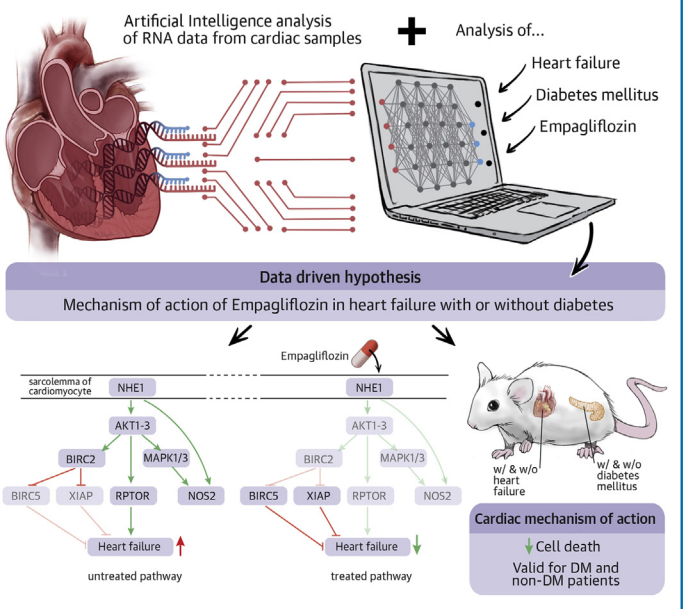
JACC: BASIC TO TRANSLATIONAL SCIENCE VOL. 4, NO. 7, 2019

PRECLINICAL RESEARCH

Unraveling the Molecular Mechanism of Action of Empagliflozin in Heart Failure With Reduced Ejection Fraction With or Without Diabetes

Oriol Iborra-Egea, MSc,[a,b,c] Evelyn Santiago-Vacas, MD,[a,b,c] Salva R. Yurista, MD,[d] Josep Lupón, MD, PhD,[a,b,c] Milton Packer, MD,[e] Stephane Heymans, MD,[f] Faiez Zannad, MD,[g] Javed Butler, MD,[h] Domingo Pascual-Figal, MD,[i] Antonio Lax, PhD,[i] Julio Núñez, MD, PhD,[j] Rudolf A. de Boer, MD, PhD,[d] Antoni Bayés-Genís, MD, PhD[a,b,c]

VISUAL ABSTRACT



Iborra-Egea, O. et al. J Am Coll Cardiol Basic Trans Science. 2019;4(7):831–40.

HIGHLIGHTS

- **Using artificial intelligence, followed by in vivo validation, this study identified the key cardiac mechanism of action of empagliflozin in heart failure in patients with or without diabetes mellitus.**
- **The most robust mechanism of action involved the NHE-1 co-transporter with 94.7% accuracy.**
- **NHE-1 blockade by empagliflozin administration in rats restored the antiapoptotic activity of XIAP and BIRC5.**
- **The beneficial reduction in cardiomyocyte cell death after empagliflozin treatment is independent of the presence of diabetes mellitus.**
- **Empagliflozin could emerge as a new treatment for heart failure patients regardless of their glycemic status.**

ISSN 2452-302X https://doi.org/10.1016/j.jacbts.2019.07.010

JACC: BASIC TO TRANSLATIONAL SCIENCE VOL. 4, NO. 7, 2019
NOVEMBER 2019:831–40

ABBREVIATIONS AND ACRONYMS

ANN = artificial neural network

DM = diabetes mellitus

HF = heart failure

HFrEF = HF with reduced ejection fraction

MI-HF = post-infarct heart failure

NHE = sodium-hydrogen exchanger

RNAseq = RNA sequencing

SGLT2i = sodium-glucose co-transporter 2 inhibitor

SUMMARY

The mechanism of action of empagliflozin in heart failure with reduced ejection fraction (HFrEF) was deciphered using deep learning in silico analyses together with in vivo validation. The most robust mechanism of action involved the sodium-hydrogen exchanger (NHE)-1 co-transporter with 94.7% accuracy, which was similar for diabetics and nondiabetics. Notably, direct NHE1 blockade by empagliflozin ameliorated cardiomyocyte cell death by restoring expression of X-linked inhibitor of apoptosis (XIAP) and baculoviral IAP repeat-containing protein 5 (BIRC5). These results were independent of diabetes mellitus comorbidity, suggesting that empagliflozin may emerge as a new treatment in HFrEF. (J Am Coll Cardiol Basic Trans Science 2019;4:831–40)

Both type 1 and 2 diabetes mellitus (DM) are major risk factors for the development of cardiovascular diseases, increasing morbidity and mortality (1). For decades, long-term clinical studies of DM have shown value in reducing glycemic levels without a substantial effect on cardiovascular outcomes and certainly no benefit in heart failure (HF) (2). The advent of a new class of agents, sodium-glucose co-transporter 2 inhibitors (SGLT2i), heralds a new era and may represent a turning point. Indeed, in the EMPA-REG OUTCOME (Empagliflozin Cardiovascular Outcome Event Trial in Type 2 Diabetes Mellitus Patients) study, patients with type 2 DM who were at high risk for cardiovascular events and who received empagliflozin in addition to standard care showed lower rates of the following compared to those who received placebo: a) primary composite cardiovascular outcome (death from cardiovascular causes, nonfatal myocardial infarction (MI) or nonfatal stroke); and b) HF-related hospitalizations; death from cardiovascular causes; and death from any cause (3). Similar results with other SGLT2is have been reported subsequently by both randomized clinical trials and observational studies (4,5).

