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RESEARCH ARTICLE

Unlocking the power of empagliflozin: Rescuing inflammation in hyperglycaemia-exposed human cardiomyocytes through comprehensive multi-level analysis

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Aims

Hyperglycaemic conditions increase cardiac stress, a common phenomenon associated with inflammation, aging, and metabolic imbalance. Sodium–glucose cotransporter 2 inhibitors, a class of anti-diabetic drugs, showed to improve cardiovascular functions although their mechanism of action has not yet been fully established. This study investigated the effects of empagliflozin on cardiomyocytes following high glucose exposure, specifically focusing on inflammatory and metabolic responses.

Methods and results

A three-part strategy was formulated: (i) a meta-analysis of selected randomized clinical trials was carried out to assess the anti-inflammatory effects of empagliflozin in diabetic patients; (ii) the impact of empagliflozin on human cardiomyocyte AC16 cells exposed to normal (5 mM) and high (33 mM) glucose concentrations for 2 and 7 days was explored by evaluating gene expression and protein levels of pivotal markers associated with cardiac inflammation, stress, endoplasmic reticulum damage, and calcium modulation; (iii) *in silico* data from bioinformatic analyses were exploited to construct an interaction map delineating the potential mechanism of action of empagliflozin on cardiac tissue. Empagliflozin reversed high-glucose mediated alterations at the transcriptional level, decreasing inflammatory, metabolic, and aging signatures. Specifically, *in vitro* experiments on human cardiomyocytes, meta-analyses of clinical data on inflammatory biomarkers from diabetic peripheral blood samples, and sequencing of pathological human heart tissues, all support that empagliflozin exerts anti-inflammatory effects both systemically and directly in cardiac tissue, on cardiomyocytes.

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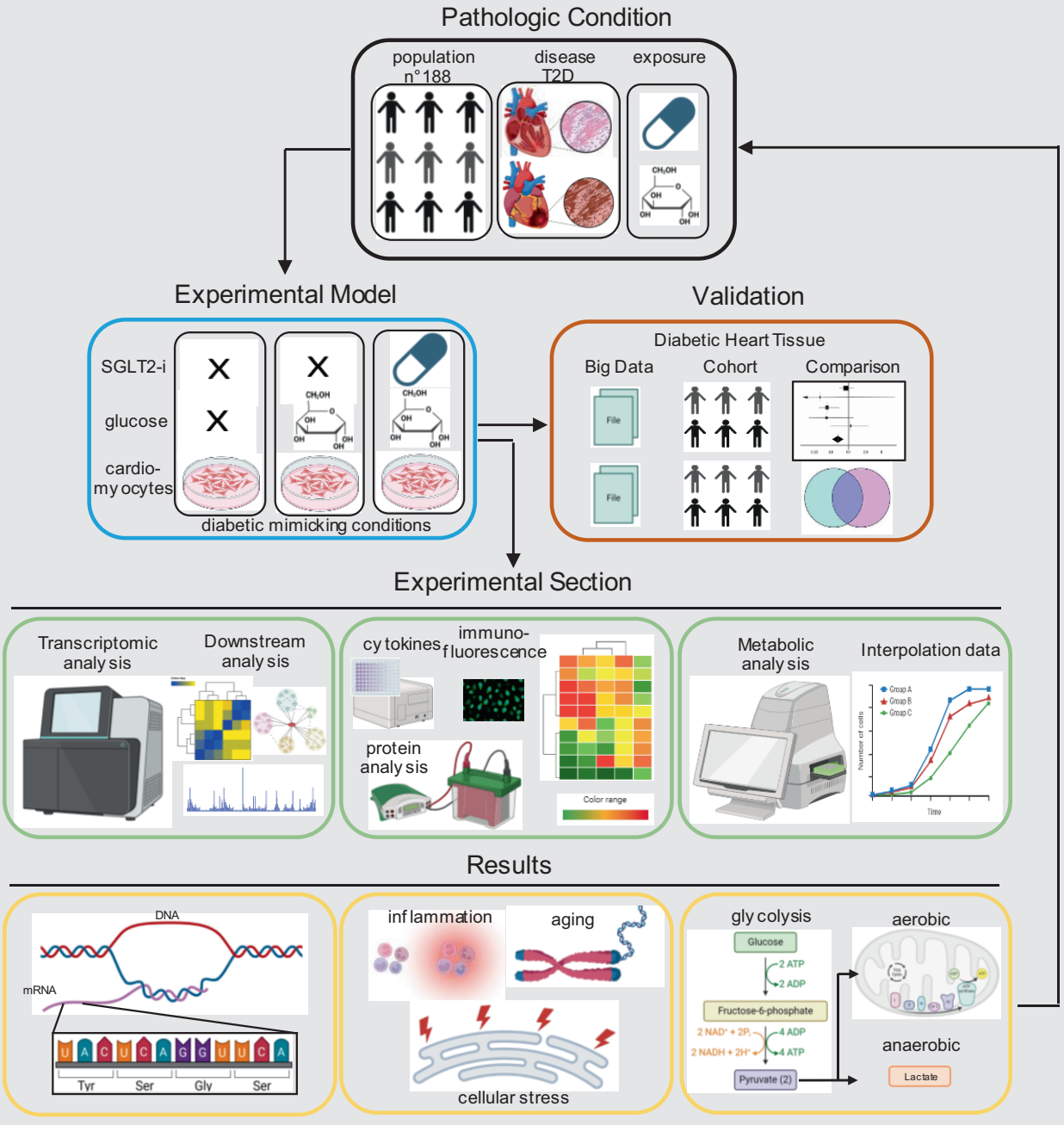
Conclusion Our study provides insights based on robust mechanistic data for optimizing heart failure management and highlights the intricate interplay between diabetes, inflammation, aging, and cardiovascular health.

Graphical Abstract



Schematic representation of the study workflow, pathological model, experimental workflow, and key findings deriving from the integration of clinical trials, public databases, and the cellular model. SGLT2-i, sodium–glucose cotransporter 2 inhibitor; T2D, type 2 diabetes.

