The sodium-glucose co-transporter-2 inhibitor ertugliflozin modifies the signature of cardiac substrate metabolism and reduces cardiac mTOR signalling, endoplasmic reticulum stress and apoptosis

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Abstract
Aim: To investigate cardiac signalling pathways connecting substrate utilization with left ventricular remodelling in a murine pressure overload model.

Methods: Cardiac hypertrophy was induced by transverse aortic constriction surgery in 20-week-old C57BL/6J mice treated with or without the sodium-glucose co-transporter 2 (SGLT2) inhibitor ertugliflozin (225 mg kg⁻¹ chow diet) for 10 weeks.

Results: Ertugliflozin improved left ventricular function and reduced myocardial fibrosis. This occurred simultaneously with a fasting-like response characterized by improved glucose tolerance and increased ketone body concentrations. While cardiac insulin signalling was reduced in response to SGLT2 inhibition, AMP-activated protein kinase (AMPK) signalling was increased with induction of the fatty acid transporter cluster of differentiation 36 and phosphorylation of acetyl-CoA carboxylase (ACC). Further, enzymes responsible for ketone body catabolism (β-hydroxybutyrate dehydrogenase, succinyl-CoA:3-oxoacid-CoA transferase and acetyl-CoA acetyltransferase 1) were induced by SGLT2 inhibition. Ertugliflozin led to more cardiac abundance of fatty acids, tricarboxylic acid cycle metabolites and ATP. Downstream mechanistic target of rapamycin (mTOR) pathway, relevant for protein synthesis, cardiac hypertrophy and adverse cardiac remodelling, was reduced by SGLT2 inhibition, with alleviation of endoplasmic reticulum (ER) stress and unfolded protein response (UPR) providing a potential mechanism for abundant reduced left ventricular apoptosis and fibrosis.

Conclusion: SGLT2 inhibition reduced left ventricular fibrosis in a murine model of cardiac hypertrophy. Mechanistically, this was associated with reduced cardiac insulin abundance.
1 | INTRODUCTION

Heart failure (HF) is a steadily increasing clinical problem attributable to diverse disease aetiologies in an ageing population with improved cardiovascular care. Adaptation to HF initiates a process of metabolic reprogramming characterized by a shift of cardiac substrate utilization from fatty acid oxidation towards glucose metabolism.1,2 This provides additional energy under stressed conditions but is limited by the occurrence of systemic insulin resistance arising in more advanced stages of HF.1,3 Restricted substrate availability and impaired mitochondrial functionality are thought to create an energetic deficit in advanced HF, further limiting cardiac contractility.4 Recent evidence suggests ketone body utilization to provide an alternative energy source in HF fuelling the heart despite insulin resistance.4,5 Application of ketone bodies was found to improve cardiac functionality in HF with reduced ejection fraction patients, suggesting metabolic interventions to provide therapeutic efficacy.6 Along these lines, the success of sodium-glucose co-transporter-2 (SGLT2) inhibition in HF therapy has been attributed to the reprogramming of cardiac metabolism.7-9 SGLT2 inhibitors were developed to lower blood glucose in patients with diabetes, inhibiting the reabsorption of primarily filtered glucose in the proximal renal tubule. Consequential glucosuria and osmotic diuresis result in caloric deficit, reduced body weight and lowering of blood pressure. Unexpectedly, SGLT2 inhibitors were also found to markedly prevent HF hospitalization in patients with diabetes.10 This observation was recently extended to HF with a preserved ejection fraction (HFpEF) patients independent of diabetes.11,12 The mechanisms relevant for these beneficial effects of SGLT2 inhibition remain incompletely understood but might relate to the modulation of cardiac metabolism. Loss of urinary glucose by SGLT2 inhibition reduces systemic carbohydrate availability and shifts substrate provision to other energetic sources with increased availability of free fatty acids, ketone bodies and glucagon—mimicking a fasting-like state.7,8,13 This change in systemic substrate availability shifts cardiac fuel utilization from glucose to fatty acid and ketone body oxidation. Importantly, this metabolic adaptation was found to occur simultaneously with improvement of left ventricular function and reduced cardiac remodelling in experimental HF models.7,8 It currently remains unknown whether the observed changes in cardiac metabolism are the cause or consequence of improved left ventricular function in response to SGLT2 inhibition. In addition, the relevant signalling networks resulting in the observed metabolic adaptations have not been explored. Here, we describe the cardiac effects of SGLT2 inhibition in the pressure overload mouse model under non-diabetic conditions.