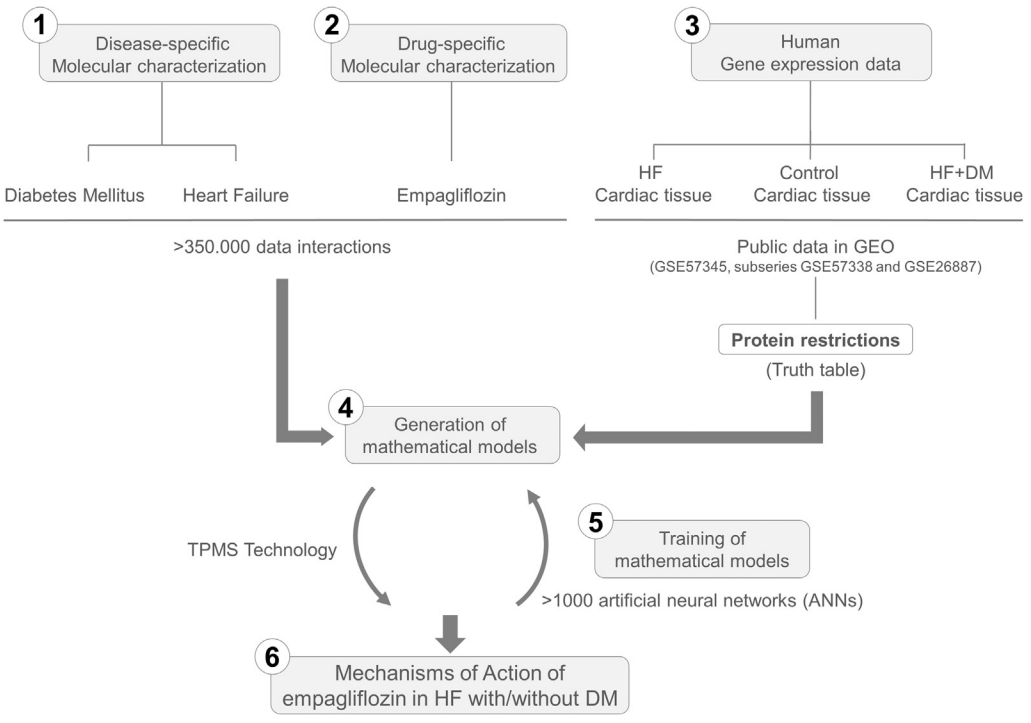
SEE PAGE 841

SGLT2 receptors are located in the proximal renal tubule and are responsible for 90% of glucose reabsorption into the bloodstream. Inhibition of SGLT2 receptors causes glucosuria and reduces glycemic levels (6). However, control of glycemic levels in response to SGLTis alone seems insufficient to explain the reported cardiovascular benefits. In fact, the EMPA-TROPISM (Are the "Cardiac Benefits" of Empagliflozin Independent of Its Hypoglycemic Activity? [ATRU-4]) trial (NCT03485222) is an ongoing trial that aims to elucidate whether the benefits obtained in the EMPA-REG OUTCOME trial were mediated, at least in part, by a glucose-

From the [a]Heart Institute, Hospital Universitari Germans Trias i Pujol, Badalona, Spain; [b]Department of Medicine, Universitat Autònoma de Barcelona, Barcelona, Spain; [c]Centro de Investigación Biomédica en Red Enfermedades Cardiovascualres (CIBERCV), Madrid, Spain; [d]Department of Cardiology, University Medical Center Groningen, University of Groningen, Groningen, the Netherlands; [e]Baylor Heart and Vascular Institute, Baylor University Medical Center, Dallas, Texas; [f]Maastricht University Medical Centre, Cardiovascular Research Institute Maastricht, Maastricht, the Netherlands; [g]Centre d'Investigation Clinique Plurithématique 1433, INSERM U1116, Université de Lorraine, Centre Hospitalier Régional et Universitaire de Nancy, French Clinical Research Infrastructure Network (F-CRIN), Investigation Network Initiative-Cardiovascular and Renal Clinical Trialists (INI-CRCT), Nancy, France; [h]Department of Medicine, University of Mississippi, Jackson, Mississippi; [i]Domingo Hospital Universitario Virgen de la Arrixaca, University of Murcia, Centro Nacional de Investigaciones Cardiovasculares, CIBERCV, Murcia, Spain; and the [j]Cardiology Department, Hospital Clínico Universitario, CIBERCV, INCLIVA, Universitat de València, València, Spain. Dr. Yurista is supported by Indonesia Endowment Fund for Education grant LPDP 20150722083422. Dr. Packer is a consultant with Abbvie, Amgen, Akcea, AstraZeneca, Bayer, Boehringer Ingelheim, Cardiorentis, Gilead, Johnson and Johnson, NovoNordisk, Pfizer, Sanofi, Synthetic Biologics, Relypsa, and Theravance. Dr. Zannad is a member of the Boehringer Ingelheim steering committee; and is a consultant for AstraZeneca, Mundipharma, NovoNordisk, and Merck. Dr. Butler is a consultant with Abbott, Adrenomed, Amgen, Array, AstraZeneca, Bayer, BerlinCures, Boehringer Ingelheim, Bristol-Myers Squibb, Cardiocell, Corvidia, CVRx, G3 Pharmaceutical, Innolife, Janssen, Lantheus, LinaNova, Luitpold, Medscape, Medtronic, Merck, Novartis, NovoNordisk, Relypsa, Roche, Sanofi, StealthPeptide, SC Pharma, V-Wave Limited, Vifor, and ZS Pharma. Dr. Núñez has received speaker fees from Boehringer Ingelheim. Dr. De Boer holds equity in scPharmaceuticals; has received honoraria from Mandalmed, Novartis, and Servier; and has received research support through his institution from AstraZeneca, Abbott, Bristol-Myers Squibb, Novartis, Roche, Trevena, and ThermoFisher GmbH. All other authors have reported that they have no relationships relevant to the contents of this paper to disclose. The authors attest they are in compliance with human studies committees and animal welfare regulations of the authors' institutions and Food and Drug Administration guidelines, including patient consent where appropriate. For more information, visit the *JACC: Basic to Translational Science* author instructions page.

Manuscript received May 20, 2019; revised manuscript received July 9, 2019, accepted July 9, 2019.

FIGURE 1 Flow Chart Depicting the Machine Learning Approach Used in This Study



See Supplemental Video 1. DM = diabetes mellitus; GEO = Gene Expression Omnibus database; HF = heart failure; TPMS = therapeutic performance mapping system.

independent mechanism (7). A recent viewpoint postulated the hypothesis that the benefit of SGLTis in HF may be mediated by the sodium-hydrogen exchanger (NHE) rather than by the effect on glucose reabsorption (8); however, a comprehensive mechanistic explanation of the extra-renal cardioprotective SGLTi effects remains elusive.

Machine learning is an interdisciplinary field based on computational and mathematical models that aims to unravel key interactions within complex biological networks (9). It is particularly well suited for investigating the mechanisms underlying the effects of drugs, including SGLTis. Accordingly, this study used deep learning analysis to investigate what the key cardiac mechanisms may be by which empagliflozin exerts its effects in HF and reduced ejection fraction (HFrEF) in patients with or without DM (Figure 1). Machine learning-driven pathways were validated in an in vivo empagliflozin-treated post-infarct HF (MI-HF) rat model.