Keywords Empagliflozin • Human cardiomyocytes • Inflammaging • Glucose

Introduction

Initially designed to regulate glucose concentration and manage diabetes by targeting the kidneys, sodium–glucose cotransporter 2 inhibitors (SGLT2i) have garnered attention for their unexpected positive impact on heart health.[1] The SGLT2i empagliflozin (EMPA) effectively reduces the risk of heart failure (HF)-related events, irrespective of diabetic status. This unexpected benefit has sparked clinical interest and was consistent across the age spectrum, encompassing patients older than 75 years.[2,3]. The EMPA-REG OUTCOME,[4,5] EMPRISE,[6] EMPEROR-Reduced,[7] and EMPEROR-Preserved[8] trials supported the efficacy of EMPA in improving outcomes for individuals with HF. Collectively, these studies demonstrate that EMPA significantly decreases the risk of HF hospitalizations and cardiovascular death in diabetic and non-diabetic patients. Very recent studies report EMPA to be beneficial in treating HF, both in the presence and absence of diabetes,[9,10] such as its protective effects were evidenced in the post-myocardial infarction phase[11,12] and as an antiarrhythmic drug.[13] EMPA improves contractile function[14] by inhibiting inflammasome activity,[15] which is particularly beneficial in both ischaemic and non-ischaemic HF and the post-ischaemic phase of myocardial infarction.[15] Additionally, the anti-inflammatory effect enhances various molecular mechanisms associated with increased nitric oxide release, downgrading oxidative stress with a parallel strength of the phosphorylation of sarcomeric proteins, leading to improved contractile capacity of cardiac myofibrils.[16,17] These mechanisms are inherently independent of diabetes, but they seem very useful in counteracting the well-known pro-inflammatory and pro-oxidative effects of diabetes mellitus.[18–20] However, the exact molecular mechanisms underlying the beneficial effect of EMPA in HF remain to be investigated. High glucose (HG) levels are reported to alter tissue and cell structure, and to disturb cell function,[21] and diabetic cardiomyopathy is described as a result of the negative long-term impact of HG on cardiomyocytes[22] that precedes overt HF. Interestingly, the genetic, epigenetic, and intracellular molecular burden driving glucose-related structural and functional cardiomyocyte derangement remains an uncharted area of research. Here, we propose that EMPA exerts its beneficial effects by targeting inflammation, which is common in both diabetes and HF, at both systemic and cardiac tissue level. Our study included: (i) an *in vivo* meta-analysis of clinical trials showing SGLT2i reduce plasma biomarkers of systemic inflammation; (ii) *in vitro* experiments in human cardiomyocytes including (a) a comprehensive investigation of gene expression and enrichment analysis, (b) real-time monitoring of aerobic and anaerobic cellular respiration, (c) quantification of cytokine and calcium release, and (d) modulation of specific inflammasome interactors at protein level; (iii) an *in silico* data comparison supporting the results obtained from the cell model.

Methods

The methods, reagents, procedures, and statistical analyses used are described in the online supplementary material. Briefly, the supporting information includes details on search inputs and databases, eligibility criteria, and statistical validation for the meta-analysis.[23–27] It also outlines the procedures for handling AC16 cells and treatment regimes[28–30]; RNA sequencing protocols, the workflow for comprehensive bioinformatic analysis[31–36]; protocols for western blot, Seahorse assays,[37] cytokine arrays, and colorimetric/fluorescence-based assays.

Results

Empagliflozin exerts anti-inflammatory effects in diabetic patients

To assess *in vivo* the anti-inflammatory role of SGLT2i, we conducted a meta-analysis where all patients diagnosed with type 2 diabetes mellitus (T2DM) were compared to placebo. The circulating biomarkers most commonly used for measuring the degree of inflammation (specifically, interleukin [IL]-6, tumour necrosis factor-alpha [TNF-α], and C-reactive protein [CRP]) were considered, and their overall anti-inflammatory effect was statistically measured. In 188 diabetic patients, SGLT2i had a highly significant overall anti-inflammatory protective effect, reducing the risk of an increase in plasma inflammation biomarker levels by 47% (*Figure 1*). CRP and IL-6 levels were significantly reduced in the treated patients, with mean changes and 95% confidence intervals of −0.49 (−0.88, −0.10) and −0.59 (−0.94, −0.24), respectively. TNF-α levels also decreased, with a median change of −0.46, although the 95% confidence interval was lower (−1.09, 0.17). Overall, all circulating biomarkers associated with inflammation were reduced by SGLT2i, with a median change of −0.53, demonstrating their efficacy in improving the clinical profile. These data support a correlation between the use of SGLT2i and the reduction in circulating inflammation-related markers in T2DM patients.

Empagliflozin reverses adverse cardiac effects associated with hyperglycaemia

We analysed gene expression profiles in human cardiomyocytes following HG stimulation for 2 days, with and without EMPA administration (online supplementary *Figure Appendix S1A*). After 2 days of hyperglycaemic conditions, several differentially expressed genes (DGEs) were either downregulated, including *ZFAND6*, *PFN2*, and *NUDT21*, or upregulated, such as *TRPV1*, *RYR1*, and *KLK14* (*Figure 2A*, left panel). Gene ontology (GO) analysis of the top 20 DGEs showed that apoptotic and cellular events, g protein-coupled receptor signalling, and calcium ion-related activity were strongly affected by HG exposure. Interestingly, EMPA restored the gene expression levels of *ZFAND6*, *PFN2*, and *SDCBP*, reversing the modulation triggered by HG in human cardiomyocytes (*Figure 2A*, right panel). Additionally, 2 days of EMPA treatment resulted in the upregulation of *SYK* and *HLA-DRA*, mainly involved in immune-related functions, and of *HSPA1B* and *PET117*, associated with protein stabilization activity and mitochondrial processes. These findings indicate that EMPA directly alleviates and potentially reverses the adverse cardiac effects associated with hyperglycaemia in human cardiomyocytes. We intersected our DGEs with published available transcriptome datasets.[35] In a cohort of seven cardiac biopsies derived from T2DM patients affected by