2 | METHODS

2.1 | Animal studies

All experiments were approved by the government of North Rhine-Westphalia (Germany), and this complied with all institutional and national requirements for the care and use of laboratory animals, and received animal care and use committee approval. A dose-finding experiment was performed under consideration of ertugliflozin-dependent glucose excretion. Male, 6-week-old C57BL/6J mice were obtained from Janvier Labs and placed in a 12-hour day–night cycle with an unlimited supply of food and water in the animal facility of the University Hospital, RWTH Aachen University. After a 1-week adaptation to our facility, mice were treated once daily with different concentrations of ertugliflozin (0.1, 0.3, 1, 10, 30 or 60 mg ml\(^{-1}\); solved in 0.5% [w/v] methylcellulose and 10% [v/v] polyethylene glycol 400) or vehicle via oral gavage for at least 10 days. Urine collection was performed in metabolic cages for 12 hours with an unlimited supply of water, and urinary glucose concentration was assessed in the chemistry department of the animal facility of the University Hospital, RWTH Aachen. This led to dietary ertugliflozin supplementation in a concentration of 225 mg kg\(^{-1}\) diet or vehicle in all other experiments, which is equivalent to a daily ertugliflozin dose of 45 mg kg\(^{-1}\) per mouse. Male C57BL/6J mice at the age of 10 weeks were obtained from Janvier Labs. At the age of 20 weeks, the mice underwent transverse aortic constriction (TAC) surgery to induce cardiac hypertrophy in comparison with sham surgery. The mice were anaesthetized with ketamine (100 mg kg\(^{-1}\)) and xylazine (10 mg kg\(^{-1}\)) and temgesic (0.1 mg kg\(^{-1}\)) was used for analgesia. After intubation, the mice were ventilated with a stroke volume of 200 μl and a respiration rate of 120 strokes per minute. The chest was opened by a small incision in the second intercostal space and aortic constriction was performed by tying a ligature against a 27G needle. Control mice underwent a sham operation in which the thread was placed, but not tied. Sham surgery (n = 22) was performed with 11 mice per group...
(sham control [ctrl.] and sham ertugliflozin [ertu.] group), while TAC surgery \((n = 52)\) was carried out with 26 mice per group (TAC ctrl. and TAC ertu. group). During surgery, one sham and nine TAC animals died, leaving 10-11 sham and 21-22 TAC animals per group. Consequently, analysis was performed using 10 ctrl. sham, 11 ertu. sham, 21 ctrl. TAC and 22 ertu. TAC mice. After surgery, the mice were fed a normal chow diet \((9 \text{kJ}\% \text{ fat}, 58 \text{kJ}\% \text{ carbohydrates and } 33 \text{kJ}\% \text{ protein}; \text{V1534-300; ssniff Spezialdiäten GmbH})\) for 10 weeks with or without ertugliflozin in a concentration of 225 mg kg\(^{-1}\), which is equivalent to a daily dose of 45 mg kg\(^{-1}\) per mouse. Urine collection was performed as described above. Seven weeks after surgery, glucose tolerance was assessed after 6 hours fasting by intraperitoneal (i.p.) injection of 2 g glucose/kg body weight.

Haemodynamics were measured by Millar catheter (Millar Instruments) after advancing the catheter across the right carotid artery through the aortic valve into the left ventricle of anaesthetized mice. Basal measurement was followed by i.p. injection of dobutamine \((5 \text{ mg kg}\(^{-1}\) body weight) as a cardiac stressor. Signals were continuously recorded and analysed using iox (emka Technologies). Finally, overnight fasted mice were sacrificed by cervical dislocation, blood glucose levels were assessed using a glucometer (Contour, Bayer) and tissue samples were snap-frozen in liquid nitrogen and stored at \(-80^\circ\text{C}\) for further analysis. Homeostatic Model Assessment of Insulin Resistance (HOMA-IR) was calculated by multiply fasted insulin \((\mu\text{U ml}\(^{-1}\)) with fasted blood glucose \((\text{mg dl}\(^{-1}\)) and all divided by 405.

### 2.2 Blood sample analysis

Serum insulin concentrations (Alpco) were determined by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer’s protocol. Serum β-hydroxybutyrate (Daisy’s Diagnostics) was measured enzymatically by the CHOD-PAP method using commercial reagents for photometric systems (Tecan).

### 2.3 Western blot analysis

Heart tissue samples were homogenized in lysis buffer \((0.25 \text{ M sucrose, } 2.2 \text{ mM Na}_2\text{EDTA, } 10 \text{ mM Tris and complemented with PhosSTOP and cOmplete from Roche})\), then separated on a 4%-15% gradient gel and transferred to a nitrocellulose membrane. All antibodies were obtained from Cell Signaling if not indicated otherwise: 4E-binding protein 1 (4E-BP1), p-4E-BP1(Ser65), acetyl-CoA carboxylase (ACC), p-ACC(Ser79), protein kinase B (AKT)1, p-AKT(Ser473), p-AKT(Thr308), AMP-activated protein kinase (AMPK)α, p-AMPKα (Thr172), activating transcription factor 4 (ATF4), activating

