

RESEARCH

Open Access



Dysregulated inflammation, oxidative stress, and protein quality control in diabetic HFpEF: unraveling mechanisms and therapeutic targets

Simin Delalat^{1†}, Innas Sultana^{1†}, Hersh Osman^{1†}, Marcel Sieme^{1†}, Saltanat Zhazykbayeva¹, Melissa Herwig¹, Heidi Budde¹, Árpád Kovács^{1,2}, Mustafa Kaçmaz¹, Eda Göztepe¹, Natalie Borgmann¹, Gelareh Shahriari¹, Benjamin Sasko³, Jan Wintrich³, Peter Haldenwang⁴, Wolfgang E. Schmidt⁵, Wiebke Fenske⁶, Muchtiar Khan⁷, Kornelia Jaquet¹, Andreas Mügge¹, Domokos Máthé^{8,9}, Viktória E. Tóth^{10,11}, Zoltán V. Varga^{10,11}, Péter Ferdinandy^{10,11}, Ibrahim El-Battrawy^{1,5}, Loek van Heerebeek^{7†} and Nazha Hamdani^{1,12,13,14,15*†}

Abstract

Background Type 2 diabetes mellitus (T2DM) represents a significant risk factor for cardiovascular disease, particularly heart failure with preserved ejection fraction (HFpEF). HFpEF predominantly affects elderly individuals and women, and is characterized by dysfunctions associated with metabolic, inflammatory, and oxidative stress pathways. Despite HFpEF being the most prevalent heart failure phenotype in patients with T2DM, its underlying pathophysiological mechanisms remain inadequately elucidated.

Objective This study aims to investigate the effects of diabetes mellitus on myocardial inflammation, oxidative stress, and protein quality control (PQC) mechanisms in HFpEF, with particular emphasis on insulin signaling, autophagy, and chaperone-mediated stress responses.

Methods We conducted an analysis of left ventricular myocardial tissue from HFpEF patients, both with and without diabetes, employing a range of molecular, biochemical, and functional assays. The passive stiffness of cardiomyocytes (F_{passive}) was assessed in demembranated cardiomyocytes before and after implementing treatments aimed at reducing inflammation (IL-6 inhibition), oxidative stress (Mito-TEMPO), and enhancing PQC (HSP27, HSP70). Inflammatory markers (NF-κB, IL-6, TNF-α, ICAM-1, VCAM-1, NLRP3), oxidative stress markers (ROS, GSH/GSSG ratio, lipid peroxidation), and components of signaling pathways (PI3K/AKT/mTOR, AMPK, MAPK, and PKG) were evaluated using western blotting, immunofluorescence, and ELISA techniques.

[†]Simin Delalat, Innas Sultana, Hersh Osman, Marcel Sieme, Loek van Heerebeek and Nazha Hamdani contributed equally to this work.

*Correspondence:
Nazha Hamdani
nazha.hamdani@rub.de

Full list of author information is available at the end of the article



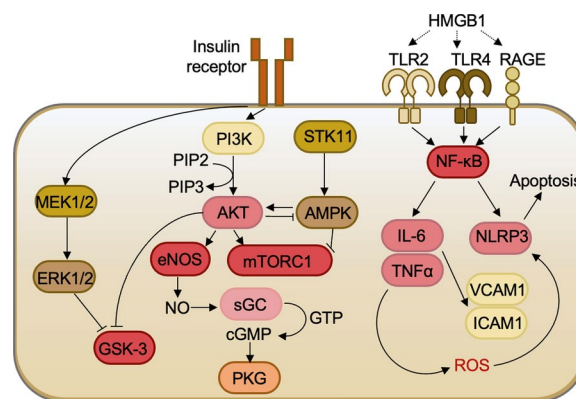
© The Author(s) 2025. **Open Access** This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by/4.0/>.