METHODS

MACHINE LEARNING IN SILICO ANALYSES. Molecular characterization of HF and DM pathology and drug treatments. First, extensive and careful review of full-length articles in PubMed was conducted to obtain information about HF and DM. When specific molecular information describing the condition was found, the articles were thoroughly reviewed to identify protein/gene candidates to feed the mathematical models. When the evidence of the implication of a candidate in the condition was judged inconsistent enough to be assigned as an effector, an additional search was performed specifically for the candidate, including all the protein names according to UniProtKB (UniProt Knowledge Base, Geneva, Switzerland). No text-mining tools were used to avoid intrinsic technical biases, and only English-language articles were included, which accounted for more than 6,509 items reviewed.

Moreover, empagliflozin pharmacological data and molecular relationships described for HF and DM were extracted by integrating massive, publicly available databases such as Drugbank, Reactome, MINT, and BioGrid. All this information accounted for more than 350,000 interactions (Figure 1). Briefly, by integrating published data, a set of molecular profiles characterizing HF and DM were defined, which were used to build the protein network and the mathematical model.

Compilation of transcriptomic data. Next, an RNA sequencing (RNAseq) dataset (public data available in Gene Expression Omnibus [GEO], accession GSE57345 and subseries GSE57338 and GSE26887) of myocardial tissue data derived from HFrEF patients with (10) or without DM (11) was thoroughly analyzed to model the molecular effects of empagliflozin in each scenario. Gene information was mapped one-to-one to proteins for introduction into the protein network, which included 280 proteins of interest (Supplemental Table 1). This collection of experimental pathophysiological signals was used as a list of principles, termed a "truth table," which apply to a specific condition (i.e., some proteins are overexpressed only in HF patients with DM) (**Figure 1**) and allow delineation of the molecular behavior of each situation (12).

Generation of mathematical models. Finally, artificial neural network (ANN)-based analyses were used to identify the most relevant pathophysiological processes implicated in HF and DM. To train the mathematical models, this large collection of well-established pathophysiological signals and clinical information relevant to the pathologies under study was reviewed. The molecular description of these input-output signals was investigated as follows:

1. Model inputs, for example, information about drugs, pathologies, and protein/gene relationships provided by public databases to assess inhibition or activation of 1 or more nodes of the protein network (their targets) triggering a perturbation through the system.
2. Model outputs, for example, experimental RNAseq data (upregulated or downregulated genes/proteins) and clinical information.
3. Hidden mechanisms of action (MoAs). Using the model's input and output data, mathematical algorithms were generated to trace a change or perturbation from 1 to another, elucidating the MoAs that would explain this connection.

The models are then able to weight the relative value of each protein (node) relationship.

Therapeutic performance mapping system technology. Details about how therapeutic performance mapping system (TPMS) (Anaxomics, Barcelona, Spain) machine learning analyses work have been reported previously (13). Briefly, TPMS incorporated massive amounts of biological information relevant to both the disease(s) and the drug under study, drawn from curated reports and from public and private databases (**Figure 1**). Next, TPMS adopted the gene expression dynamics stored in the truth table as a reference framework to shape all the biological information. Finally, TPMS generated mathematical models to form complex relationships between datasets or to find patterns in the data (14,15). The system built an ANN, which was trained with 1,000 iterations. During this process, the models generated different MoAs, which progressively increased in accuracy by correcting itself with already-known clinical and biomedical information, and cross-validation with independent sets of data (**Figure 1**, Supplemental Video 1). Eventually, the algorithms reached a plateau which yielded the final accuracy performance (maximum = 100%) to explain the MoA of empagliflozin in HFrEF (**Figure 1**).

POST-INFARCT HEART FAILURE IN VIVO RAT MODEL. A total of 74 rats were used for the experimental validations. The nondiabetic, post-infarct heart failure (MI-HF) study was performed in male Sprague Dawley rats weighing 250 to 280 g, obtained from Envigo (Huntingdon, United Kingdom). The diabetic MI-HF study (MI-HF-DM) was performed in male Wistar rats weighing 160 to 200 g obtained from Envigo (Barcelona, Spain).

Animals were housed in groups under standard laboratory condition of 12L:12D cycle. All animals received food and water ad libitum during the study (n = 7 to 12 per group). All experiments were conducted in accordance with U.S. National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Committee for Animal Experiments.

Induction of diabetes mellitus in Wistar rats. DM was induced by a single intraperitoneal injection of streptozotocin (STZ) (50 mg/kg of body weight) (catalog number S0130, Sigma-Aldrich, St. Louis, Missouri) dissolved in freshly prepared 0.1 M citrate buffer (pH 4.5, catalog number 71402, Sigma-Aldrich). In order to prevent hypoglycemia in the first 24 h after STZ injection, rats were allowed to have free access to water with 5% glucose. Three days after STZ injection, rats with blood glucose levels >300 mg/dl were considered diabetic.

Induction of myocardial infarction. Initially, animals were anesthetized using intraperitoneal ketamine (75 mg/kg) and medetomidine (0.5 mg/kg) before being intubated and ventilated under 2% isoflurane anesthesia. Rats were randomly allocated to MI or sham surgery, as described elsewhere (16). Left-sided thoracotomy was performed by a small incision between the third and fourth intercostal spaces. The pericardial sac surrounding the heart was cut open, but the heart was not exteriorized. The infarction was performed by ligation of the left anterior descending coronary artery. Visible blanching and cyanosis of the anterior wall of the left ventricle and swelling of the left atrium were taken as

indicative of successful ligation. Sham surgery was identical to the MI surgery but without any ligation.

Experimental design and study protocol. Two weeks after surgery, nondiabetic rats (n = 40) were fed either with standard rat chow (product R/M-H V1534-70, Ssniff-Spezialdiäten, Soest, Germany) or chow containing empagliflozin (product BI 10773, in concentration of 200 mg/kg per day to reach a dose of 30 mg/kg per day), and this was continued for a period of 12 weeks. After 12 weeks, hearts were rapidly excised, weighed, and processed for further analysis. Animals were divided into 4 groups: group 1 (n = 7) were sham rats receiving vehicle treatment; group 2 (n = 10) were sham rats receiving empagliflozin; group 3 (n = 12) were infarcted rats receiving vehicle treatment; group 4 were infarcted rats receiving empagliflozin.