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**FIGURE 1** Ertugliflozin improves glucose metabolism. Twenty-week-old male C57BL/6J mice underwent transverse aortic constriction (TAC) surgery to induce cardiac hypertrophy as a pressure overload model. After 7 weeks of ertugliflozin treatment, glucose tolerance (A: Ctrl. sham vs. ertu. sham \(n = 5-6\) per group; and B: Ctr. TAC vs. ertu. TAC \(n = 10-13\) per group), C: Visualized as area under the curve (AUC), and D: Insulin sensitivity \((n = 10\) sham or \(n = 18\) TAC per group) were improved, while E: Circulating ketone bodies \((n = 9-10\) sham or \(n = 15-19\) TAC per group) were increased in response to ertugliflozin at the 10-week time point. Results are expressed as mean ± SD. Two-way ANOVA with Dunnett post hoc test was used when comparing each group with the ctrl. TAC group; \(*P < .05\) and \(**P < .01\)
transcription factor 6 (ATF6), caspase 3 (CASP3), C/EBP homology protein (CHOP), eIF2α, p-eIF2α(Ser51), glyceraldehyde 3 phosphate dehydrogenase (GAPDH), glucose transporter type 4 (GLUT4), interleukin 1β (IL-1β), mechanistic target of rapamycin (mTOR), p-mTOR(Ser2481), p70 S6 kinase (p70S6K), p-p70S6K(Thr389), phosphoenolpyruvate carboxykinase (PCK), Raptor, p-Raptor(Ser792), tuberous sclerosis complex 2 (TSC2), p-TSC2(Ser1387), p-TSC2(Ser939), Unc-51–like autophagy activating kinase (ULK1), p-ULK1(Ser757) (all concentration 1:1.000), branched-chain alpha-keto acid dehydrogenase (BCKDHA), p- BCKDHA(Ser293), brain natriuretic peptide (BNP), dihydrolipoamide branched chain transacylase (DBT) (all concentration 1:500) (Abcam), cluster of differentiation 36 (CD36) (concentration 1:100) (Novus Biologicals), acetoacetyl-CoA acetyltransferase 1 (ACAT1), beta-hydroxybutyrate dehydrogenase 1 (BDH1), succinyl-CoA:3-oxoacid-CoA transferase (SCOT) (concentration 1:500) (Thermo Fisher), collagen 1 (COL1) (concentration 1:1000) (Southern Biotech) and Grp78 and Grp94 (KDEL) (concentration 1:1000) (Enzo) and anti-rabbit or anti-mouse IgG, Horseradish Peroxidase-linked antibodies were used as secondary antibody (both concentration 1:1.000). Western blots were detected by Chemi DocTM MP Imaging System (BioRad) and analysed with the software Image Lab 5.0 (BioRad).

2.4 | Histological analysis

Heart tissue was fixed in 4% paraformaldehyde overnight and embedded in paraffin. The heart was cut in the direction from top to apex and 4-μm sections were collected after the mitral valve. Collagen was visualized by Gomori's one-step trichrome stain and analysed with the software Image-Pro Plus 7.0 (Media Cybernetics).

To analyse the size and area of cardiomyocytes, tissue sections were rehydrated and heat-induced antigen retrieval was performed in citrate buffer. Slides were incubated with fluorescein-coupled wheat germ agglutinin (1:100; Vector Laboratories) and counterstained with 4’,6-Diamidin-2-phenylindol. Slides were digitalized using a Aperio Versa200 whole slide scanner (Leica Biosystems). In each mouse, 150-200 cross-cut cardiomyocytes were measured and analysed using Image J (NIH).

2.5 | Metabolome analysis

Flash-frozen tissues from ctrl. TAC and ertu. TAC mice (n = 8 per group) were homogenized using lysing matrix tubes and 300 μl of extraction buffer (80% methanol). After homogenization, the samples...
were centrifuged for 15 minutes at 20 000 g at 4°C. The supernatant was transferred to mass spectrometry vials for analysis.