Results Hearts from diabetic HFpEF patients exhibited significantly heightened inflammation, characterized by the upregulation of NF- κ B, IL-6, and the NLRP3 inflammasome. This increase in inflammation was accompanied by elevated oxidative stress, diminished nitric oxide (NO) bioavailability, and impaired activation of the NO-sGC-cGMP-PKG signaling pathway. Notably, dysregulation of insulin signaling was observed, as indicated by decreased AKT phosphorylation and impaired autophagy regulation mediated by AMPK and mTOR. Additionally, PQC dysfunction was evidenced by reduced expression levels of HSP27 and HSP70, which correlated with increased cardiomyocyte passive stiffness. Targeted therapeutic interventions effectively reduced P passive, with IL-6 inhibition, Mito-TEMPO, and HSP administration leading to improvements in cardiomyocyte mechanical properties.

Conclusion The findings of this study elucidate a mechanistic relationship among diabetes, inflammation, oxidative stress, and PQC impairment in the context of HFpEF. Therapeutic strategies that target these dysregulated pathways, including IL-6 inhibition, mitochondrial antioxidants, and chaperone-mediated protection, may enhance myocardial function in HFpEF patients with T2DM. Addressing these molecular dysfunctions could facilitate the development of novel interventions specifically tailored to the diabetic HFpEF population.

Keywords Heart failure with preserved ejection fraction, Type 2 diabetes, Inflammation, Oxidative stress, Protein quality control, Insulin resistance, Cardiomyocyte stiffness, Autophagy, Heat shock proteins

Graphical abstract



Introduction

Type 2 diabetes mellitus (T2DM), a significant comorbidity associated with cardiovascular disease (CVD), has seen an exponential increase over the past few decades, presently impacting approximately 8% of the global population [1, 2]. Projections for the year 2019 estimated that there were 463 million cases of diabetes, with prevalence expected to double by the year 2045 [3].

Currently, CVD remains a predominant cause of mortality in individuals with T2DM, largely attributable to conditions such as coronary artery disease (CAD), ischemic cardiomyopathy, and heart failure (HF) [4–6]. Patients diagnosed with diabetes are three times more likely to develop HF, contributing to what has become a global health crisis characterized by an increasing prevalence [7]. While HF has traditionally been associated with systolic dysfunction and reduced ejection fraction—termed heart failure with reduced ejection fraction (HFrEF) [8, 9], over 50% of patients, particularly women and the elderly, exhibit symptoms such as dyspnea, fatigue, fluid retention, and exercise intolerance. This condition, referred to as heart failure with preserved

ejection fraction (HFpEF), currently represents the most prevalent cause of hospitalization among individuals aged 65 years and older and is anticipated to become the most common form of heart failure [10, 11].

Evidence suggests the existence of two distinct HF phenotypes in diabetic patients: HFrEF, which is more commonly associated with Type 1 diabetes, and HFpEF, which is linked to T2DM and often accompanied by obesity, indicating a more complex underlying pathophysiology [12].

The biochemical mechanisms underlying T2DM and its association with insulin resistance result in a hyperglycemic and hypertriglyceridemic state. The impairment of glucose uptake by cardiomyocytes leads to the excessive utilization of fatty acids (FAs) as a source of energy to sustain metabolic functions. This phenomenon results in the overproduction of reactive oxygen species (ROS), reduced nitric oxide (NO) bioavailability, increased levels of pro-inflammatory cytokines, mitochondrial dysfunction, and dysregulation of autophagy and protein quality control (PQC) [13, 14].

PQC strictly regulates complex processes such as protein folding, post-translational modifications, protein trafficking, calcium (Ca^{2+}) homeostasis, and degradation of misfolded proteins. Through these processes, newly synthesized polypeptides are transformed into functional proteins, thereby maintaining overall protein quality [15]. Disruption of protein folding, whether due to intracellular or extracellular stressors, triggers PQC activation via the unfolded protein response in the endoplasmic reticulum (UPRER), heat shock proteins (HSPs), autophagy-lysosomal pathways, and the ubiquitin–proteasome system (UPS), all of which play critical roles in maintaining protein quality [16].