Immediately after diabetes was induced, the Wistar rats were treated with empagliflozin (10 mg/kg per day) (n = 34). This treatment was maintained for 4 weeks before MI was induced and was continued for a period of 4 weeks after MI. After 4 weeks, hearts were rapidly excised, weighed, and processed for further analysis. Animals were divided into 4 groups: group 1 (n = 8) were diabetic sham rats receiving phosphate buffered saline (PBS) for 56 days; group 2 (n = 9) were diabetic sham rats receiving empagliflozin for 56 days; group 3 (n = 7) were diabetic infarcted rats receiving PBS for 56 days; group 4 (n = 10) were diabetic infarcted rats receiving empagliflozin for 56 days.

Real time PCR. Total RNA from tissues were isolated by the TRIzol RNA isolation protocol. cDNA was further isolated from RNA by using the QuantiTecht RT kit (Qiagen, Venlo, the Netherlands), following the manufacturer's instructions. Quantitative real-time polymerase chain reaction (qRT-PCR) was performed in a model CFX384 real-time system (Bio-Rad, Philadelphia, Pennsylvania) using SYBR Green mix (Thermo Fisher Scientific, Waltham, Massachusetts) was used to determine the relative gene expression. Gene expression was determined by correcting the reference gene (*36B4*), and the calculated values are expressed relative to the control group per experiment. The primers for BIRC5 and XIAP qRT-PCR used in this study can be found in Supplemental Table 2.

DATA INTEGRATION AND STATISTICAL ANALYSIS. The RNAseq data were compiled and processed using the GEO2R tool, using the neqc method for normalization, allowing identification of differential expression levels and calculation of fold changes. The p values obtained for each RNA probe were adjusted using the Benjamini-Hochberg false discovery rate at a significance level of 0.01. Only genes with an adjusted p value of <0.01 and log fold changes of >0.25 were considered. In the in vivo analysis, gene expression was determined by correcting the reference gene (*36B4*), and the calculated values were expressed relative to the control group per experiment. The $2\text{-}\Delta\Delta_{CT}$ method for comparing relative expression results between treatments in real-time PCR was applied. To compare normally distributed parameters, one-way analysis of variance (ANOVA) followed by Tukey post hoc test was used. When data were not normally distributed, a nonparametric Kruskal-Wallis test followed by a Mann-Whitney *U* test with correction for multiple comparisons was used. To compare empagliflozin with vehicle treatment, an independent *t*-test or a Mann-Whitney *U* test was used where appropriate. Two-sided test results yielding a p value of <0.05 were considered statistically significant. SPSS statistics (version 23.0, IBM, Armonk, New York) for Windows (Microsoft, Redmond, Washington) was used to perform all statistical analysis.

TABLE 1 Mechanisms Involved in Heart Failure and Associated Conditions Considered in the Mechanistic Study

Pathology	Pathological Mechanisms	Proteins Implicated*
Heart failure		**106**
	Heart hypertrophy	33
	Cardiomyocyte cell death	47
	Inefficient myocardial fuel metabolism	7
	Oxidative stress	8
	Inflammation	11
Obesity		**140**
	Hyperphagia and dysregulated appetite	135
	White adipose tissue formation	5
Hypertension		**42**
	Retention of sodium	42
Hyperuricemia		**16**
	Decreased renal excretion of uric acid	16

Values are n. **Bold** numbers indicate the total number of proteins implicated in the general condition, later dissected by more specific motives. *Number of proteins implicated in each mechanism.

RESULTS

EMPAGLIFLOZIN ACTS UPON A SPECIFIC HF-RELATED PATHOLOGICAL SIGNATURE. First, the protein signatures of HFrEF and empagliflozin were defined and an ANN analysis identified the processes most likely associated with the beneficial effects of empagliflozin observed in HFrEF (Supplemental Table 1). Obesity, hypertension, and hyperuricemia contributed the most to the HF pathological signature and encompassed the possible effects of empagliflozin, with 140, 42, and 16 proteins of interest, respectively. For each of these 3 disorders, a series of mechanisms and the involved proteins were

TABLE 2 Proteins Known to Be Modulated by Empagliflozin

Gene Name	Protein Name	Reference (PMID)
STAT3	Signal transducer and activator of transcription 3	29311992
NOS2	Nitric oxide synthase, inducible	29311992
IL6	Interleukin-6	29311992
BDH1	D-beta-hydroxybutyrate dehydrogenase, mitochondrial	27289126
IFNG	Interferon gamma	29311992
ALDH2	Aldehyde dehydrogenase, mitochondrial	29311992
GCG	Glucagon	26590679
INS	Insulin	27289126
ACE2	Angiotensin-converting enzyme 2	26880444
BDNF	Brain-derived neurotrophic factor	25344694
HDAC1	Histone deacetylase 1	27829948
HDAC2	Histone deacetylase 2	27829948
HDAC3	Histone deacetylase 3	27829948
HDAC8	Histone deacetylase 8	27829948

PMID = unique identifier number used in PubMed for each article.

established (Table 1). The efficacy analysis identified the 3 following complementary strategies: 1) Consider sodium/glucose cotransporter 2, Na(+)/glucose cotransporter 2 (SGLT2); 2) consider sodium/hydrogen exchangers 1 and 3 (NHE1 and NHE3); and 3) considering empagliflozin bioflags; those proteins known to be modulated by the drug (Table 2).

ANN EVALUATES THE RELATIONSHIPS AMONG PROTEIN SETS INSIDE THE NETWORK, PROVIDING A PREDICTIVE SCORE. Once the pathological signatures were identified, the possible relationships between empagliflozin and heart failure, including associated processes, were evaluated by ANN analyses. Thus, the study of the MoA of empagliflozin was focused on the following specific pathways (motives) of the diseases affected by the treatment:

1) None of the evaluated diseases and motives are strongly related to SGLT2 according to the established criterion ($p > 0.3$).
2) Considering NHE1 and NHE3 as effectors, 2 conditions appear to be highly related (>75%) to empagliflozin's complete target profile:
   Hypertension: retention of sodium is the main motive identified, with a predictive score of 80%.
   HF: heart hypertrophy and cardiomyocyte cell death are the main motives identified, with a predictive score of 75.6%.
3) Three conditions appear to be highly related (>75%) to empagliflozin's bioflags:
   Hyperuricemia: decreased renal excretion of uric acid is the motive most related to empagliflozin, with 77% of predictive score.
   Obesity: the most related motives are hyperphagia and dysregulated appetite and white adipose tissue formation.
   HF: the most related motives are energy-inefficient myocardial fuel metabolism, oxidative stress, and inflammation.