Ten microlitres of each sample was loaded into a Dionex UltiMate 3000 LC System (Thermo Scientific Bremen) equipped with a C-18 column (Acquity UPLC HSS T3 1.8 μm; 2.1 × 150 mm; Waters) coupled to a Q Exactive Orbitrap mass spectrometer (MS; Thermo Scientific) operating in negative ion mode. A step gradient was carried out using solvent A (10 mM TBA and 15 mM acetic acid) and solvent B (100% methanol). The gradient started with 0% of solvent B and 100% of solvent A and remained at 0% of B until 2 minutes postinjection. A linear gradient to 37% of B was carried out for 7 minutes then increased to 41% until 14 minutes. Between 14 and 26 minutes, the gradient increased to 100% of B and remained at 100% of B for 4 minutes. At 30 minutes, the gradient returned to 0% of B. The chromatography was stopped at 40 minutes. The flow was kept constant at 250 μl min⁻¹ and the column was maintained at 25°C throughout the analysis. The MS was operated in full scan mode (m/z range: 70-1050) using a spray voltage of 3.2 kV, capillary temperature of 320°C, sheath gas at 10.0 and auxiliary gas at 5.0. The AGC target was set at 36e6 with a maximum IT fill time of 512 ms. Data collection was performed using Xcalibur software (Thermo Scientific). The data analyses were performed by integrating the peak areas (El-Maven—Polly—Elucidata). Metabolites’ concentrations were normalized to an internal standard and adjusted to heart tissue weight. Data are displayed as fold changes of ctrl. TAC versus ertu. TAC mice (n = 8 per group) and statistical analysis was performed using multiple t test with a desired false discovery rate (FDR) (Q) of 30%.

2.6 | Gene expression analysis by RT-PCR

Total ribonucleic acid (RNA) was isolated with the RNeasy Mini Kit and RNA preparation was followed by DNase digestion and reverse transcription into complementary DNA (cDNA; Invitrogen). Gene expression was quantified by the use of SYBR or TaqMan reagents with a ViiATM 7 Real-Time PCR System (Applied Biosystems). Measurements were conducted in duplicate under standard reaction conditions and normalized to Actb (β-actin) or Gapdh (glyceraldehyde-3-phosphate dehydrogenase). Genes were selected concerning their pathophysiological or physiological function (HF: Bnp [natriuretic peptide type B]) and branched-chain amino acid (BCAA) catabolism:

![Figure 3](image-url)

**FIGURE 3** Ertugliflozin treatment causes a switch of cardiac metabolic pathways. At the final time point, protein expression of cardiac tissue was analysed by western blot (n = 4 per group), suggesting a switch of substrate utilization from Glucose A and D, towards Fatty acids B and E, and Ketone bodies C and G, while BCAA catabolism was not affected (B and E: Protein expression; and F: mRNA expression [n = 5 sham and n = 10-11 TAC per group]). Metabolic changes were supported by a targeted metabolomic analysis (I and J: Comparing ctrl. TAC against ertu. TAC [n = 8 per group]; visualized as fold change). Results are expressed as mean ± SD, except for metabolome analysis, where values are shown as fold change (ctrl. TAC vs. ertu. TAC mice; n = 8 per group). Two-way ANOVA with Dunnett post hoc test was used when comparing each group with the ctrl. TAC group, and for metabolome analysis, statistical analysis was performed using multiple t test with a desired FDR (Q) of 30%; *P < .05 and **P < .01
Bckde2 (branched chain ketoacid dehydrogenase E2), Bckd (branched chain ketoacid dehydrogenase) kinase, branched chain amino acid transaminase 2 (Bcat2), Bckde1b (branched chain ketoacid dehydrogenase E1b) and Ppm1k (protein phosphatase 1 K). Primers were obtained from Eurofins Genomics or Thermo Fisher Scientific, and all primer sequences or assay IDs are presented in Tables S1 and S2.

2.7 | Statistical analysis

All data are presented as mean ± SD, except for metabolome data, which are presented as fold change. All statistical data analyses and graph preparations were performed using GraphPad Prism (version 9). Statistical analysis was performed by two-way ANOVA with Dunnett post hoc test and comparing each group with the ctrl. TAC group. For metabolome analysis, statistical analysis was performed using multiple t test with a desired FDR (Q) of 30%, and one-way ANOVA with Bonferroni post hoc test was used for urinary glucose excretion (Figure S1A).

3 | RESULTS

As expected, the SGLT2 inhibitor ertugliflozin increased urinary glucose excretion in a dose-dependent manner in non-diabetic C57BL/6J mice (Figure S1A). To investigate the effects of ertugliflozin on cardiac structure and function, we used TAC surgery as a pressure overload model in comparison with the sham procedure. Ertugliflozin improved glucose tolerance (Figure 1A–C), insulin sensitivity (Figure 1D) and circulatory ketone concentrations under TAC conditions, while no difference was found in sham-treated animals (Figure 1E).

During a 10-week period after TAC surgery, less animals died in the ertugliflozin-treated group compared with control TAC animals, which did not reach statistical significance (ctrl. TAC = 77.3% vs. ertu.
FIGURE 4  Ertugliflozin treatment leads to reduced cardiac mTOR signalling, endoplasmic reticulum stress, unfolded protein response and apoptosis. A–C: Cardiac protein expression was analysed by western blot (n = 4 per group), indicating, D–F: Increased AMPK activation and inhibition of mTOR signalling with less ER stress, UPR, apoptosis, inflammation, fibrosis and BNP expression in response to ertugliflozin treatment. Results are expressed as mean ± SD. Two-way ANOVA with Dunnett post hoc test was used when comparing each group with the ctrl. TAC group; *P < .05, **P < .01 and ***P < .001.

