive effect on migration, while spironolactone modifies the signalling pathways activated by SGLT2i and its beneficial effects of biomarkers of HF.

Generating primary cell culture from cardiac fibroblast of healthy and diseased specimens may have great potential for cardiac disease modelling. They can retain some phenotypic characteristics and the gene expression profile associated with the turnover of ECM pro-fibrotic cytokines.[23,26] In our experimental model, cardiac fibroblasts were explanted from the heart tissue of patients with HFrEF who underwent cardiac transplant and kept in culture under standard conditions. Under basal conditions, we observed that fibroblasts isolated from hearts of HFrEF patients have a higher migration rate (~20% increase in the AUC, $P = 0.012$) than cells isolated from hearts of healthy donors. Furthermore, HFrEF fibroblasts show a 3- to 6-fold increase in the production of ECM components such as COL1A1 and FN1 and an up-regulation of the pro-inflammatory cytokines IL-1β and IL-6 relative to control cells. These results reinforce the potential of using diseased cardiac fibroblast to search and validate therapeutic strategies for heart failure.

SGLT2i is a relatively new hypoglycemiant agent that has demonstrated additional cardiovascular protective effects independently of serum glucose reduction in patients with and without diabetes mellitus. A systematic review and meta-analysis of large-scale clinical trials describe that SGLT2i can reduce cardiovascular death or hospitalization for HF in HFrEF regardless of diabetes mellitus status.[27] Moreover, a secondary analysis of nondiabetic HFrEF patients enrolled in the EMPA-TROPISM study described significant improvement in epicardial adiposity, MIF, and aortic stiffness after treatment with Empagliflozin, which was associated with a significant reduction in inflammatory biomarkers.[11] Nonetheless, despite the growing evidence, the mechanisms behind the direct effects of SGLT2i in HFrEF remain largely unknown. In this study, we primarily sought to determine the effects and mechanisms associated with SGLT2 inhibition in cardiac fibroblasts that could account for the direct benefits of SGLT2i in HF.

When we treated the fibroblasts from HFrEF patients with dapagliflozin, we observed a concentration-dependent decrease in the migration rate of these cells. In addition, the dapagliflozin-treated HFrEF cells showed a marked reduction

Figure 3 Association of SGLT2i with LZC696 or spironolactone on heart failure biomarkers. Box and whiskers plot shows the distribution and the mean of levels (pg/mL) of the heart failure biomarkers fibronectin (FN1); galectin 3 (GAL3); matrix metalloproteinase-3 (MMP-3) and -9 (MMP-9); and interleukins (IL) IL-6 and IL-1β in cardiac fibroblasts of HFrEF patients, as indicated. The levels of biomarkers were determined in cells treated with vehicle (NT), 100 nM SGLT2 inhibitor (SGLT2i), the combination of 100 nM SGLT2i + 100 nM LCZ696 or the combination of 100 nM SGLT2i + 100 nM Spironolactone. Brown Forsythe and Welch ANOVA analysed the dependence of data on the type of treatment with Dunnett's T3 post hoc analysis for multiple comparisons. *P*-values and comparisons are expressed on top of box plots. Significance is considered when $P < 0.05$.