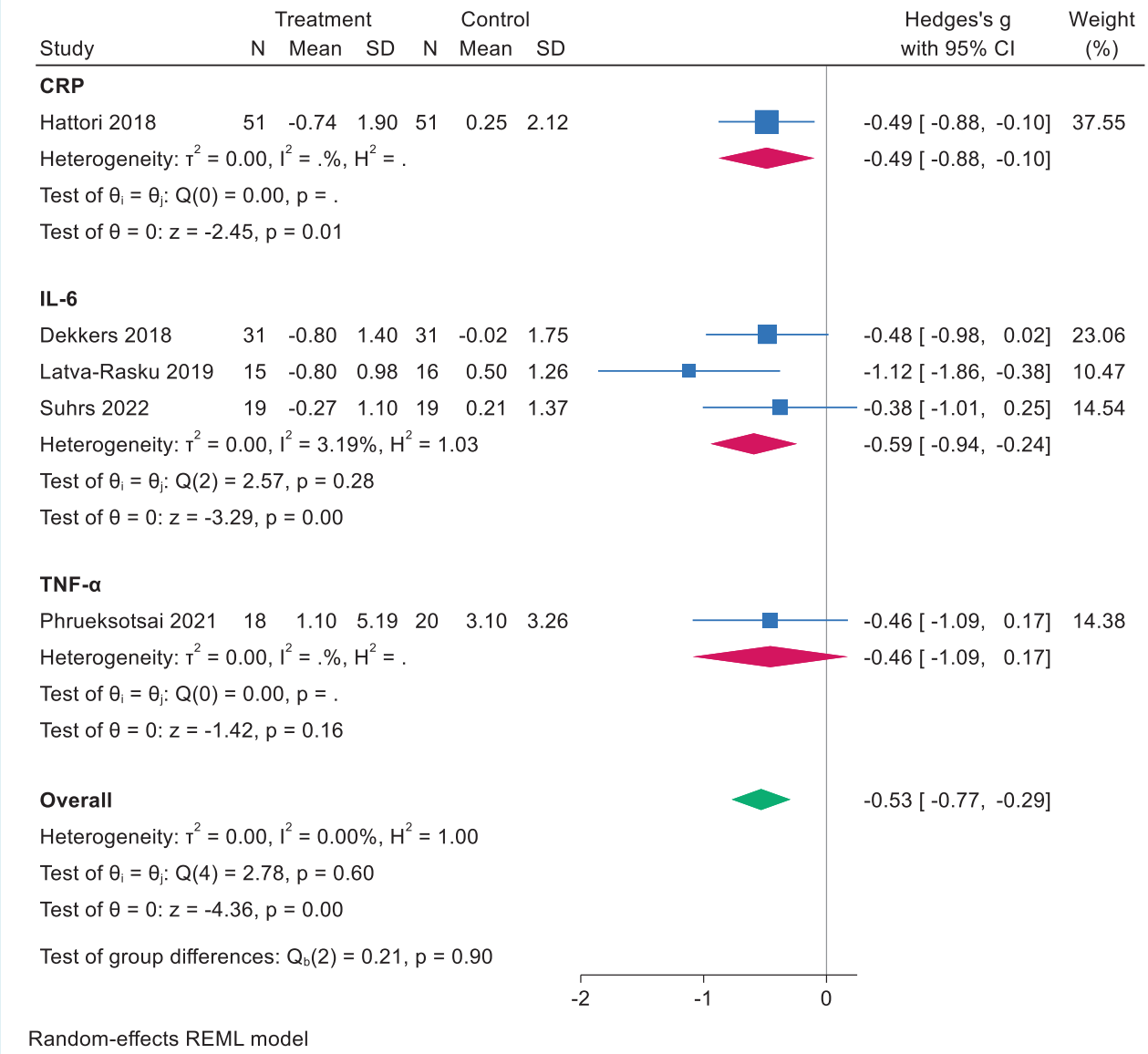


Figure 1 Forest plot of diabetic patients untreated or treated with sodium–glucose cotransporter 2 inhibitors. Data are shown as mean change in biomarkers to compare differences between groups. Hattori (trial registration: UMIN Clinical Registry, UMIN000021552), Dekkers (Netherlands Trial Register, NTR 4439), Latva-Rasku (trial registration: ClinicalTrials.gov, NCT02426541), Suhrs (EU Clinical Trials Register, 2017-000240-17), and Phrueksotsai (clinicaltrials.in.th, TCTR20170511001). CI, confidence interval; CRP, C-reactive protein; IL-6, interleukin-6; SD, standard deviation; TNF-α, tumour necrosis factor-alpha.

dilated hypokinetic post-ischaemic cardiomyopathy (D-HF) and five healthy controls (online supplementary *Figure Appendix S1B*), transcriptome analyses revealed genes differentially expressed in D-HF patients. Diabetic cardiomyopathy and post-ischaemic dilated cardiomyopathy in diabetic patients share key pathophysiological features, including: (i) impaired glucose metabolism and insulin resistance lead to increased fatty acid (FA) oxidation and toxic lipid accumulation, worsening cardiac dysfunction; (ii) high oxidative stress and systemic inflammation damage myocardial cells, exacerbating fibrosis and contributing to overall dysfunction; (iii) persistent hyperglycaemia causes fibrosis and remodelling, stiffening the heart muscle and impairing diastolic and systolic function; and (iv) endothelial dysfunction limits vasodilatation, while (v) mitochondrial damage leads to energy deficits, both furthering HF progression. In essence, the diabetic condition accelerates dysfunction in both cardiomyopathies; however, post-ischaemic cases are further complicated by ischaemic injury from coronary artery disease, affecting prognosis more severely. By aligning published gene list with our results in AC16, we identified a substantial set of genes highly expressed both in healthy individuals and in EMPA-treated

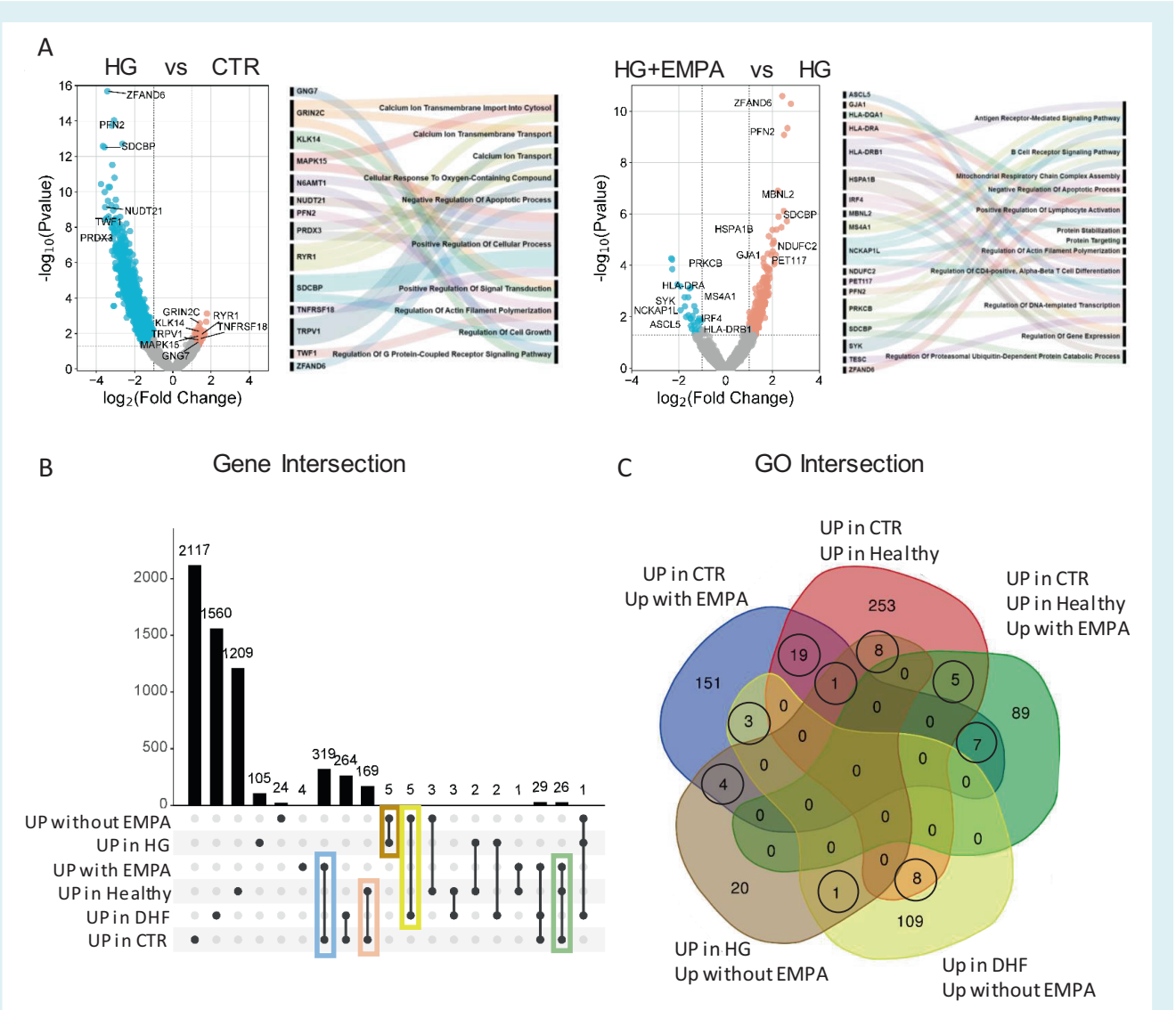


Figure 2 (*A*) Volcano plots displaying differentially expressed genes (DGEs) from normalized RNA-seq reads. The x and y axes indicate fold-change resulting from comparisons expressed in log2 and *p*-value in −log10, respectively; alluvial plots showing gene ontology (GO) for biological processes of the top 20 DGEs (high glucose [HG] vs. control [CTR] conditions, left; HG + empagliflozin [EMPA] vs. HG conditions, right). (*B*) Gene intersection across DGEs from HG versus CTR and HG + EMPA versus HG comparisons with DGEs from diabetic and non-diabetic patients affected by post-ischaemic heart failure. (*C*) Venn diagram showing the intersection of GO biological processes from common genes of gene intersection analysis.