FIGURE 5  Schematic illustration of ertugliflozin-dependent cardiac signalling. Ertugliflozin seems to cause a switch of the enzymes involved in cardiac metabolic pathways with reduced insulin and increased AMPK signalling, leading to reduced mTOR signalling, endoplasmic reticulum stress, unfolded protein response, apoptosis and fibrosis. FFA: free fatty acid; LV: left ventricular.
TAC = 90.5%; \( P = .265 \) (Figure 2A), while none of the sham animals died (data not shown). After 10 weeks, the TAC procedure significantly reduced left ventricular systolic contractility under stressed conditions, which was ameliorated by SGLT2 inhibition (ctrl. TAC = 960.07 ± 3621.7 mmHg s\(^{-1}\) vs. ertu. TAC = 12 083.7 ± 2078.9 mmHg s\(^{-1}\); \( P = .0053 \)) (Figure 2B). TAC surgery did not impair cardiac relaxation as an indicator of diastolic function; however, this was significantly improved by SGLT2 inhibition under stressed conditions (ctrl. TAC = -8086.37 ± 2897.3 mmHg s\(^{-1}\) vs. ertu. TAC = -10 323.3 ± 2558 mmHg s\(^{-1}\); \( P = .0118 \)) (Figure 2C). Importantly, heart rate was not affected by SGLT2 inhibition but, surprisingly, was reduced by TAC surgery in the control group (Figure 2D).

Analysing heart structure and morphology we observed TAC-dependent cardiomyocyte hypertrophy (ctrl. TAC = 309.4 ± 18.2 \( \mu \)m\(^2\) vs. ertu. TAC = 249.8 ± 34.6 \( \mu \)m\(^2\); \( P = .0163 \)) (Figure 2E,F), septal thickness (ctrl. TAC = 386.6 ± 79.5 \( \mu \)m vs. ertu. TAC = 334.8 ± 34.2; \( P = .0683 \)) (Figure 2H) and left ventricular fibrosis (ctrl. TAC = 4.8% ± 2.4% vs. ertu. TAC = 2.3% ± 1.8%; \( P = .0046 \)) (Figure 2E,G), which was prevented by treatment with the SGLT2 inhibitor. Further, cardiac Bnp mRNA expression, as an indicator of HF, was reduced by SGLT2 inhibition (ctrl. TAC vs. ertu. TAC: 0.647-fold reduction; \( P = .0499 \)) (Figure 2I).

Investigating cardiac substrate metabolism, we observed the TAC procedure to increase cardiac insulin signalling, as indicated by a significant increase of AKT phosphorylation at Thr308, expression of the promoting enzyme PCK\(^{15} \), which were all significantly reduced by SGLT2 inhibition (Figure 3A,D). Further, ertugliflozin significantly increased expression of the fatty acid transporter CD36 and phosphorylation of ACC, promoting fatty acid oxidation (Figure 3B,E). No difference was observed in enzymes relevant for BCAA catabolism (BCKDH/phosphorylation, DBT [Figure 3B,E] in addition to expression of branched chain ketoacid dehydrogenase E2 and E1b [Bckdh1e2 and Bckdh1e1b], Bckd kinase, Bcat2 and Ppm1k [Figure 3F]). By contrast, enzymes relevant for ketone body catabolism (BDH1, SCOT and ACAT1; Figure 3H) were significantly induced in response to ertugliflozin treatment under pressure overload conditions (Figure 3C,G).

Performing targeted metabolomic analyses from ctrl. TAC versus ertu. TAC mice, we found cardiac palmitic acid content to be increased in response to SGLT2 inhibition, while the abundance of hexose and glycolytic end products (pyruvate and lactate) remained unaltered (Figure 3I,J). This occurred in conjunction with an increased cardiac abundance of tricarboxylic acid (TCA) cycle metabolites with significant induction of succinate (Figure 3J). Carriers of cellular energy (ATP, nicotinamide adenine dinucleotide [NAD]) and nicotinamide adenine dinucleotide phosphate [NADPH]) were significantly or by trend induced in response to SGLT2 inhibition (Figure 3I). This could indicate a shift of cardiac substrate utilization from glucose to fatty acids and ketone bodies in response to SGLT2 inhibition with increased TCA cycle flux providing additional energy under hypertrophic TAC conditions.