Under normal physiological conditions, the expression of HSPs is relatively low. However, in the context of T2DM, pathological mechanisms lead to a significant increase in ROS levels, resulting in an excessive accumulation of oxidatively damaged proteins within cells. Under these circumstances, HSPs play a critical role in modulating oxidative stress, exhibiting various functions at multiple levels [17, 18]. As a highly conserved protein family, HSPs are essential components of the cellular stress response, serving as the primary checkpoint for PQC. Their primary functions include the elimination of misfolded proteins, the facilitation of proper protein folding, and the prevention of protein aggregation. Specifically, heat shock protein 70 (HSP70) and its co-chaperones are instrumental in targeting aberrant proteins for degradation through the proteasome and lysosome pathways. In certain instances, these chaperones possess the unique capacity to refold misfolded proteins, restoring them to their functional form [19]. Furthermore, other chaperones, such as heat shock protein 27 (HSP27) and alpha B-crystallin (αB -crystallin), are known to inhibit the apoptotic cascade, thereby promoting cellular survival and preventing apoptosis [20].

Autophagy, recognized as the second checkpoint in PQC, initiates the lysosomal digestion process, facilitating the generation of nutrients and energy necessary for sustaining cellular functions under metabolic stress. During this process, damaged macromolecules and organelles are hydrolyzed to preserve energy [21]. Autophagy with dual function has beneficial or detrimental effects on a diabetic heart [22]. Dysregulated autophagy within diabetic hearts has been associated with glucose intolerance and insulin resistance [23]. A primary focus of investigation is the role of autophagy in modulating insulin sensitivity. Evidence indicates that enhanced autophagy improves insulin sensitivity in cardiomyocytes and reduces apoptosis [24]. Increased autophagy in the heart is correlated with the production of ROS, which contributes to insulin resistance but may also function as an endogenous self-protective mechanism downstream of the PI3K/AKT insulin signaling pathway [25]. Under

conditions of nutrient deficiency and low intracellular AMP/ATP ratio, AMP-activated protein kinase (AMPK) positively regulates the activation of autophagy [26]. The activation of AMPK leads to the suppression of the mammalian target of rapamycin (mTOR) activity [27]. The inhibition of mTOR activity facilitates the upregulation of stress-responsive mechanisms, including a reduction in protein synthesis, enhanced autophagy, cell cycle arrest, and DNA repair, ultimately limiting cell death [28]. The AMPK–mTOR pathway plays a crucial role in protecting against cardiovascular complications associated with diabetes through the maintenance of mitochondrial function and the suppression of oxidative stress, inflammation, excessive autophagy, and myocardial cell death or apoptosis [29–31]. Persistent hyperglycemia markedly increases the activity of the nuclear factor kappa-light-chain-enhancer of activated B-cells (NF- κB) [32]. This activation results in the heightened transcription of inflammatory genes, including pro-inflammatory cytokines such as interleukin-6 (IL-6), IL-1 β , IL-18, and tumor necrosis factor (TNF- α). This chronic inflammatory state compromises the ubiquitin–proteasome system (UPS) and autophagy, ultimately contributing to cardiomyocyte damage [33]. Therefore, our study aimed to elucidate the underlying mechanisms of diabetic mellitus-induced inflammation, oxidative stress, autophagy, stress signaling and PQC system, and their contribution to the cardiac dysfunction in human HFpEF.

Methods

Human studies

HFpEF patients were referred for cardiac catheterization and endomyocardial biopsy procurement because of clinical suspicion of a cardiomyopathy ($n=25$) see Table 1. They all had been admitted to the hospital because of worsening HF. Subsequent histological examination ruled out a cardiomyopathy/infiltrative myocardial disease. Coronary angiography showed the absence of significant coronary artery stenosis or graft vasculopathy. All patients satisfied the criteria as proposed by the European study group on HFpEF, i.e. signs and symptoms of congestive HF, LV ejection fraction (EF) $\geq 50\%$ and abnormal LV end-diastolic pressure at rest > 12 mmHg. All patients had one or more predisposing risk factors for diastolic LV dysfunction. Endomyocardial biopsies were obtained from the LV and were snap frozen in liquid nitrogen and stored at -80°C until processing. Samples were collected from HFpEF diabetic patients ($n=10$, average age 70 years) and non-diabetic patients ($n=15$, average age 75 years). In total, we have used 25 biopsies throughout the manuscript in different experiments. Due to the limited availability of the tissues, we did not use the same biopsies in the all experiments. Clinical, echocardiographic, and medication characteristics are provided