cardiomyocytes (*Figure 2B*). The similar trend in healthy controls and in the HG + EMPA group was corroborated and strengthened by intersecting the biological processes (*Figure 2C*). These findings demonstrate that our experimental model replicates the diabetic condition that predisposes individuals to HF or diabetic cardiomyopathy. Furthermore, our data suggest that EMPA treatment in T2DM patients could rebalance the expression of a set of specific genes, enhancing cardiac function and modulating inflammatory response. Genes whose expression is restored by EMPA treatment in HG conditions include *PFN2*, reported to alleviate myocardial infarction and improve cardiac function[38]; *UGCG*, found to be involved in contractile capacity in cardiomyocytes and to preserve normal heart function[39]; and *FAM3C*, associated with metabolic disorders and diabetes.[40]

Empagliflozin rescues cardiomyocytes from high glucose-induced stress

We performed a gene set enrichment analysis to corroborate the detrimental effects of HG on AC16 and to investigate the beneficial impact of EMPA treatment. Our analysis revealed that 2 days of HG stimulation led to the activation of inflammatory events and decreased insulin responsiveness, cardiac muscle potential, and cardiomyocyte differentiation pathways (*Figure 3A,B*). After 2 days

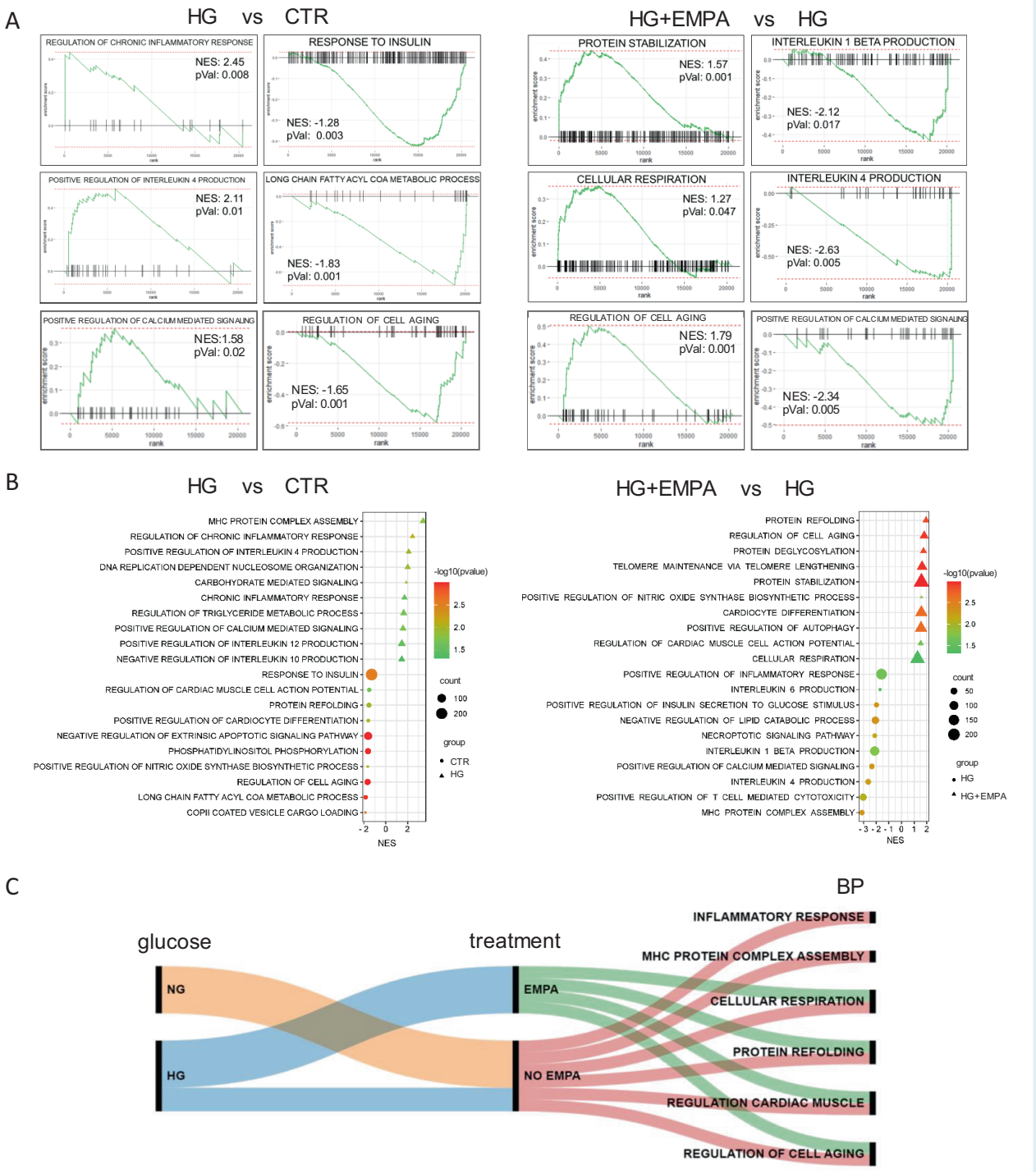


Figure 3 (*A*) Enrichment plots displaying processes altered upon high glucose (HG) stimulation with (left) and without (right) empagliflozin (EMPA). Normalized enrichment score (NES) and *p*-value indicate differential expression in pathway enrichment across comparisons and related statistical significance, respectively. (*B*) Significant processes from enrichment analysis in HG versus control (CTR) and HG + EMPA versus HG. Terms enriched are significant for $p < 0.5$, reported transformed as −log10 in the label. NES indicates the value of enrichment. The size of each term is reported as gene count in the label. (*C*) Alluvial plot illustrating the main pathways altered by HG and EMPA. The plot shows that EMPA rebalances gene expression in the direction of normal glucose (NG).

in HG conditions, chronic inflammation pathways increased, but EMPA treatment reversed this, boosting protein refolding, cardiac potential, differentiation, telomere maintenance, aging, and respiration. Considering the data, we postulated that HG exposure alters cardiomyocyte homeostasis and leads to global changes in RNA expression. Conversely, EMPA reverts the HG-mediated expression profile, mitigating tissue inflammation and cellular aging processes, which can both contribute to the progressive decline in cardiac function (*Figure 3C*).