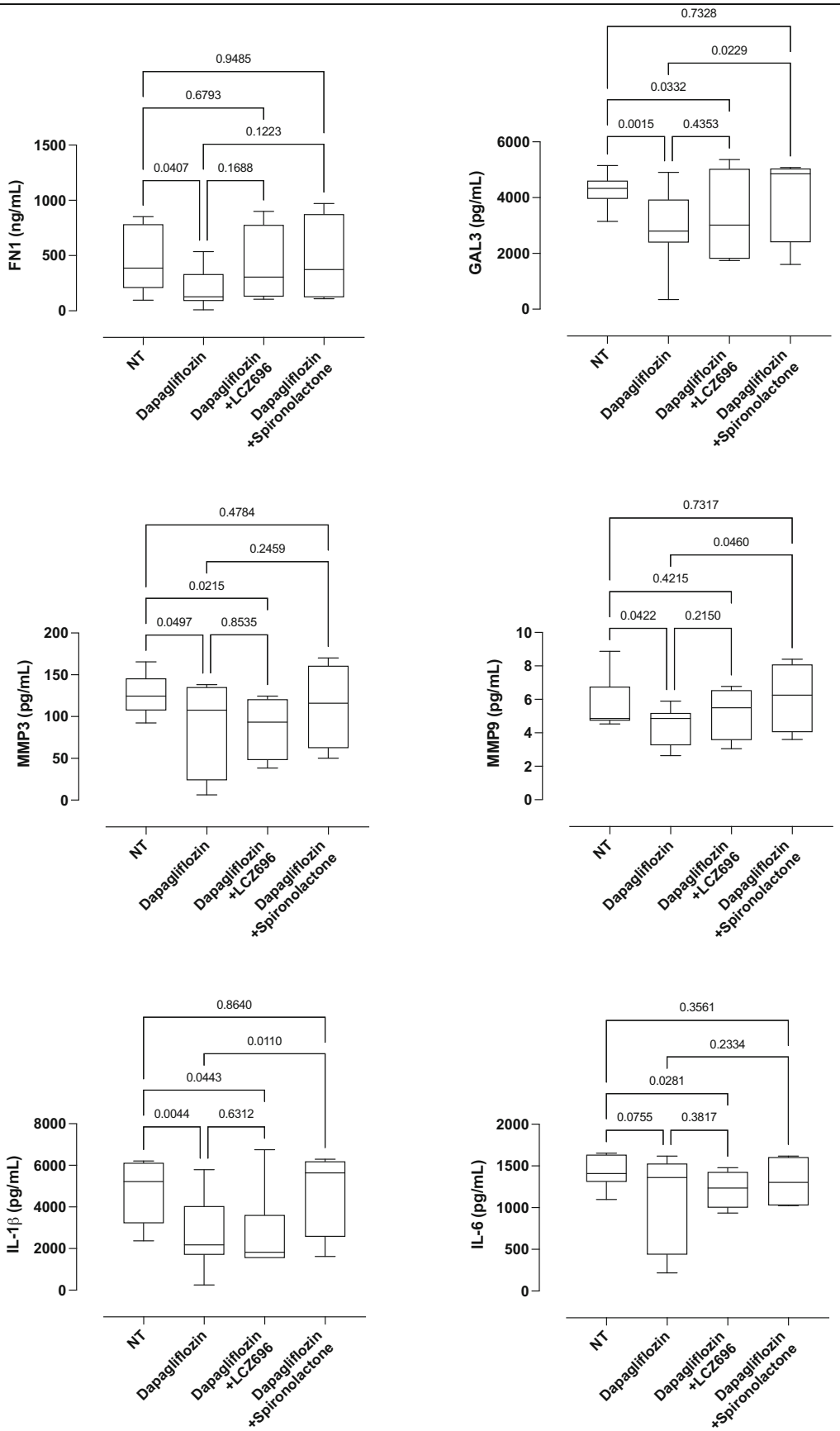
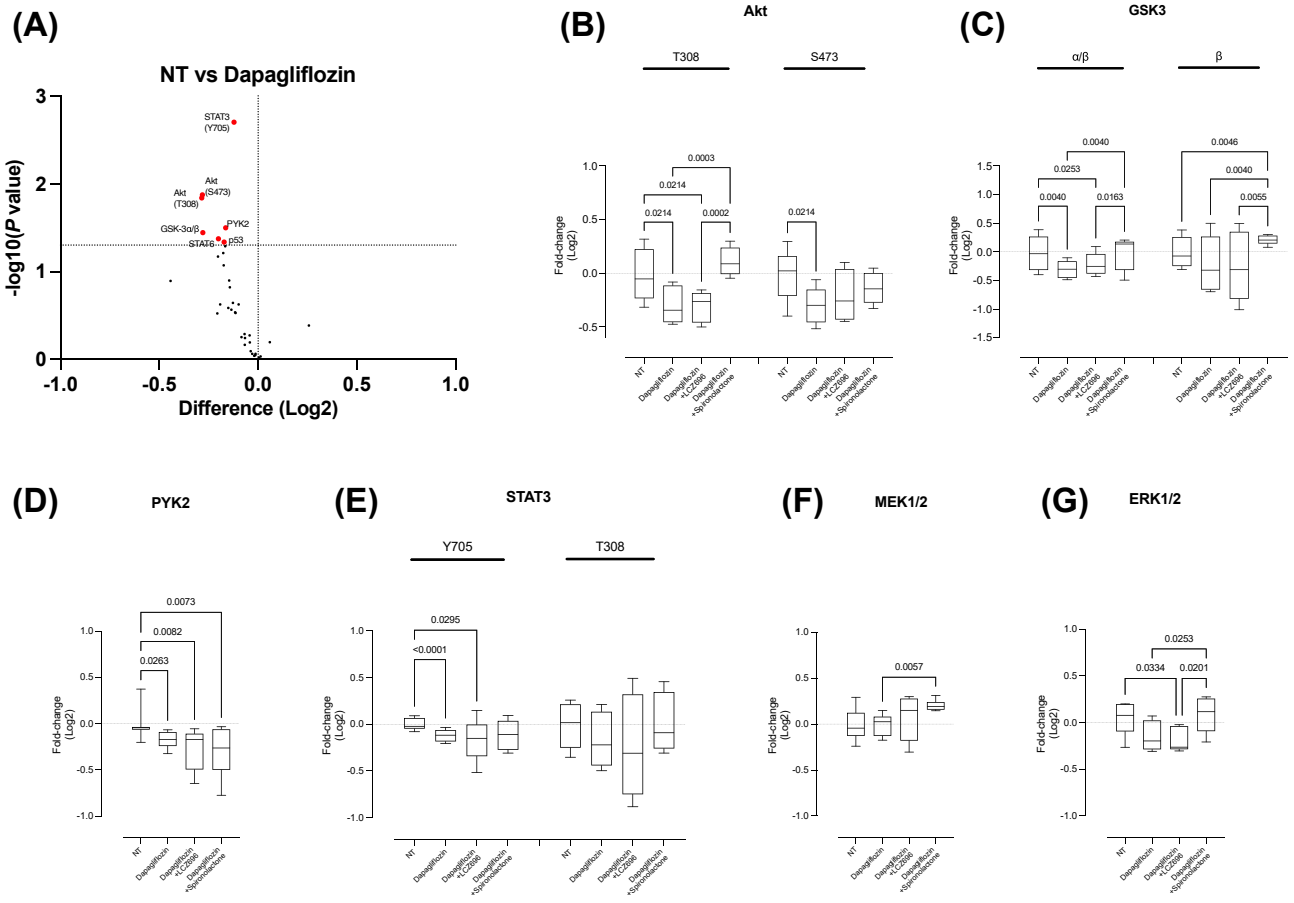