Table 1 Clinical, echocardiographic, and medication characteristics of HFpEF patients with and without diabetes mellitus

HF phenotype	HFpEF-non DM	HFpEF-DM
number of the Patient	n = 15	n = 10
Age	75.27 ± 2.06	70.88 ± 2.08
Gender % (M = male)	M = 13.3%	M = 10%
BMI (kg/m ²)	32.96 ± 2.26	32.35 ± 2.16
SBP (mmHg)	145.73 ± 4.47	146.38 ± 7.16
DBP (mmHg)	77.13 ± 2.24	75.75 ± 2.96
Hb (g/dL)	8.62 ± 0.19	7.81 ± 0.25
Creatinin (mmol/L)	80.73 ± 5.35	115.38 ± 29.54
eGFR (CKD-EPI) (mL/min/1.73 m ²)	64.86 ± 3	54.29 ± 11.54
NTproBNP (pmol/L)	34.07 ± 6.47	53.88 ± 24.88
CRP (mg/L)	4.26 ± 1.11	2.83 ± 0.93
LVMI (g/m ²)	84.86 ± 5.79	91.63 ± 8.21
LVEF (%)	59.69 ± 2.86	59.64 ± 2.94
E/e' mean ratio	11.95 ± 0.89	14.04 ± 1.3
LAVI max (mL/m ²)	53.13 ± 5.75	45.8 ± 3.59
Loop diuretic	9/15	5/10
Thiazide diuretic	2/15	0/10
MRA	6/15	4/10
Bblocker	6/15	6/10
ACEi	2/15	1/10
ARB	8/15	4/10
SGLT2i	14/15	7/10
Statin	8/15	7/10
Oral anti-glycaemic agent	0/15	6/10
Insulin	0/15	4/10

Number of patient (n), Age (years), gender % (M = male), Body mass index (BMI, kg/m²), Systolic blood pressure (SBP, mmHg), Diastolic blood pressure (DBP, mmHg), Hemoglobin (Hb, g/dL), Creatinine (μmol/L), Estimated glomerular filtration rate (eGFR, mL/min/1.73 m²), N-terminal pro-brain natriuretic peptide (NT-proBNP, pmol/L), C-reactive protein (CRP, mg/L), Left ventricular mass index (LVMI, g/m²), Left ventricular ejection fraction (LVEF, %), E/e' mean (ratio of early mitral inflow velocity to mitral annular early diastolic velocity), Maximum left atrial volume index (LAVI max, mL/m²), Loop diuretic, Thiazide diuretic, Mineralocorticoid receptor antagonist (MRA), Beta-blocker (Bblocker), Angiotensin-converting enzyme inhibitor (ACEi), Angiotensin II receptor blocker (ARB), Sodium-glucose cotransporter-2 inhibitor (SGLT2i), Statin, Oral anti-glycaemic agent (OAG), Insulin. Values are given as mean ± SEM or number

in Table 1. Tissues were collected in a cardioplegic solution and stored in liquid nitrogen until use. All samples were obtained with informed consent and approval from the local Ethics Committee. All procedures were performed according to the Declaration of Helsinki and were approved by the local ethics committee. Biopsies were obtained for the primary purpose of diagnosis following Ethics Committee approval numbers WO18.026, 20–6976 BR, 20–6976-1- BR and informed consent.

Force measurements on isolated de-membranated cardiomyocytes:

Force measurements were conducted on single-skinned cardiomyocytes (n = 30–36/5 cardiomyocytes/hearts per group) as described before [34, 35]. To summarize, LV samples were de-frozen in a relaxing solution (containing

in mM: 1.0 free Mg²⁺; 100 KCl; 2.0 EGTA; 4.0 Mg-ATP; 10 imidazole; pH 7.0), mechanically disrupted, and incubated for 5 min in a relaxing solution supplemented with 0.5% Triton X-100 (all from Sigma-Aldrich). The cell suspension was washed 5 times in relaxing solution. Single cardiomyocytes were selected under an inverted microscope (Zeiss Axiovert 135, 40× objective; Carl Zeiss AG Corp, Oberkochen, Germany) and attached with silicone adhesive between a force transducer and a high-speed length controller (piezoelectric motor) as part of a "Permeabilized Myocyte Test System" (1600A; with force transducer 403A; Aurora Scientific, Aurora, Ontario, Canada).