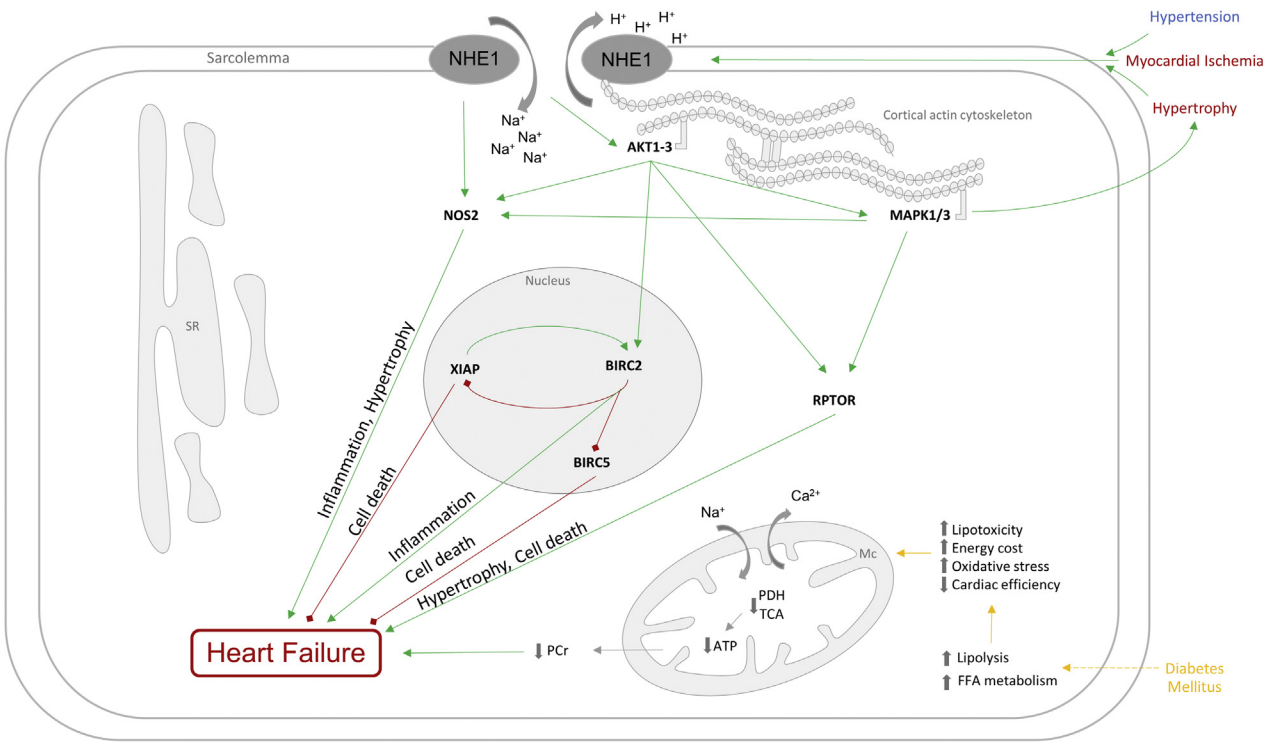
NHE1 IS RESPONSIBLE FOR THE CARDIAC EFFECTS OF EMPAGLIFLOZIN IN HFrEF WITH OR WITHOUT DM. By using the differentially expressed proteins found to be related to HF and the specific motives affected during the pharmacological treatment, the mathematical models determined the molecular mechanisms involved in the heart-focused beneficial effects of empagliflozin relative to heart failure in DM and non-DM patients.

The most robust MoA identified in this study involved the cardiac sodium/hydrogen exchanger 1 (NHE1) with 94.7% accuracy (Figure 2, Supplemental Table 3), which was similar for DM and non-DM patients, indicating a DM-independent mechanism (Table 3). This approach describes mainly the mitigation of cell death but also the prevention of heart hypertrophy and the improvement upon an inefficient myocardial fuel metabolism as the potential heart-mediated mechanisms by which empagliflozin may counteract heart failure.

Direct empagliflozin-driven NHE1 blockade ameliorates cardiomyocyte cell death. In this scenario, the amelioration of cardiomyocyte cell death was established as the main driver of empagliflozin beneficial effects in HFrEF. In the absence of empagliflozin, protein kinases B (AKTs; referred to as AKT1 to -3 in Figure 2) were identified as downstream effectors of NHE1 activation responsible for the induction of baculoviral IAP repeat-containing protein 2 (BIRC2), which in turn induced degradation of proteasome-mediated X-linked inhibitor of apoptosis (XIAP) and BIRC5 and prompted the progression of HFrEF. In empagliflozin-enriched models, by inhibiting NHE1, repressed AKT1-3 and BIRC2 allowed the expression of the antiapoptotic mediators XIAP and BIRC5, ultimately halting HFrEF progression.

To validate these data obtained in silico, mRNA levels of XIAP and BIRC5, as last effectors of the pathway, were examined in vivo in an empagliflozin-treated MI-HF rat model. Confirming in silico findings, gene expression of *XIAP* and *BIRC5* fell 45% and 36%, respectively, in vehicle-treated MI-HF animals compared to sham controls ($p < 0.01$ for both, respectively). Remarkably, empagliflozin-treated MI-HF animals without diabetes showed upregulation of *XIAP* and *BIRC5* mRNA expression similar to that in sham controls and significantly higher than vehicle-treated MI-HF counterparts ($p = 0.003$ and $p = 0.05$, respectively) (Figure 3). More importantly, these changes in gene expression occurred ubiquitously regardless of DM presence, further strengthening the

FIGURE 2 **NHE1-Identified Signaling Pathways in Cardiomyocytes in Heart Failure**



Each relationship represents a mechanism that may directly or indirectly (via downstream effectors) impact on HF, either through the activation (green arrows) or inhibition (red arrows) of downstream proteins. AKT1 = RAC-alpha serine/threonine-protein kinase 1; AKT2 = RAC-beta serine/threonine-protein kinase 2; AKT3 = RAC-gamma serine/threonine-protein kinase 3; ATP = adenosine triphosphate; BIRC2 = baculoviral IAP repeat-containing protein 2; BIRC5 = baculoviral IAP repeat-containing protein 5; LEP = leptin; MAPK1 = mitogen-activated protein kinase 1; MAPK3 = mitogen-activated protein kinase 3; MC = mitochondria.;NHE1 = sodium/hydrogen exchanger 1; NOS2 = nitric oxide synthase = inducible; PCr = phosphocreatine. ; PDH = pyruvate dehydrogenase; RPTOR = regulatory-associated protein of mTOR; SR = sarcoplasmic reticulum; TCA = tricarboxylic acid; XIAP = E3 ubiquitin-protein ligase XIAP.

in silico predictions. Indeed, empagliflozin-treated MI-HF-DM animals showed upregulation of *XIAP* and *BIRC5* mRNA expression similar to sham controls and significantly higher than vehicle-treated MI-HF-DM counterparts ($p = 0.01$ and $p = 0.005$, respectively) (**Figure 3**). Thus, empagliflozin-driven NHE1 inhibition ultimately counteracted reduced function of *XIAP* and *BIRC5*.