Figure 4 Phosphorylation array. Volcano plot (A) shows the values of fold change (Log2, *x*-axis) by the FDR-adjusted *P*-value (−Log10 of *P*-value, *y*-axis) in phosphorylation signal of cells treated with SGLT2i relative to the densitometric signal of untreated samples. Highlighted in red are phosphorylation signals that were significantly down-regulated ($P < 0.05$). Box and whiskers plots show the distribution and the mean fold change (Log2) in the phosphorylation signals of AKT kinase at Threonine 308 and Serine473 site (B), glycogen synthase kinase-3 (GSK3) at subunits alpha and beta (C), protein-tyrosine kinase 2 (PYK2) (D), signal transducer and activator of transcription 3 (STAT3) (E), and components of the mitogen-activated protein kinase cascade, MEK1/2 (F) and ERK1/2 (G). Phosphorylation signals were determined in lysate of cardiac fibroblasts from HFrEF treated with vehicle (NT), 100 nM SGLT2 inhibitor (SGLT2i), the combination of 100 nM SGLT2i + 100 nM LCZ696 or the combination of 100 nM SGLT2i + 100 nM Spironolactone. Brown Forsythe and Welch ANOVA analysed the dependence of data on the type of treatment with Dunnett's T3 post hoc analysis for multiple comparisons. *P* values and comparisons are expressed on top of box plots. Significance is considered when $P < 0.05$.