Cardiomyocyte Ca²⁺-independent passive force (F_{passive}) was measured in relaxing buffer at room temperature (RT) within a sarcomere length (SL) range between 1.8 and 2.4 μm. Force values were normalized to myocyte cross-sectional area calculated from the diameter of the cells, assuming a circular shape. Subsequently cardiomyocytes were incubated for 30 to 40 min in relaxing solution supplemented with either (1) an inhibitor of interleukin 6 (IL-6) (Siltuximab, 0.015 mL/L, EUSA Pharma Ltd, Hemel Hempstead, Hertfordshire, UK), (2) the mitochondria-targeted superoxide dismutase mimetic MitoTEMPO (10 μM; Sigma-Aldrich Merck KGaA, St. Louis, MO, USA), (3) heat shock protein (Hsp) (1 mg/mL; Sigma-Aldrich Merck KGaA, St. Louis, MO, USA), or (4) HSP70 (1 mg/mL; Sigma-Aldrich Merck KGaA, St. Louis, MO, USA). Cardiomyocyte F_{passive} was thereafter measured within a SL range between 1.8 and 2.4 μm as described above. All treatments with Mito-TEMPO, HSPs, and IL-6 inhibitor were done in intact tissues.

Immunofluorescence imaging

As we described before, immunofluorescence imaging was performed on the frozen tissue slide [15]. The frozen LV tissue slides were air-dried for 10 min, rehydrated with PBS, and fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 5 min. The slides were then washed three times for 5 min with PBS. Next, the tissue was incubated with wheat germ agglutinin (WGA 555 conjugate, invitrogen, W32464) for 5 min, followed by another three washes for 5 min with PBS (5 min each). Next, the slides were blocked with 5% BSA/PBS and 0.1% Triton for 1 h at room temperature. After washing three times with PBS, the primary antibody was diluted in 5% BSA/PBS and incubated overnight at 4 °C (Table 2). After incubation with the primary antibody and washing with PBS three times, the slides were incubated with the secondary antibody diluted in 5% BSA/PBS overnight at 4 °C (Table 2). The slides were then washed again three times with PBS and then covered and sealed with Mowiol mounting medium and ultrathin glass

Table 2 Corresponding antibody for Immunofluorescence imaging

Antibody	Product No	Company	Dilution
IL6	P620	Thermo Fisher Scientific	1:150
IL6R	Ab275982	Abcam	1:150
NE	Sc-55549	Santa Cruz Biotechnology	1:100
MPO	14569s	Cell Signaling Technology	1:100
Anti-mouse	70765	Cell Signaling Technology	1:10000
Anti-Rabbit	70745	Cell Signaling Technology	1:10000
Alexa Fluor Plus 488 (Anti-Rabbit)	A32731	Thermo Fisher Scientific	1:150
Alexa Fluor Plus 647 (Anti-Mouse)	A32728	Thermo Fisher Scientific	1:150

Table 3 Corresponding Antibody list for western blot experiment

Antibody	Product NO	Company	Dilution
TNF- α	AMC3012	Invitrogen	1:500
IL-6	Sc.32296	Santa Cruz Biotechnology	1:1000
IL-6 Receptor	MA5-29721	Invitrogen	1:1000
eNOS	32027	Cell Signaling Technology	1:1000
HSP-27	ab2790	Abcam	1:1000
HSP-70	ab2787	Abcam	1:1000
PKG	13511	Cell Signaling Technology	1:1000
p38 MAPK	92125	Cell Signaling Technology	1:1000
NF- κ B	8242	Cell Signaling Technology	1:1000
p65(D14E12)	4695	Cell Signaling Technology	1:1000
ERK (1/2) p44/42	4691	Cell Signaling Technology	1:1000
MAPK			
AKT pan (C67E7)	4691	Cell Signaling Technology	1:1000
Phospho-AKT (S473) (736E11)	3787	Cell Signaling Technology	1:1000
AMPK (D5A2)	5831	Cell Signaling Technology	1:1000
Phospho-AMPK (Thr172) (40H9)	2535	Cell Signaling Technology	1:1000
mTOR (7C10)	2983	Cell Signaling Technology	1:1000
Phospho-mTOR (Ser2448)	2971	Cell Signaling Technology	1:1000
NLRP3 (D4D8T)	15101	Cell Signaling Technology	1:1000
PI3 Kinase p110 α (C73F8)	4249	Cell Signaling Technology	1:1000
GSK-3 β	MA5-1509	Thermo Fisher Scientific	1:1000
GAPDH-anti mouse	97166	Cell Signaling Technology	1:10000
GAPDH-anti rabbit	2118	Cell Signaling Technology	1:10000