Empagliflozin improves cardiac function and exhibits anti-inflammatory activity at transcriptional level

To gain a more mechanistic understanding of the impact of EMPA on cardiac function, we focused on 374 genes with different expression profiles in HG and EMPA conditions (*Figure 4A*). GO analysis revealed contrasting enrichments in biological processes (*Figure 4B*). Specifically, HG exposure decreased the negative regulation of apoptotic processes, phenomena ameliorated by EMPA. This restorative effect involved genes such as *ZFAND6*, *CAV1*, *DKK1*, and *CDK1* (*Figure 4C*). In addition, the positive regulation of cell proliferation involving genes such as *SDCBP*, *TTK*, *GJA1*, and *MDM2* (online supplementary *Table S3*) was altered under HG conditions and rescued by EMPA. Furthermore, EMPA restored several mitochondrial functions, including ATP synthesis coupled with electron transport and cardiac muscle activity. Additionally, we compared the 374 genes regulated by EMPA with an inflammaging signature, consisting of 1070 genes involved in both inflammation and aging pathways. The results identified 19 genes involved in inflammaging whose expression was significantly reduced under HG stimulation and restored with EMPA treatment (*Figure 4D*). Notably, this set of 19 genes is implicated in cardiac inflammation and several pivotal metabolic pathways (*Figure 4E*). Treatment with EMPA restored the expression profiles of these 19 genes to the baseline of normal glucose (NG) conditions, thereby fully counteracting the transcriptional induction observed under HG. GO analysis associated the functions of these genes with the regulation of cardiac muscle (*CAV1*, *IL6ST*, and *GJA1*), lipid metabolic processes (*MBTPS1* and *MBTPS2*), cellular response to increased oxygen levels (*ATP6AP2* and *CAV1*), and negative regulation of the apoptotic process (*GCLC*, *HSPA1B*, and *MDM2*). Our findings suggest that EMPA improves specific cardiac functions and exhibits anti-inflammatory activity in the cardiac context. These molecular insights may explain the clinically observed enhancement in cardiac performance in non-diabetic and T2DM patients treated with EMPA.

Empagliflozin reduces glucose-induced inflammation and stress by impacting on calcium and fatty acid equilibrium

We confirmed the HG-induced inflammatory reaction by measuring cytokine release from cardiomyocytes. HG caused a steady increase in cytokine levels, particularly those of pro-inflammatory cytokines such as IL-1β, IL-4, and IL-6. However, when EMPA was present, cytokine secretion decreased (*Figure 5A*). Further inflammation pathway activation was examined after 2 days of HG induction and EMPA treatment (*Figure 5B*). Specifically, we found that IL-6 levels surged upon HG stimulation, whereas EMPA treatment restored IL-6 content to control levels. We also examined expression levels of NLRP3 and ASC, crucial components of the inflammasome complex.[41] After 2 days of HG exposure, NLRP3 showed a slight upregulation, with EMPA treatment impacting its expression. This finding corroborates the activation of the inflammatory process during hyperglycaemia and underscores the efficacy of EMPA in alleviating inflammation. GLUT1, a network marker of inflammation, aging, and metabolic dysregulation,[42] increased in HG conditions, while EMPA treatment promoted its reduction. We also assessed the HG-induced inflammatory condition by investigating total and phosphorylated forms of NF-κB, a key regulator of the inflammatory process, under acute (2 days) and chronic (7 days) hyperglycaemic conditions; the longer time frame was selected to evaluate any potential long-term or non-transcriptional effects. EMPA administration reduced NF-κB activation in both short- and long-term treatments (*Figure 5C*). Transcriptional regulation induced by HG and subsequently by EMPA had functional effects on human cardiomyocytes, corroborated by the intracellular dynamics of Ca^{2+} and FAs. In this context, intracellular Ca^{2+} levels, taken as a readout of cell stress, were evaluated upon HG induction for 2 and 7 days. Ca^{2+} levels showed a significant increase, mainly following long-term HG stimulation, while prolonged EMPA treatment significantly reduced intracellular Ca^{2+} levels (*Figure 5D*). The total quantity of FAs (a readout of metabolic impairment) increased after HG 2 days, while this increase and the rebalancing effect induced by EMPA were even more pronounced after 7 days (*Figure 5E*). Overall, these results indicate that modulable effects occur after both short- and long-term EMPA treatment, suggesting that EMPA reduces glucose-induced inflammation and cellular stress with acute transcriptional and consequently chronic regulatory effects.

Empagliflozin boosts mitochondrial activity, reducing glycolytic function upon sirtuin 1 activation in high glucose conditions

We tested, in real-time, the synergistic effect of EMPA on aerobic and anaerobic metabolic function in cardiomyocytes exposed to HG upon sirtuin 1 (SIRT1) activation, as a metabolic stress effector[43] (online supplementary *Figure S2A,B*). HG exposure increased basal respiration and glycolysis (*Figure 6A*), while EMPA significantly counteracted this effect. Interestingly, basal respiration increased independently of EMPA treatment upon SIRT1 activation in HG conditions, while basal glycolysis was reduced with administration of EMPA. The metabolic phenotype was defined using oxygen consumption rate and extracellular acidification rate values (*Figure 6B*). Globally, EMPA treatment mainly reduced the glycolytic activity and this effect was amplified upon SIRT1 activation, resulting in a boost of aerobic respiration and a strong reduction in glycolytic function. Indeed, maximal respiration did not change as a result of EMPA