at the level of ECM macromolecules and inflammatory biomarkers. Those effects were not seen in control fibroblasts (healthy donors). Our results corroborate with experimental models of HF that demonstrate a reduction in fibrosis and inflammation markers in cardiac tissue by SGLT2i.[11,28] However, these models do not discriminate the metabolic from a direct effect of SGLT2 inhibition on cardiac tissue. In one of the few studies in cell culture, Uthman et al.[29] have shown a direct effect of distinct SGLT2i (empagliflozin, dapagliflozin, and canagliflozin) on various cardiac cell types, including fibroblasts and endothelial cells. Their data agree with our results that support how SGLT2i induce anti-inflammatory responses, although they have not determined their contribution to the generation of ECM components.[29]

The latest guidelines from the European Society of Cardiology recommended adding SGLT2i in the pharmacotherapy of all patients with HFrEF already treated with ARNI, a beta-blocker, and MRA, regardless of whether they have diabetes or not.[1] Few clinical trials have addressed the synergic effect of the combination of SGLT2i with ARNI or MRA in the outcomes of patients with HF.[17–20] Moreover, to the best of our knowledge, no study has addressed this association in cell function at the molecular level. In this regard, this study provides a first step to increase our knowledge about the effects of SGLT2i interactions with drugs that are the pillars of therapy in HF, including ARNI and MRA.

Following the 48 h incubation time, the combination of dapagliflozin with sacubitril/valsartan further decreased

HFrEF cardiac fibroblasts migration. Although this association did not modify the effects of SGLT2i on the expression of most biomarkers studied, we observed a modest but significant decrease in IL-6 expression when combining dapagliflozin with sacubitril/valsartan. A systematic review of randomized controlled trials evaluating SGLT2i and ARNI alone or in combination concluded that each drug treatment isolated provides similar benefits in HFrEF, while their association results in a better cardiovascular protective effect.[27] In basic research studies, treatment with sacubitril/valsartan has shown significant inhibitory effects on the migration and growth of cardiac fibroblasts of rats, which parallels with lower cardiac remodelling after treatment in experimental rat models.[30–32] Moreover, a recent study comparing the effects of dapagliflozin and sacubitril/valsartan in a rat model of nondiabetic stress-induced heart failure has shown that each treatment alone effectively reduced the cumulative risk of death, myocardial fibrosis, and the pro-inflammatory cytokines IL-1β and IL-6 levels in cardiac tissue.[33] Although the authors established a direct action of dapagliflozin in cardiac remodelling, the interaction of SGLT2i and ARNI was not addressed.

Contrary to ARNI, dapagliflozin and spironolactone association did not change the migration rate of fibroblasts but significantly altered the response of SGLT2i on the expression of MMP9, GAL3, and IL-1b. These results are not aligned with the sub-analysis of the EMPEROR-Reduced study, which showed that the use of MRAs did not influence the effect of SGLT2i to reduce adverse heart failure outcomes. However, the magnitude of the benefit of MRAs may differ among subgroups of patients with HF, and those differences can be extrapolated to the benefits of MRAs interaction with other drugs.[34,35] In the EMPA-REG Outcomes trial, the beneficial effect of SGLT2 inhibition with empagliflozin on heart failure outcomes was attenuated in patients receiving spironolactone at the start of the study.[34] Although the molecular mechanisms for the attenuated effect of these interactions are unclear, they may be linked to the ability of both SGLT2i and MRA to interact with the same site of action, the Sodium/hydrogen exchanger 1 (NHE1). Studies have proposed that both drugs are effective when NHE1 is up-regulated, as in heart failure and diabetes. Conversely, the benefits of SGLT2i may be minimized if the NHE1 is already inhibited by MRA.[35]