Additionally, the analyses also suggested that empagliflozin could further ameliorate cardiomyocyte cell death by inhibiting the AKT-dependent regulation- associated mTOR protein (RPTOR) and by down-regulation of nitric oxide synthase (NOS2)-inducible actions (**Figure 2**).

DISCUSSION

The EMPA-REG OUTCOME trial reported that empagliflozin exerted cardiovascular benefits which did not depend on its effects on blood glucose. This observation led to the hypothesis that empagliflozin exerted an effect on the myocardium independent of its inhibition of SGLT2 in the kidneys, thereby benefiting HF patients (6). The exact mechanism remains unknown, but it was postulated to have a direct action on the heart. The present study used artificial intelligence and machine learning, further validated in an in vivo animal model, to investigate whether empagliflozin could exert direct effects on the heart and whether these effects were the same for patients with and without DM.

This study has 2 main findings. First, these results validate the fact that empagliflozin interacts with the cardiac Na^+/H^+ exchanger NHE1 directly and identifies a mechanistic pathway acting primarily by reducing cardiomyocyte cell death, the main effector of its cardioprotective effects. Indeed, the activity of cardiac NHE1 seems to be increased in HFrEF patients, and previous data in experimental models (17–21) showed that NHE1 inhibition attenuated cardiomyocyte injury, remodeling, systolic dysfunction, and ultimately HF. Second, these data suggest that

TABLE 3 Mathematical Models Generated in This Study*

Mathematical Models	Stimulus	Response	Restrictions
HF with DM	Cardiac NHE1	Cardiomyocyte cell death Hypertrophy Inflammation	Truth Table + GSE57345 subseries GSE57338 dataset
HF without DM	Cardiac NHE1	Cardiomyocyte cell death Hypertrophy Inflammation	Truth Table + GSE26887 dataset

*The stimulus, response, and restrictions included in each model are shown.

DM = diabetes mellitus; GSE designations = Gene Expression Omnibus accession and subseries; HF = heart failure; NHE1 = sodium-hydrogen exchanger-1.

the cardiac effects of empagliflozin are independent of the presence of DM.

Uthman et al. (22) reported that SGLT2i reduced cardiac cytosolic Na^+ and cytosolic Ca^{2+} concentrations by inhibiting NHE1 in mouse cardiomyocytes. Cardiac NHE1 activity and cytosolic Na^+ were measured in the presence of clinically relevant concentrations of empagliflozin (1 μmol/l), dapagliflozin (1 μmol/l), and canagliflozin (3 μmol/l). All 3 SGLT2is bound with high affinity to the extracellular Na+-binding site of NHE1 (23). Recently, Baartscheer et al. (23) proposed a possible relationship between empagliflozin and NHE1 inhibition in rats and rabbits. In their study, an increase in extracellular glucose produced an increase in cytosolic levels of Na^+ and Ca^{2+}, an effect that was inhibited by empagliflozin. The effects of empagliflozin were strongly reduced after cells were pre-treated with the NHE1 inhibitor cariporide. In addition, empagliflozin affected cytosolic Na^+ and NHE1 flux in the absence of extracellular glucose, which demonstrated its putative effects in normoglycemic conditions.

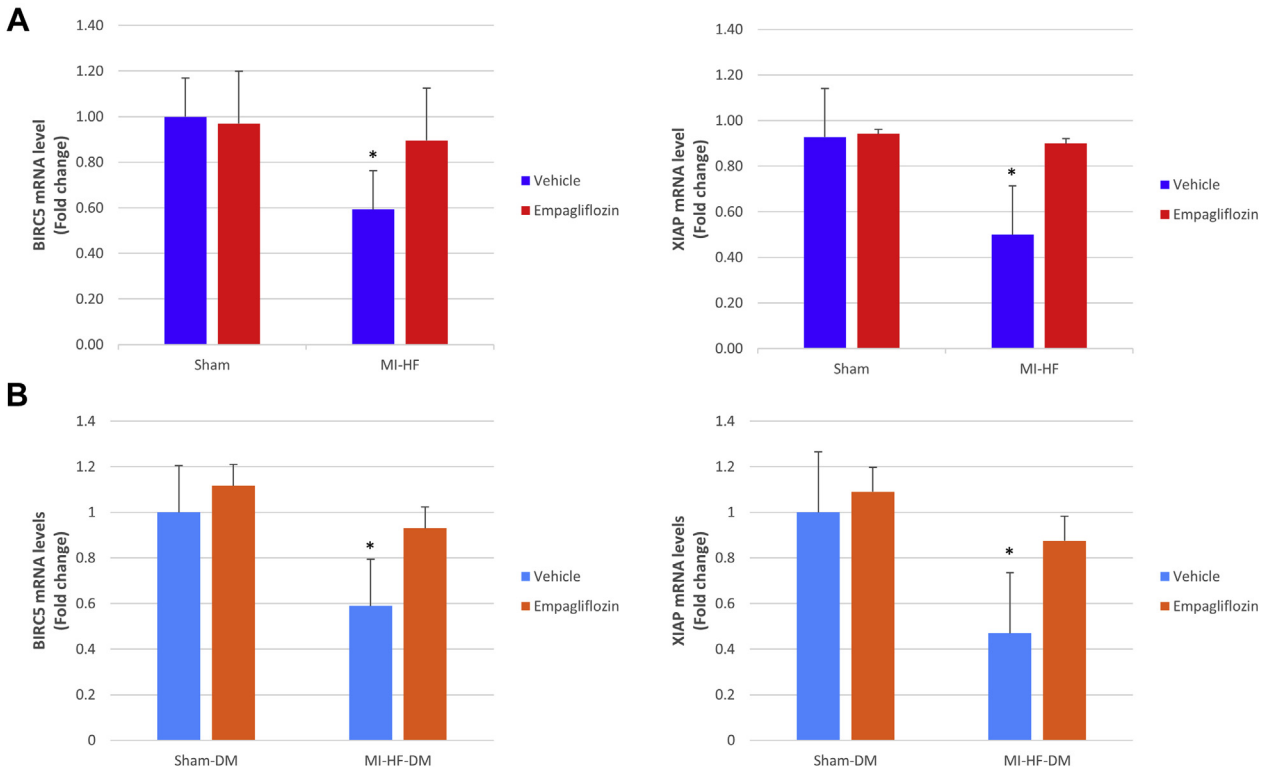
These mechanistic studies have the power to address specific mechanisms in detail, but the unravelling of the global molecular effects of empagliflozin in HFrEF with or without DM requires the integration of complex big data, best handled by artificial intelligence and deep learning analyses. Considering all possible empagliflozin-derived cardiac mechanisms, the present analysis pinpointed NHE1 modulation as the most likely effector of the beneficial effects. Moreover, amelioration of cardiomyocyte cell death was found to be the most important effect of empagliflozin in HF.