To understand the underlying mechanisms, we investigated pathways connecting energy metabolism with left ventricular functionality and remodelling. Ertugliflozin significantly increased the activating phosphorylation of cardiac AMPK at Thr172 (Figure 4A,B). This led to downstream inhibition of the mTORC1 (Figure 4A,B) pathway as a consequence of AMPK-dependent Raptor-phosphorylation Ser792 (Figure 4A,B). Additional inhibition of mTOR could be attributable to reduced cardiac insulin signalling with significantly less AKT-dependent TSC2 (Figure 4A,B) and mTOR activation (Figure 4A,C), resulting in reduced downstream phosphorylation of mTOR targets including p70S6K, eukaryotic translation initiation factor 4E-BP1 and ULK1 (Figure 4A,C).

The mTOR signalling pathway critically mediates cardiac hypertrophy relevant for protein synthesis, cellular growth and workload adaptation. Although providing an initial adaptive response, sustained mTOR signalling is known to cause endoplasmic reticulum (ER) stress by unfolded protein response (UPR) endangering for apoptosis and adverse cardiac remodelling.\(^{16} \) We consistently found pressure overload to induce ER stress, as indicated by KDEL expression, which was significantly attenuated by ertugliflozin treatment (Figure 4D,E). SGLT2 inhibition further mitigated the activation of different indicators of ER stress, including ATF6 with eukaryotic initiation factor α (eif2α)-phosphorylation (Figure 4D,E) and downstream ATF4 and CHOP signalling (Figure 4D,E). This led to a significant decrease of caspase 3, collagen I and IL-1β expression, as indicative of less apoptosis, fibrosis and left ventricular remodelling by SGLT2 inhibition, together with downregulation of BNP expression (Figures 4D,F and 5).

4 | DISCUSSION

In this study, we found SGLT2 inhibition to reduce myocardial hypertrophy and adverse cardiac remodelling in a murine model of pressure overload that led to improved left ventricular contractility under dobutamine stress conditions. Analysis of targeted metabolomics and cardiac metabolic signalling suggested a shift of myocardial substrate utilization from glucose to fatty acids and ketones with increased TCA cycle flux. This might provide additional energy for cardiac contractility. Investigating the underlying metabolic network, we found cardiac insulin signalling to be reduced, while AMPK signalling was increased with inhibition of the mTOR pathway, and resolution of ER stress as a yet unappreciated cardioprotective mechanism of SGLT2 inhibition.

Urinary glucose excretion by SGLT2 inhibition lowers systemic glucose availability, favouring a fasting-like response with reduced circulating insulin concentrations and increased ketone body provision.\(^{13,17} \) A consequential shift of myocardial substrate utilization has been suggested to overcome energetic deficiency present under HF conditions.\(^{7,8} \) Consistent with others, we found SGLT2 inhibition to reduce myocardial glucose utilization while increasing fatty acid uptake, TCA cycle flux metabolites and myocardial energetics in a pressure overload HF environment.\(^{7,8} \) The mechanisms mediating this shift of myocardial substrate utilization remain incompletely understood but might relate to reduced systemic insulin concentrations and a fasting-like response of the organism.\(^{13} \) Insulin acts as a relevant growth factor providing
cellular energy while stimulating protein synthesis mediated by mTOR activation.\textsuperscript{19} mTOR is a master regulator of cell growth, metabolic control and autophagy.\textsuperscript{19} Broad functional relevance of negative mTOR regulation by SGLT2 inhibition was suggested in our study by modulation of multiple downstream targets including p70S6K and 4E-BP1—both stimulating protein synthesis—and ULK1-inhibiting autophagy.\textsuperscript{19} Cardiac mTOR signalling is required for organ development, with mTOR deletion being embryonic lethal.\textsuperscript{16} Persistent mTOR activation during HF progression, however, favours left ventricular hypertrophy and adverse cardiac remodelling, while partial mTOR inhibition prevents left ventricular fibrosis in models of hypertrophic, metabolic and age-related cardiomyopathy.\textsuperscript{16,19} Cardioprotective effects of mTOR inhibition have been attributed to reduction of anabolic energy-demanding processes, while stimulating catabolic energy-providing reactions in conjunction with increased autophagic organelle turnover, less UPR and reduced ER stress.\textsuperscript{17,20,21} We consistently observed SGLT2 inhibition to reduce expression of Grp78 and Grp94, both chaperones induced by ER stress facilitating the degradation of misfolded proteins in conjunction with reduced induction of ER stress sensors including ATF6, protein kinase RNA-like endoplasmic reticulum kinase (PERK) and Inositol-requiring enzyme 1 (IRE1).\textsuperscript{22} These pathways provide cellular protection by counterbalancing misfolded protein accumulation but lead to apoptosis if overwhelmed by sustained insufficient protein synthesis. Consistently, we found apoptosis (by caspase 3 expression) and fibrosis (by collagen expression) to be increased in response to the TAC procedure and reduced by SGLT2 inhibition. These observations provide an as of yet unappreciated signalling network regulated by SGLT2 inhibition integrating cellular metabolism with cell growth and survival pathways. Interestingly, these cardioprotective signalling events by SGLT2 inhibition were only found under pressure overload, but not under healthy conditions. This suggests that stress-induced cardiac alteration is required for therapeutic efficacy of SGLT2 inhibition, which will require further investigation.