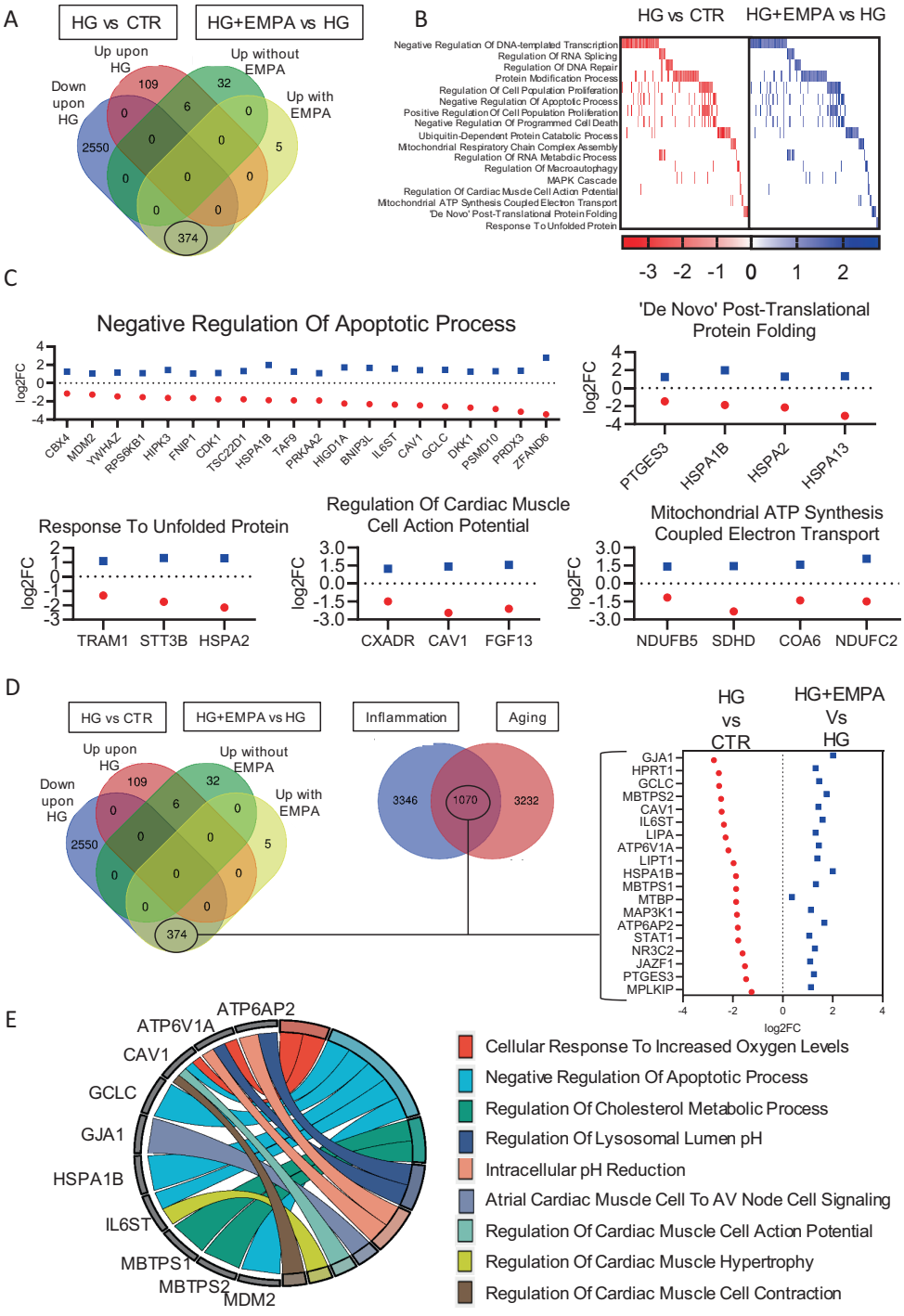


Figure 4 (*A*) Venn diagram showing the intersection between genes recovered upon empagliflozin (EMPA) treatment in high glucose (HG) conditions. (*B*) Heatmaps showing gene ontology analysis of biological processes from common differentially expressed genes downregulated upon HG exposure from the HG versus control (CTR) comparison (red) and upregulated with EMPA from the HG + EMPA versus HG comparison (blue). Data are reported as the fold-change from each comparison in log2. (*C*) Dot plots showing biological processes with opposite patterns of expression and related gene signature expression. Data are reported as the fold-change from each comparison in log2. (*D*) Venn diagrams showing the intersection between genes recovered upon EMPA treatment in HG conditions and the inflammaging signature. Dot plot showing significant genes involved in inflammaging reduced with HG and recovered upon EMPA treatment. Data are reported as the fold-change from each comparison in log2. (*E*) Gene ontology chart indicating significant biological processes associated with common genes involved in inflammaging.

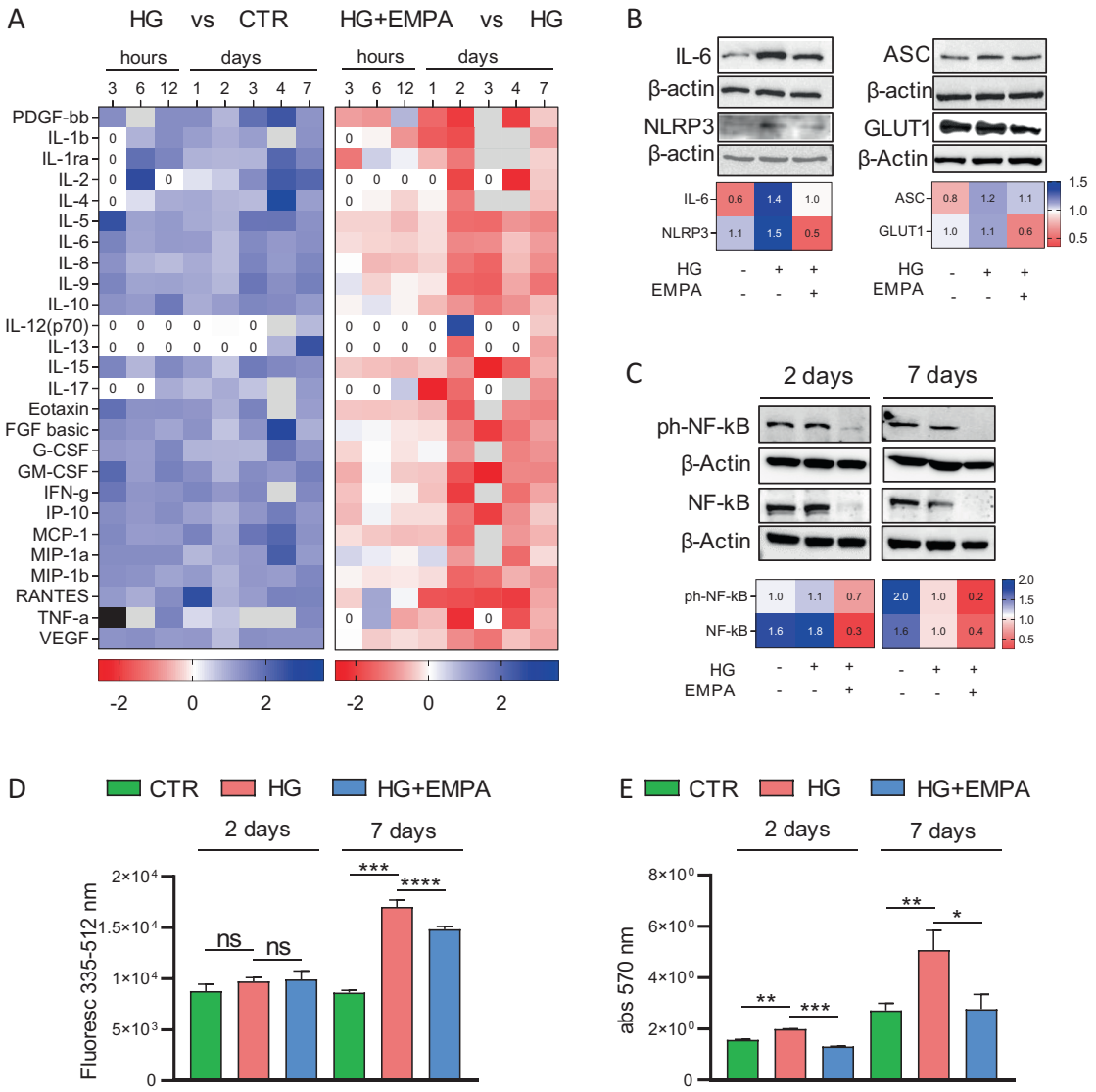


Figure 5 (*A*) Heatmaps illustrating cytokine content in a time course setting (3, 6, and 12 h and then 1, 2, 3, 4, 7 days) upon high glucose (HG) exposure with/without empagliflozin (EMPA). Data are reported as log2 ratio normalized on protein content. (*B*) Western blot analysis of interleukin-6 (IL-6), NLRP3, ASC, and GLUT1 in normal and high glucose conditions for days, untreated or treated with EMPA at 0.5 μM. (*C*) Western blot analysis of total and phosphorylated form of NF-κB in normal and high glucose conditions for 2 and 7 days, untreated or treated with EMPA at 0.5 μM. (*D*) Histogram showing Fura-2 AM fluorescence values at a wavelength of 335 nm for excitation and 512 nm emission resulting from HG exposure with/without EMPA for 2 and 7 days. Statistical significance was calculated with unpaired *t*-test and is reported as follows: *<0.05, **<0.01, ***<0.001. (*E*) Histogram showing fatty acid accumulation as absorbance values at 570 nm resulting from HG exposure with/without EMPA for 2 and 7 days. Statistical significance was calculated with unpaired *t*-test and is reported as follows: *<0.05, **<0.01, ***<0.001.