Most importantly, the algorithm predicted a modulation of AKTs resulting in an inability of BIRC2 to degrade the antiapoptotic factors XIAP and BIRC5. XIAP expression was found to be decreased in failing human hearts, suggesting that this potent inhibitor of apoptosis may play a key role in protecting the heart from cell damage (24,25). Using an adenoviral-associated vector, Piacentino et al. (24) increased XIAP expression in neonatal rat cardiomyocytes, which inhibited caspase-3/7 activity, showing a reduction of myocardial injury. Relative to BIRC5, Lee et al. (26) found that overexpression of this pathway may attenuate the progression of LV systolic dysfunction in doxorubicin-induced cardiomyopathy. Furthermore, Levkau et al. (27) showed that cardiac-specific deletion of BIRC5 pathway resulted in premature cardiac death caused by a dramatic reduction in total cardiomyocyte numbers. Again, using adenoviral vectors to restore this pathway (through restoration of survivin) expression in cardiomyocytes inhibited doxorubicin-induced apoptosis, induced DNA synthesis, and promoted cell cycle progression (27). Following this rationale and, to further confirm the present study's in silico data, mRNA levels of both *XIAP* and *BIRC5* were measured in an in vivo MI-HF rat model. In agreement with existing studies, both expression levels of XIAP and BIRC5 were substantially lower in vehicle-treated animals with dysfunctional myocardium. Remarkably, in the empagliflozin-treated groups, expression levels of both XIAP and BIRC5 returned to normal, thus they were able to exert their antiapoptotic effects.

The present study was designed to investigate whether the MoA of empagliflozin in HF in patients with DM was different from that in patients without DM. Surprisingly, the most robust MoA identified here was identical in both groups, suggesting that empagliflozin may have value in treating HFrEF patients regardless of their glycemic status, acting to ameliorate adverse cardiac remodeling. In other words, empagliflozin, currently indicated only for the treatment of DM, may eventually be repurposed to treat patients with HFrEF. Several ongoing randomized clinical trials are testing the value of SGLTi in HF patients (both with reduced and preserved ejection fraction) with and without DM.

The mathematical models reported here were robust, and each link described is validated in the existing research publications. However, there is an ongoing discussion about the mathematical algorithms used in artificial intelligence, often termed "black boxes," because sometimes it may not be easy to follow the determination of the output, no matter how good or reliable this may be. This study's analysis took a massive amount of collected information and analyzed it for hidden patterns in the data that would be otherwise inaccessible to human stand-alone analysis, to understand how the drug could lead to a specific clinical outcome. These algorithms are widely used in other fields, and the techniques are among the best understood and developed, with time-tested characteristics which ensure an accurate

FIGURE 3 mRNA Levels of Antiapoptotic Proteins XIAP and BIRC5 In Vivo



(A) Upper panels show nondiabetic rats. **(B) Lower panels** show diabetic rats. Data are mean ± SEM. *p < 0.05 vs. empagliflozin-MI and Vehicle-Sham. One-way ANOVA followed by Tukey post hoc test was used for normally distributed data, whereas Kruskal-Wallis followed by Mann-Whitney *U* test was used for non-normally distributed data. Abbreviations as in Figure 2.

application. A key differentiating feature of deep learning compared with other subtypes of artificial intelligence is the ability to operate without external guidance to draw conclusions. The neural network is not designed by humans, rather, the number of layers is determined by the data itself. Deep ANNs have used primarily supervised learning, with training from known patterns and labeled input data (truth table) to prospect reliable insights in large datasets. At the same time, all the outputs from the algorithm are restricted by the current scientific and medical standards. Taken together, all this limits human bias, and the algorithm can act as a hypothesis-free, independent data-driven analysis (28).

STUDY LIMITATIONS. However, this study has some limitations. The same premise that makes the models robust also serves as a potential downside. Specifically, the information considered here has already been described or uploaded in public repositories, which in turn limits the capacity of the analyses to incorporate data that have not been yet collected. The data reported here are valid for HFrEF and cannot be extrapolated to HF with preserved ejection fraction (HFpEF). Further research is required to better understand the MoA of empagliflozin in HFpEF.

CONCLUSIONS

Deep learning in silico analyses, together with in vivo validation, allowed deciphering the MoA of empagliflozin in HFrEF. Our data suggest that empagliflozin interacts and blocks the NHE1 co-transporter at the cardiomyocyte level, triggering a signaling cascade that halts detrimental cell death. These results were independent of DM comorbidity, suggesting that empagliflozin may emerge as a new treatment in HFrEF.

ACKNOWLEDGMENTS The authors thank Boehringer Ingelheim for providing animal control subjects and chow containing empagliflozin.

ADDRESS FOR CORRESPONDENCE: Dr. Antoni Bayes-Genis, Heart Institute, Hospital Universitari Germans Trias i Pujol, Carretera de Canyet s/n, 08916 Barcelona, Spain. E-mail: abayesgenis@gmail.com.

PERSPECTIVES

COMPETENCY IN MEDICAL KNOWLEDGE: The MoA of empagliflozin in HFrEF was deciphered by using deep learning in silico analyses together with in vivo validation. The most robust MoA involved the sodium-hydrogen exchanger-1 (NHE1), which was similar for diabetics and nondiabetics. Notably, direct NHE1 blockade by empagliflozin ameliorated cardiomyocyte cell death.

TRANSLATIONAL OUTLOOK: Empagliflozin, in addition to its renal effects, has direct cardioprotective effects at the cardiomyocyte level. These effects were independent of DM comorbidity, suggesting that empagliflozin may emerge as a new treatment in HFrEF.