It is tempting to speculate SGLT2 inhibition to orchestrate a systemic fasting-like response mediated by reduced insulin signalling and glucose availability.\textsuperscript{13,17} Insulin signalling has been found to have deteriorating effects in experimental HF models.\textsuperscript{23} Depletion of insulin or its myocardial receptor improved pressure overload-induced cardiac dysfunction.\textsuperscript{24} Further, treatment of type 1 diabetic mice with insulin promoted cardiomyocyte death and HF, despite improving systemic glucose metabolism.\textsuperscript{24} In addition, insulin treatment independently predicted adverse prognostic in HF trials.\textsuperscript{25,26} Reducing circulating insulin concentrations by SGLT2 inhibition might therefore explain some of the cardiac benefits of this drug class.

A relevant limitation of our study remains its descriptive nature, which is unable to prove causal relevance of metabolic signalling for inhibition of left ventricular remodelling by SGLT2 inhibition. In line with this, the observed metabolomic signature does only provide a snapshot, without allowing conclusions on metabolic flux and substrate utilization. However, the coordinated regulation of different signalling events interconnecting energy metabolism with left ventricular remodelling provides additional evidence for the cardioprotective relevance of metabolic reprogramming by SGLT2 inhibition.

In conclusion, we found the SGLT2 inhibitor ertugliflozin to improve left ventricular remodelling in a murine model of cardiac pressure overload. Mechanistically, this was associated with a modulation of cardiac metabolic pathways with reduced cardiac insulin and increased cardiac AMPK signalling, potentially leading to reduced cardiac mTOR signalling, ER stress and adverse cardiac remodelling.

**AUTHOR CONTRIBUTIONS**

JM: experimental design and performance; and wrote the first draft of the manuscript. PAM: experimental performance; and edited the first draft of the manuscript. BAK: helped with metabolome analysis, critical discussion of results and editing of the manuscript. FK: critical discussion of results and editing of the manuscript. BMK: helped with histological analyses; and edited the first draft of the manuscript. PB: critical discussion of results and editing of the manuscript. RK: critical discussion of results and editing of the manuscript. BG: performed metabolome analysis, critical discussion of results and editing of the manuscript. NM: critical discussion of results and editing of the manuscript. ML: experimental design, wrote the final manuscript and is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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**CONFLICT OF INTEREST**

NM has given lectures for Boehringer Ingelheim, Sanofi-Aventis, MSD, BMS, AstraZeneca, Lilly and Novo Nordisk; has received unrestricted research grants from Boehringer Ingelheim, and has served as an advisor for Bayer, Boehringer Ingelheim, Sanofi-Aventis, MSD, BMS, AstraZeneca and Novo Nordisk; and in addition, has served in trial leadership for Boehringer Ingelheim and Novo Nordisk. ML received grants and personal fees from Boehringer Ingelheim, MSD and Novo Nordisk, and personal fees from Amgen, Sanofi, Astra...
Zeneca, Bayer, Lilly, Daiichi Sankyo and Novartis. RK receives unrelated research funding from Galapagos, Chugai/Roche and Traverge Therapeutics and serves as an advisor for Bayer. JM, PAM, BAK, FK, BMK, BG and PB have nothing to disclose.

**PEER REVIEW**
The peer review history for this article is available at [https://publons.com/publon/10.1111/dom.14814](https://publons.com/publon/10.1111/dom.14814).

**DATA AVAILABILITY STATEMENT**
The data that support the findings of this study are available from the corresponding author upon reasonable request.