coverslips overnight at 4 °C. For the imaging, the confocal laser scanning microscopy (cLSM) (Nikon Eclipse Ti-E inverted Microscope System, Nikon Instruments, Nikon Corp, Shinagawa, Tokyo Japan) was used.

Protein analysis with western blot

SDS-gel electrophoresis was performed to detect proteins as we described before [36, 37]. The LV tissue samples were solubilized in sample buffer (8% SDS, 40% Glycerin, 200 mM DTT, 0.4% Bromphenol blue, 253 mM Tris/HCl pH 6.8). The samples were then boiled for 3 min at 97 °C, followed by centrifugation for 3 min at 13,000 rpm. Protein concentration was determined using the Pierce 660 nm Protein Assay (22660 Thermo Fisher). Samples were separated 10–15% SDS-PAGE. Gels were run at 90 V for 20 min followed by 125 V for about 90 min (running buffer; 24.76 mM Tris, 192 mM Glycine, 3.47 mM SDS). After electrophoresis, proteins were transferred onto a PVDF membrane (Immobilon-P transfer membrane, 0.45 μ m, Merck Millipore) using the Trans-Blot Turbo system from Bio-Rad (Anode buffer 300 mM Tris, 100 mM Tricin, pH 8.8; cathode buffer 300 mM Aminoheptanoic Acid, 30 mM Tris, pH 8.7 pH). The membrane was activated with methanol for 1 min before the transfer. Afterwards, the blots were blocked with 5% BSA in TBST (20 mM TRIS, 137 mM NaCl, 0.1% Tween 20) for 1 h at RT and subsequently with primary antibody overnight at 4 °C. After washing with TBST, the secondary antibody was applied for 1 h at room temperature in 5% BSA in TBST and the signal was detected with the ChemiDoc MP imaging system from Bio-Rad. For the analysis, the signal was normalized to GAPDH using Multi Gauge V3.2 and BioRad ImageLab V6.1 software (Table 3).

Quantification of tissue oxidative and metabolic stress and inflammation

Myocardial levels (n = 10 samples/group) of inflammatory and oxidative stress markers were measured with enzyme-linked immunosorbent assay (ELISA) and colorimetric assay kits according to the manufacturer's instructions. The following kits were used in this study: high mobility group box protein 1 (HMGB1) ELISA kit (MBS701378; MyBioSource), Toll-like receptor 2 (TLR2) ELISA kit (RAB0744-1KT; Sigma-Aldrich), TLR4 ELISA kit (RAB1088-1KT; Sigma-Aldrich), receptor for advanced glycation end products (RAGE) ELISA Kit (ab190807; Abcam), NOD-like receptor protein 3 (NLRP3) ELISA Kit (ab190807; Abcam), IL-6 ELISA kit (ab100772; Abcam) and 3-nitrotyrosine ELISA kit (ab116691; Abcam), lipid peroxidation (malondialdehyde; LPO) ELISA Kit (ab118970; Abcam), tumor necrosis factor (TNF α) ELISA kit (ab108913; abcam), Intercellular adhesion molecule 1 (ICAM-1) ELISA kit (ERICAM1;

Thermo Fisher), vascular cell adhesion molecule 1 (VCAM-1) ELISA kit (KHT0601; Thermo Fisher).