The graphical abstract illustrates our understanding of the pathways accounting for the altered function in dapagliflozin-treated fibroblasts. Although SGLT2 expression has not been described in cardiac cells, its cardioprotective effects have been primarily mediated through interference with the NHE1.[35–37] Deep learning in vitro analysis with in vivo validation have established with 95% accuracy that NHE1 blockage is the most robust mechanism of action in the cardioprotection by SGLT2i.[38] Additionally, this computational analysis also suggested the inhibition of AKT-dependent signalling pathways as a downstream mechanism of NHE1 blockage.[38]

In the present study, results on the phosphoprotein array confirm the algorithm prediction about AKT modulation by SGLT2i. Our data show that treatment with dapagliflozin reduced the degree of AKT phosphorylation at both the serine and threonine sites and of its downstream effector glycogen synthase kinase 3 (GSK3). GSK3 is one of the few protein kinases activated by dephosphorylation and is a negative regulator of cardiac hypertrophic gene expression and fibrosis.[39,40] Other regulators of cardiac remodelling that have also shown a lower degree of phosphorylation after dapagliflozin treatment were the kinase PYK2 and the transcription factors STAT3 and STAT6.[40] In response to cytokines and growth factors, both PYK2 and STAT3 are phosphorylated and trigger signalling associated with matrix reorganization, cell migration, and inflammation.[41] The increase in IL-6 and IL-1β observed in HFrEF fibroblasts could be responsible for activating the PY2K and STAT pathways. Although this study has not determined the direct correlation between interleukin increase and PY2K and STAT phosphorylation, both effects are prevented by treatment with SGLT2i. Therefore, a decrease in the phosphorylation of AKT/GSK3 and PYK2 and STAT pathways by dapagliflozin could contribute to the more favourable phenotype observed in cardiac fibroblasts from HFrEF patients after treatment with dapagliflozin.

As observed in protein expression studies, the association of dapagliflozin with sacubitril/valsartan did not modify the phosphoprotein profile induced by SGLT2 inhibition alone. On the other hand, SGLT2i and MRA association reversed the phosphorylation of the kinases AKT and GSK3 and increased the phosphorylation of the mitogen-activated protein kinases MEK1/2 and ERK1/2. These results reinforce the idea of crosstalk between SGLT2i and MRA.

In conclusion, our data show that the SGLT2i dapagliflozin has direct and beneficial effects in cardiac cells of patients with HFrEF by a mechanism that involves a decrease in the phosphorylation of AKT/GSK3 and PYK2 and STAT signalling pathways. Although SGLT2 inhibition does not affect healthy cells, in diseased cardiac fibroblasts, dapagliflozin has anti-migration, anti-inflammatory, and ECM turnover regulating effects and may represent an essential target in the therapeutic benefit observed in nondiabetic HF. Furthermore, the interaction of SGLTi with sacubitril/valsartan may promote further benefits by enhancing the anti-migratory and anti-inflammatory effects of dapagliflozin. Conversely, the association of dapagliflozin with the MRA spironolactone deserves more attention, as it interferes with the effects of SGLT2i. Although clinical trials have described benefit or null effects on this interaction, future studies should be designed to further determine the molecular mechanisms and potential effects of this interaction.