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**REFERENCES**
1. Bertero E, Maack C. Metabolic remodelling in heart failure. *Nat Rev Cardiol*. 2018;15:457-470.
2. Ardeshi H, Sabbath HN, Burke MA, et al. Targeting myocardial substrate metabolism in heart failure: potential for new therapies. *Eur J Heart Fail*. 2012;14:120-129.
3. Mann PA, Lehrke M. Cardiac substrate utilization in heart failure: Where is the relevance of SGLT2 inhibition? *J Thorac Cardiovasc Surg*. 2021. doi: [10.1016/j.jtcs.2021.02.092](https://doi.org/10.1016/j.jtcs.2021.02.092).
4. Bedi KC, Jr., Snyder NW, Brandimarto J, et al. Evidence for Intramyocardial disruption of lipid metabolism and increased myocardial ketone utilization in advanced human heart failure. *Circulation*. 2016;133:706-716.
5. Aubert G, Martin OJ, Horton JL, et al. The failing heart relies on ketone bodies as a fuel. *Circulation*. 2016;133:698-705.
6. Nielsen R, Moller N, Gormsen LC, et al. Cardiovascular effects of treatment with the ketone body 3-Hydroxybutyrate in chronic heart failure patients. *Circulation*. 2019;139:2129-2141.
7. Yurista SR, Sillije HHW, Oberdorr-Maass SU, et al. Sodium-glucose co-transporter 2 inhibition with empagliflozin improves cardiac function in non-diabetic rats with left ventricular dysfunction after myocardial infarction. *Eur J Heart Fail*. 2019;21:862-873.
8. Santos-Gallego CG, Requena-Ibanez JA, San Antonio R, et al. Empagliflozin ameliorates adverse left ventricular remodeling in nondiabetic heart failure by enhancing myocardial energetics. *J Am Coll Cardiol*. 2019;73:1931-1944.
9. Kappel BA, Lehrke M, Schutt K, et al. Effect of empagliflozin on the metabolic signature of patients with type 2 diabetes mellitus and cardiovascular disease. *Circulation*. 2017;136:969-977.
10. Zelniker TA, Wiviott SD, Raz I, et al. SGLT2 inhibitors for primary and secondary prevention of cardiovascular and renal outcomes in type 2 diabetes: a systematic review and meta-analysis of cardiovascular outcome trials. *Lancet*. 2019;393:31-39.
11. Zannad F, Ferreira JP, Pocock SJ, et al. SGLT2 inhibitors in patients with heart failure with reduced ejection fraction: a meta-analysis of the EMPEROR-reduced and DAPA-HF trials. *Lancet*. 2020;396:819-829.
12. Anker SD, Butler J, Filippatos G, et al. Empagliflozin in heart failure with a preserved ejection fraction. *N Engl J Med*. 2021;385:1451-1461.
13. Packer M. SGLT2 inhibitors produce cardiorenal benefits by promoting adaptive cellular reprogramming to induce a state of fasting mimicry: a paradigm shift in understanding their mechanism of action. *Diabetes Care*. 2020;43:508-511.
14. Moellmann J, Klinkhammer BM, Droste P, et al. Empagliflozin improves left ventricular diastolic function of db/db mice. *Biochim Biophys Acta Mol Basis Dis*. 2020;1866:165807.
15. Ma H, Yu S, Liu X, et al. Lin28a regulates pathological cardiac hypertrophic growth through Pck2-mediated enhancement of anabolic synthesis. *Circulation*. 2019;139:1725-1740.
16. Sciarretta S, Forte M, Frati G, Sadoshima J. The complex network of mTOR signaling in the heart. *Cardiovasc Res*. 2021;118:424-439.
17. Packer M. Autophagy stimulation and intracellular sodium reduction as mediators of the cardioprotective effect of sodium-glucose co-transporter 2 inhibitors. *Eur J Heart Fail*. 2020;22:618-628.
18. Xu L, Brink M. mTOR, cardiomyocytes and inflammation in cardiac hypertrophy. *Biochim Biophys Acta*. 2016;1863:1894-1903.
19. Sciarretta S, Forte M, Frati G, Sadoshima J. New insights into the role of mTOR signaling in the cardiovascular system. *Circ Res*. 2018;122:489-505.
20. Ren J, Bi Y, Sowers JR, Hetz C, Zhang Y. Endoplasmic reticulum stress and unfolded protein response in cardiovascular diseases. *Nat Rev Cardiol*. 2021;18:499-521.
21. Bielecka-Dabrowa A, Ebner N, Dos Santos MR, Ishida J, Hasenfuss G, von Haelening S. Cachexia, muscle wasting, and frailty in cardiovascular disease. *Eur J Heart Fail*. 2020;22:2314-2326.
22. Wang S, Binder P, Fang Q, et al. Endoplasmic reticulum stress in the heart: insights into mechanisms and drug targets. *Br J Pharmacol*. 2018;175:1293-1304.
23. Chaanine AH, Hajjar RJ. AKT signalling in the failing heart. *Eur J Heart Fail*. 2011;13:825-829.
24. Shimizu I, Minamino T, Toko H, et al. Excessive cardiac insulin signaling exacerbates systolic dysfunction induced by pressure overload in rodents. *J Clin Invest*. 2010;120:1506-1514.
25. Cosmi F, Shen L, Magnoli M, et al. Treatment with insulin is associated with worse outcome in patients with chronic heart failure and diabetes. *Eur J Heart Fail*. 2018;20:888-895.
26. Shen L, Rorth R, Cosmi D, et al. Insulin treatment and clinical outcomes in patients with diabetes and heart failure with preserved ejection fraction. *Eur J Heart Fail*. 2019;21:974-984.

**SUPPORTING INFORMATION**
Additional supporting information can be found online in the Supporting Information section at the end of this article.

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