Stable hydrogen peroxide (H₂O₂) accumulation was also assessed in myocardial homogenates and mitochondrial fractions using a colorimetric assay (Sigma Aldrich). The GSSG/GSH ratios, LPO and H₂O₂ levels were determined both in myocardial homogenates and in the mitochondrial fraction. For fractionation, a subcellular protein fractionation kit (78840; Thermo Fisher Scientific) was used according to the manufacturer's instructions.

Quantification of nitric oxide (NO) level

The levels of NO were measured by means of a colorimetric assay kit (BioVision Inc, Milpitas, CA, USA) providing the measurement of total nitrate/nitrite as previously described [36, 38]. NO production was measured in tissue homogenates. Briefly, LV tissue samples (n = 10 LV samples/group) were treated with trichloroacetic acid (8 g in 80 mL acetone; Sigma-Aldrich) and washed with 1 mL 0.2% dithiothreitol (DTT). Tissue samples were homogenized in 1% SDS sample buffer (Tri-distilled water: 8.47 mL; glycerol: 2.1 mL; 10% SDS: 1.4 mL; 0.5 M Tris-HCl (pH 6.8): 1.75 mL; brome-phenol blue: 0.28 mL; DTT: 32.4 mg; all from Sigma-Aldrich). After homogenization, tissue samples underwent sonication and were subsequently centrifuged at 14,000 g for 15 min at 2–8 °C. Supernatants containing equal amounts of total protein were analysed for NO concentration. sGC was measured in 100 mg tissues, which were rinsed and homogenised in PBS and stored overnight in –20 °C.

Measurement of soluble guanylyl cyclase (sGC) activity

The activity of sGC was assessed by means of a colorimetric assay kit (MyBioSource) as we described before [36]. In brief, 100 mg of tissue (n = 10 LV samples/group) were rinsed and homogenised in PBS and stored overnight in –20 °C. These tissue samples underwent sonication and were subsequently centrifuged at 14,000 g for 15 min at 2–8 °C. Supernatants containing equal amounts of total protein were analysed for sGC activity according to manufacturer's instructions. The absorbance of samples was measured at 570 nm using a plate reader.

Measurement of myocardial cyclic guanosine monophosphate (cGMP) level

Myocardial cGMP levels were measured according to previous protocols [39, 40]. Briefly, cGMP was determined in LV homogenates (n = 10 samples/group) by means of a parameter cGMP assay immunoassay kit (R&D Systems, Minneapolis, MN, United States), in which cGMP present in the homogenate competes with a fixed amount of HRP-labeled cGMP for sites on a rabbit polyclonal antibody. These homogenates were diluted in cell lysis buffer, and 100 µL of 0.025 µg/µL protein

aliquots were measured according to the manufacturer's instructions. The results of duplicate determinations were averaged and expressed as µg/µL.

Measurement of myocardial protein kinase G (PKG) activity

LV tissue samples (n = 10 samples/group) were homogenized in 25 mM Tris-HCl (pH 7.4), 1 mM EDTA, 2 mM EGTA, 5 mM DTT, 0.05% Triton X-100, and protease inhibitor cocktail (all from Sigma-Aldrich) and centrifuged for 5 min. Supernatants containing equal amounts of total protein were analysed for PKG activity as described previously [39]. In brief, reaction mixtures were incubated at 30 °C for 10 min. Reaction mixtures contained 40 mM Tris-HCl (pH 7.4), 20 mM Mg(CH₃COO)₂, 0.2 mM [32P] adenosine triphosphate (ATP) (500–1,000 cpm pM⁻¹; Amersham PLC, Little Chalfont, UK), 113 mg/mL heptapeptide (RKRSRAE) and 3 µM cGMP (both from Promega Corp, Madison, WI, USA), and a highly specific inhibitor of cyclic adenosine monophosphate-dependent protein kinase (5–24; Calbiochem, San Diego, CA, USA). The reaction was terminated by spotting 70 µL onto Whatman P-81 filters (MACHEREY-NAGEL). Samples were subsequently incubated and washed with 75 mM H₃PO₄ for 5 min to remove unbound ATP. Filters were then washed with 100% ethanol and air dried before quantification. PKG activity was quantified using a Wallac 1409 Liquid Scintillation Counter (Hidex Oy, Turku, Finland). The specific activity of PKG was expressed as pM of 32P incorporated into the substrate (pM/min/mg protein).