Perspectives

Competency in medical knowledge

Sodium-glucose cotransporter 2 inhibitors (SGLT2i) are a relatively new family of oral hypoglycemiant drugs. They inhibit the renal reabsorption of glucose, thus favouring its renal excretion. SGLT2i reduces plasma glucose levels and has shown substantial improvement in cardiovascular outcomes in patients with type 2 diabetes (T2DM). Clinical trials on SGLT2i have shown an impressive reduction in the incidence and progression of heart failure in patients with T2DM. Although these exciting results have been primarily associated with glycemia regulation, recent studies have described that SGLT2i can reduce cardiovascular death or hospitalization for HF regardless of diabetes mellitus status, suggesting a pleiotropic action of SGLT2i in the cardiac tissue. At this time, there are few data to support this hypothesis and few molecular studies to understand the associated mechanisms. This study provides new insights into the cardioprotective mechanisms of SGLT2i. We used primary cell culture from cardiac fibroblast of healthy and HFrEF patients as cardiac disease modelling to determine the direct effects of SGLT2i on cardiac cell specimens, excluding the effects of glucose metabolism in the effects observed.

Translational outlook

Regardless of the aetiology, the pathophysiology of HF comprises disturbances of collagen turnover (increased generation and deposition of collagen type I fibres and decreased degradation) and inflammatory signalling, which leads to fibrosis and remodelling of cardiac tissue. Emerging evidence suggests that cardiac fibroblasts are main effectors in cardiac remodelling. When activated by neurohumoral stimuli, these cells acquire a proliferative and secretory phenotype that favours the increase of collagen deposition and the production of pro-inflammatory cytokines, chemokines, and growth factors. In this regard, the pharmacological targeting of cardiac fibroblasts to control their secretory phenotype may be of great clinical interest. Recent efforts in HF therapy aim to reverse acute hemodynamic abnormalities and relieve symptoms. The objective of targeting the myocardial interstitial fibrosis (MIF) to improve the treatment of HF is still poorly fulfilled. To address this knowledge gap, we provide new insights into the direct effect that a new pharmacological treatment (dapagliflozin) has on MIF, regulating the phenotype of cardiac fibroblasts' phenotype and decreasing their migratory capacity and the secretion of inflammatory molecules and those involved in collagen turnover. Furthermore, considering that the treatment of HFrEF requires a multimodal treatment with a combination of several drugs to foster symptomatic and prognostic improvements, our study will advance our knowledge about the effects and likely consequences of the association of SGLT2i with drugs used as conventional therapeutic strategies in HF, such as ARNI or MRA.

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Conflicts of interest

The Cardiology Department of Hospital Clínic Barcelona has received unrestricted grants from AstraZeneca.

Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. Time course of mean migratory rate (% Healing) in cardiac fibroblasts of healthy controls. Migration rate was analysed in control fibroblasts (n = 4) treated with vehicle (NT) and increasing concentrations (1 nM to 1 μM) of SGLT2 inhibitor (SGLT2i), as shown. The extra sum-of-squares F determined differences in the fit of time-course curves. *P* values and comparisons are expressed next to each time-course curve. Significance is considered when $P < 0.05$.

Figure S2. Time course of mean migratory rate (% Healing) in cardiac fibroblasts of patients with heart failure with reduced ejection fraction (HFrEF). Migration rate was analysed in HFrEF fibroblasts (n = 5) treated with vehicle (NT) and increasing concentrations (1 nM to 1 μM) of SGLT2 inhibitor (SGLT2i), as shown. The extra sum-of-squares F determined differences in the fit of time-course curves. *P* values and comparisons are expressed next to each time-course curve. Significance is considered when $P < 0.05$.

Figure S3. Time course of mean migratory rate (% Healing) in cardiac fibroblasts of patients with heart failure with reduced ejection fraction (HFrEF). Migration rate was analysed in HFrEF fibroblasts (n = 5) treated with vehicle (NT) and LCZ696 (100 nM) or Spironolactone (100 nM), as shown. The extra sum-of-squares F determined differences in the fit of time-course curves. *P* values and comparisons are expressed next to each time-course curve. Significance is considered when $P < 0.05$.

Table S1. Media Formulation - DMEM/F-12 + 10% FBS +

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