treatment upon SIRT1 activation in HG conditions, whereas compensatory glycolysis and post-2-deoxyglucose acidification were both significantly reduced (*Figure 6C*). Total ATP production did not change significantly (*Figure 6D*), such as spare respiratory capacity, non-mitochondrial oxygen consumption, proton leak, and coupling efficiency (*Figure 6E*). These findings indicate that EMPA improves mitochondrial function by reducing glycolysis, and that the SIRT1 activator, when present, exerts an additive or synergistic effect.

Empagliflozin tempers endoplasmic reticulum oxidative stress response in high glucose conditions and its action is boosted by sirtuin 1 activation

Western blot analysis revealed increased expression of the phosphorylated form of eIF2α, crucial for initiating translation in response to environmental stress, under HG conditions, indicating

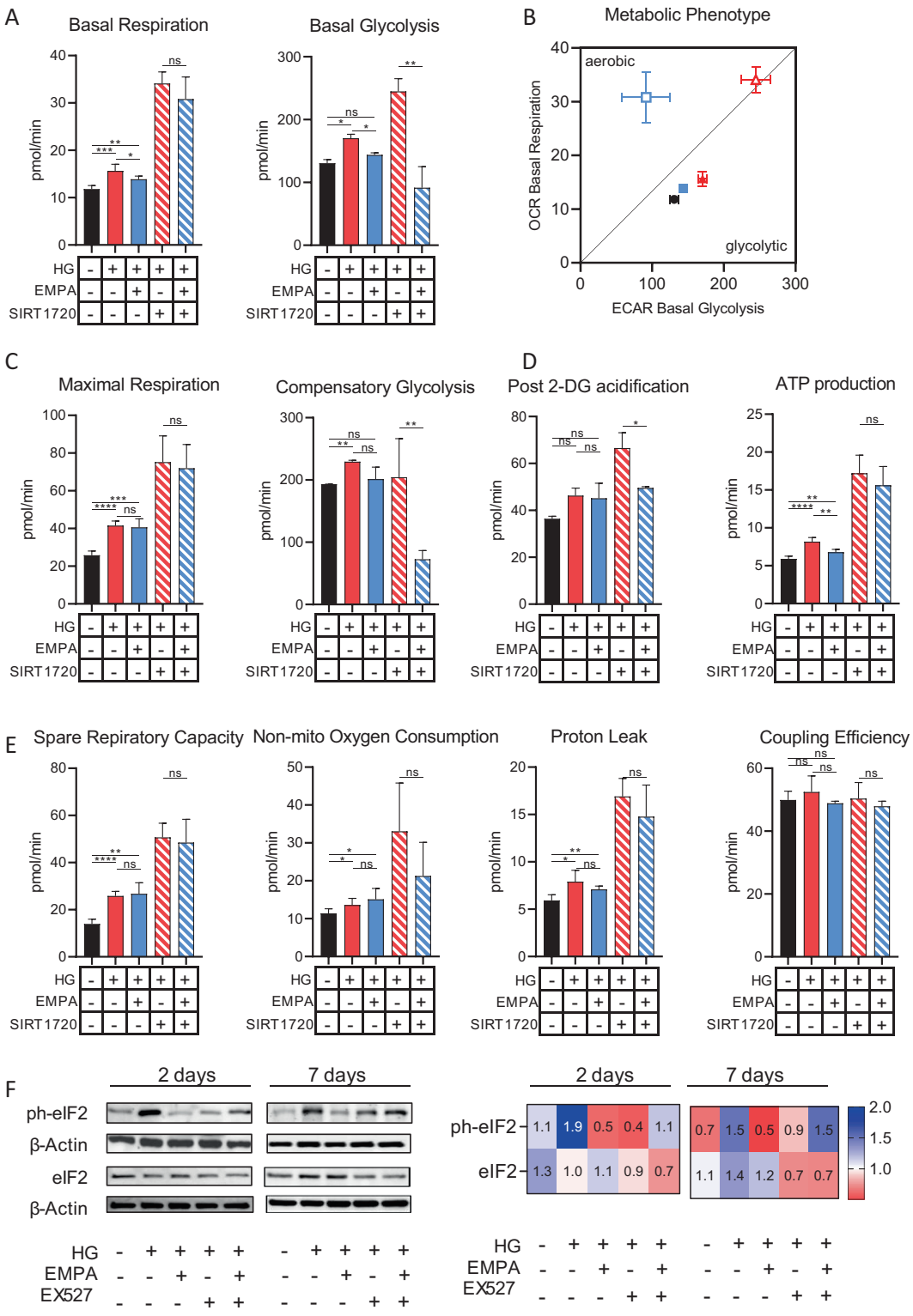


Figure 6 (*A–E*) Metabolic parameters based on oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) values upon high glucose (HG) exposure with/without empagliflozin (EMPA). Statistical significance was calculated with unpaired *t*-test and is reported as follows: *<0.05, **<0.01, ***<0.001. Metabolic phenotypes based on basal respiration and glycolysis. (*F*) Western blot analysis of total and phosphorylated form of eIF2α in AC16 cells in normal and high glucose conditions for 2 and 7 days, untreated or treated with EMPA at 0.5 μM, EX-527 at 10 μM, and combo treatment (EMPA 0.5 μM + EX-527 10 μM).

elevated stress (*Figure 6F*). Conversely, EMPA treatment reduced phosphorylation, restoring conditions similar to the control group. To further validate the role of SIRT1 in adaptation to nutrient stress, we used the well-known SIRT1 inhibitor EX-527, alone or in combination with EMPA. The results showed that EX-527 counteracted the effects of EMPA treatment, leading to increased levels of eIF2α phosphorylation. This finding highlights the functional interplay between EMPA and SIRT1, underscoring their cooperative role in alleviating endoplasmic reticulum stress. In response to increased phosphorylated eIF2α (p-eIF2α) levels, which impair translational initiation, stress granules (SGs), composed of untranslated mRNAs and associated proteins, are formed. We assessed SG production using confocal microscopy and immunofluorescence to monitor G3BP1 and TIAR key markers of SG formation. HG conditions did not contribute to SG accumulation, regardless of acute or chronic exposure (online supplementary *Figure S3*). However, our data on metabolic activity and cellular stress response strongly suggest that EMPA positively mitigates the harmful effects of hyperglycaemia. Additionally, EMPA treatment, enhanced by SIRT1 activation, shows potential for restoring metabolic balance following stress induced by hyperglycaemic conditions.