REFERENCES

1. Emerging Risk Factors Collaboration, et al. Association of cardiometabolic multimorbidity with mortality. JAMA 2015;314:52–60.

2. Gerstein HC, Bosch J, Dagenais GR, et al., for the ORIGIN Trial Investigators. Basal insulin and cardiovascular and other outcomes in dysglycemia. N Engl J Med 2012;367:319–28.

3. Zinman B, Wanner C, Lachin JM, et al., for the EMPA-REG OUTCOME Investigators. empagliflozin, cardiovascular outcomes, and mortality in type 2 diabetes. N Engl J Med 2015;373:2117–28.

4. Wiviott SD, Raz I, Bonaca MP, et al., for the Sabatine MS; DECLARE-TIMI 58 Investigators. Dapagliflozin and cardiovascular outcomes in type 2 diabetes. N Engl J Med 2019;380:1881–2.

5. Cavender MA, Norhammar A, Birkeland KI, et al., for the CVD-REAL Investigators and Study Group. SGLT-2 inhibitors and cardiovascular risk: an analysis of CVD-REAL. J Am Coll Cardiol 2018;71:2497–506.

6. Zelniker TA, Braunwald E. Cardiac and renal effects of sodium-glucose co-transporter 2 inhibitors in diabetes: JACC State-of-the-Art Review. J Am Coll Cardiol 2018;72:1845–55.

7. Flores E, Santos-Gallego CG, Diaz-Mejia N, Badimon JJ. Do the SGLT-2 inhibitors offer more than hypoglycemic activity? Cardiovasc Drugs Ther 2018;32:213–22.

8. Packer M, Anker SD, Butler J, Filippatos G, Zannad F. Effects of sodium-glucose cotransporter 2 inhibitors for the treatment of patients with heart failure proposal of a novel mechanism of action. JAMA Cardiol 2017;2:1025–9.

9. Waltemath D, Wolkenhauer O. How modeling standards, software, and initiatives support reproducibility in systems biology and systems medicine. IEEE Trans Biomed Eng 2016;63:1999–2006.

10. Greco S, Fasanaro P, Castelvecchio S, et al. MicroRNA dysregulation in diabetic ischemic heart failure patients. Diabetes 2012;61:1633–41.

11. Liu Y, Morley M, Brandimarto J, et al., for the MAGNet consortium. RNA-Seq identifies novel myocardial gene expression signatures of heart failure. Genomics 2015;105:83–9.

12. Irving H. Peirce's truth-functional analysis and the origin of the truth table. History and Philosophy of Logic 2012;33:87–97.

13. Iborra-Egea O, Gálvez-Montón C, Roura S, et al. Mechanisms of action of sacubitril/valsartan on cardiac remodeling: a systems biology approach. NPJ Syst Biol Appl 2017;3:12.

14. Romeo-Guitart D, Forés J, Herrando-Grabulosa M, et al. Neuroprotective drug for nerve trauma revealed using artificial intelligence. Sci Rep 2018;8:1879.

15. Enderton H. A Mathematical Introduction to Logic. 2nd edition. New York: Harcourt Academic, 2001.

16. Yurista SR, Silljé HHW, Oberdorf-Maass SU, et al. Sodium-glucose co-transporter 2 inhibition with empagliflozin improves cardiac function in nondiabetic rats with left ventricular dysfunction after myocardial infarction. Eur J Heart Fail 2019;21:862–73.

17. Kilić A, Huang CX, Rajapurohitam V, Madwed JB, Karmazyn M. Early and transient sodium-hydrogen exchanger isoform 1 inhibition attenuates subsequent cardiac hypertrophy and heart failure following coronary artery ligation. J Pharmacol Exp Ther 2014;351:492–9.

18. Aker S, Snabaitis AK, Konietzka I, et al. Inhibition of the Na+/H+ exchanger attenuates the deterioration of ventricular function during pacing-induced heart failure in rabbits. Cardiovasc Res 2004;63:273–82.

19. Baartscheer A, Schumacher CA, van Borren MM, Belterman CN, Coronel R, Fiolet JW. Increased Na+/H+-exchange activity is the cause of increased [Na+]; and underlies disturbed calcium handling in the rabbit pressure and volume overload heart failure model. Cardiovasc Res 2003;57:1015–24.

20. Engelhardt S, Hein L, Keller U, Klämbt K, Lohse MJ. Inhibition of Na+/H+ exchange prevents hypertrophy, fibrosis, and heart failure in beta 1-adrenergic receptor transgenic mice. Circ Res 2002;90:814–9.

21. Kusumoto K, Haist JV, Karmazyn M. Na+/H+ exchange inhibition reduces hypertrophy and heart failure after myocardial infarction in rats. Am J Physiol Heart Circ Physiol 2001;280:H738–74.

22. Uthman L, Baartscheer A, Bleijlevens B, et al. Class effects of SGLT2 inhibitors in mouse cardiomyocytes and hearts: inhibition of Na+/H+ exchanger, lowering of cytosolic Na+ and vasodilation. Diabetologia 2018;61:722–6.

23. Baartscheer A, Schumacher CA, Wüst RC, et al. empagliflozin decreases myocardial cytoplasmic Na+ through inhibition of the cardiac Na+/H+ exchanger in rats and rabbits. Diabetologia 2017;60:568–73.

24. Piacentino V III, Milano CA, Bolanos M, et al. X-linked inhibitor of apoptosis protein-mediated attenuation of apoptosis, using a novel cardiac-enhanced adeno-associated viral vector. Hum Gene Ther 2012;23:635–46.

25. Haider N, Arbustini E, Gupta S, et al. Concurrent upregulation of endogenous proapoptotic and antiapoptotic factors in failing human hearts. Nat Clin Pract Cardiovasc Med 2009;6:250–61.

26. Lee PJ, Rudenko D, Kuliszewski MA, et al. Survivin gene therapy attenuates left ventricular systolic dysfunction in doxorubicin cardiomyopathy by reducing apoptosis and fibrosis. Cardiovasc Res 2014;101:423–33.

27. Levkau B, Schäfers M, Wohlschlaeger J, et al. Survivin determines cardiac function by controlling total cardiomyocyte number. Circulation 2008;117:1583–93.

28. Topol EJ. High-performance medicine: the convergence of human and artificial intelligence. Nat Med 2019;25:44–56.

KEY WORDS empagliflozin, heart failure, machine learning

APPENDIX For supplemental tables and a video, please see the online version of this paper.