Discussion

In this study, we investigated the anti-inflammatory properties of EMPA in T2DM patients using a three-step approach (*Graphical Abstract*). Firstly, we performed a meta-analysis of clinical trials investigating the effect of EMPA in T2DM patients. Secondly, we thoroughly investigated the impact of EMPA on structural and functional changes in human AC16 cardiomyocytes exposed to HG, simulating both acute (2 days) and chronic (7 days) diabetic conditions. Lastly, we intersected our results with a published available dataset of T2DM patients with HF. This data integration approach underscored a positive correlation between DGEs and the anti-inflammatory effects of EMPA, reflecting what we observed in the meta-analysis for T2DM patients treated with this SGLT2i. By reducing IL-6, TNF-α, and CRP, circulating biomarkers of systemic inflammation, EMPA induced an anti-inflammatory effect describing the protective effect on clinical outcome. Experimentally, exposure of cardiomyocytes to HG was detrimental to controlling biological processes, interfering with transcriptional regulation and the acquired expression profile. *ZFAND6* was strongly downregulated at mRNA level upon HG exposure. Very few studies have explored this target, which is known to be involved in cardiomyocyte apoptotic processes. *ZFAND6* is in fact a negative regulator of the apoptotic process and regulates I-κB kinase/NF-κB signalling, preventing inflammation triggered by mitochondrial damage/impaired mitochondrial homeostasis.[44] However, its expression was found downregulated after myocardial infarction,[45] with an altered methylation profile in T2DM. *ZFAND6* was also associated with the downregulation of insulin secretion in T2DM *in vitro* pancreatic cells,[46] with a polymorphic variant rs11634397 predisposing to pre-diabetes and T2DM susceptibility.[47] Based on our RNA-seq data, the downregulation of *ZFAND6* under HG conditions confirmed the activation of pro-inflammatory pathways in hyperglycaemic conditions in cardiac tissue, while its upregulation upon EMPA treatment confirmed the anti-inflammatory efficacy of this SGLT2i. The same regulatory trend was also observed for other genes, including *PFN2* and *TRPV1*, both involved in stress response processes and cardiovascular diseases.[38,48,49] To corroborate our observations and translate our findings into a clinically relevant context, we compared our RNA-seq data with a publicly available dataset for non-end-stage failing hearts, specifically contrasting D-HF patients with similar haemodynamic impairment. Indeed, diabetic cardiomyopathy and post-ischaemic dilated cardiomyopathy in diabetic patients represent conditions with a common denominator: the metabolic damage caused by hyperglycaemia.[50] This integrated analysis of our RNA-seq with publicly available dataset data revealed that HG conditions in cardiomyocytes *in vitro* and D-HF conditions share a cohort of similarly expressed genes. A subset of this group of newly identified genes can be modulated by EMPA treatment and plays a fundamental role in cardiac function, such as PFN2,[38] UGCG,[39] and FAM3C.[40] Given the functional perspective that hyperglycaemia may systemically induce inflammation and aging processes, and directly in cardiac tissue, we sought to determine the extent of overlap between genes regulated by NG, HG, and HG + EMPA conditions and the inflammaging signature. Strikingly, we observed a substantial overlap, showing that the mechanisms emphasized by EMPA are indeed associated with the inflammatory process and direct cardiac homeostasis (e.g. metabolism, replicative capacity, calcium signalling, and reduction of oxidative stress). Overall, our data corroborate and strengthen the role of glycaemic stress as a driving force for biological function impairment in cardiomyocytes and, in parallel, underscore how EMPA is able to transcriptionally regulate cardiomyocytes, almost nullifying the effect of HG. We also evaluated the functional impact of EMPA on cardiac tissue, beyond its transcriptional effects. Our *in vitro* experiments, complementary to single-cell RNA-seq analysis, substantiated the direct influence on the release of inflammatory cytokines and the expression of proteins involved in inflammasome assemblies, such as NLRP3, ASC, and IL-6.[51] EMPA also induced the release of cellular calcium and caused a decrease in the intracellular accumulation of FAs, accompanied by a decrease in NF-κB and its phosphorylated form. Acting as a second messenger, Ca^{2+} initiates signalling cascades in response to stress stimuli, thereby contributing to inflammation. The accumulation of Ca^{2+} in the cardiac system during stress conditions is tightly regulated, influencing myocardial contractility and facilitating adaptation to heightened workload.[52] However, Ca^{2+} accumulation in hyperglycaemic conditions can impair mitochondrial function, leading to reduced ATP production and increased release of reactive oxygen species, which contribute to HF.[53] A similar dual role is exhibited by FAs. While lipid metabolism is crucial for cardiomyocytes, under hyperglycaemic conditions, excessive FAs are associated with an elevated risk of cardiovascular disease.[54] In our model, FA levels increase under hyperglycaemic conditions, and EMPA effectively reduces FA accumulation, particularly after 7 days of HG exposure. Consequently, EMPA exerts a protective effect against lipotoxicity.[55]

This study also investigated the impact of EMPA on SIRT1 modulation. Sirtuins, including SIRT1, are nutrient-sensitive metabolic sensors,[56] which might also have a role in enhancing cardiac metabolism in hyperglycaemic conditions. The EMPA-induced

reduction in glycolytic function was enhanced when SIRT1 was activated, increasing aerobic respiration. Notably, maximal respiration remained unchanged, while compensatory glycolysis was reduced. To further corroborate the role of SIRT1 in nutritional stress adaption, we showed that blocking SIRT1 is able to counteract the anti-stress effects mediated by EMPA. Indeed, in HG conditions SIRT1 inhibition restores oxidative stress indicators, specifically eIF2 phosphorylation. These findings highlight the functional relationship between EMPA and SIRT1 activation, corroborating their synergistic role in reducing oxidative stress in cardiac cells. EMPA is then emerging as a promising therapeutic agent for comprehensively modulating inflammatory biomarkers, and particularly for attenuating cardiac tissue damage associated with elevated glucose levels in T2DM patients. This investigation provides valuable insights supported by robust mechanistic evidence for optimizing HF treatment strategies and elucidates the complex interrelationship between diabetes, inflammation, and cardiovascular diseases.

Study limitations

The observed direct effects of specific organs on systemic inflammation underscore the need for a more sophisticated investigational model than what was employed. We constrained our focus to cardiomyocytes and the chronic pathology of diabetes, potentially oversimplifying the complexity of systemic interactions. Furthermore, the absence of commercially available human cardiomyocyte cell lines, aside from AC16, limits our ability to validate findings across different *in vitro* human models. Additionally, the literature predominantly features data from liquid biopsies or untreated tissues, with only a limited number of studies providing expression profiles of cardiomyocytes exposed to hyperglycaemia in *ex vivo* patient samples. This scarcity of relevant data hampers more comprehensive comparative analyses.

Supplementary Information

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

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