RNA isolation and quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

In the initial stage, human tissue samples were carefully weighed, ranging between 50 to 100 mg. The entire procedure was executed to preserve RNA activity under the application of liquid nitrogen. For homogenization, one stainless steel bead and 1 mL of QIAzol Lysis Reagent (Qiagen, 79306) were added to each tissue sample on ice under a safety bench. The samples were homogenized in the Precellys Evolution Touch Homogenizer (Berlin Technologies) for 2 cycles of 5 min at 5000 rpm with cooling. After incubation on ice for 5 min, the samples were centrifuged at 13,000 rpm for 10 min at 4 °C. The supernatant was carefully transferred to a clean tube, and 200 µL of chloroform was added. The mixture was vortexed, incubated at RT for 3 min, and then centrifuged at 13,000 rpm for 15 min to facilitate phase separation. The RNA-containing aqueous phase was subsequently carefully transferred to a new tube.

Next, 360 µL of 100% isopropanol and 1 µL of Glyco-gen azure (Sigma Aldrich, G5510) were added, mixed, and incubated for 10 min at RT. After centrifugation at 13,000 rpm for 10 min, the supernatant was removed,

and the RNA pellet was washed four times with 1 mL of 75% ethanol, followed by centrifugation at 7500 rpm for 5 min each time. After the final wash, the pellet was air-dried and resuspended in 10 µL of RNase-free water. RNA concentrations were measured using spectrophotometry (Implen Nanophotometer® N60).

cDNA synthesis

The working solution was prepared using the Sensifast cDNA synthesis kit (Bioline, BLI 65053), by mixing 4 µL of 5× TransAmp Buffer and 1 µL of reverse transcriptase/reaction. The 0.2 ml RNase free Eppendorf tubes were filled with 5 µL of the working solution, 0.5 µg RNA per sample, and nuclease-free water to a final volume of 20 µL. Tubes were processed in a thermal cycler for cDNA synthesis. Afterward, cDNA purity was checked using spectrometry, and samples were stored at -80 °C for subsequent qRT-PCR analysis.

The primers for the experiments were designed using the Primer-BLAST tool provided by the National Center for Biotechnology Information (NCBI). Gradient PCR measurements were carried out on the primers to determine their annulation temperature. Quality control of the primer products was performed using agarose gel electrophoresis after the PCR reactions.

Initially, the primers were dissolved, and their concentration adjusted to a final concentration of 100 µM with nuclease-free water. Reaction mixtures were prepared by combining 7.5 µL/reaction of SensiFAST SYBR® NO-ROX Kit (Bioline, BIO-98020), 4.85/reaction µL of nuclease-free water, and 0.075 µL/reaction of forward and reverse primer solutions, respectively. Subsequently, 12.5 µL of the reaction mixture was dispensed into each well of a 384-well LC Multiwell Plate (Roche), followed by 2.5 µL of the respective samples in duplicates. The plate was sealed, centrifuged at 1500 rpm for 2 min, and placed onto the LightCycler® 480 qPCR machine (Roche).

The program was initiated using the LightCycler® 480 SW 1.5.1 software, with data analysis completed after approximately 1.5 h. The results were calculated using the 2 - ΔΔCp method. Peptidylprolyl isomerase A (PPIA) and ribosomal protein L13A (RPL13A) functioned as house-keeping genes (Table 4).

Statistics

We used estimation plots which show on the left axis the data as box and whisker plots (median, 25th to 75th percentiles, minimum and maximum) in order to present individual points in each group. The effect size meaning the difference between the means (mean ± SEM) is shown on the right axis. The *P*-values are from unpaired Student's *t*-test; * *P* < 0.05, ** *P* < 0.01, *** *P* < 0.001. For the analysis of force measurements, which involves parametric data comparing more than two groups, one-way ANOVA was used. *P*-values were adjusted for multiple comparisons using the Tukey method; significant comparisons include * *P* < 0.05 HFpEF-DM vs. HFpEF + DM, † *P* < 0.05 HFpEF-DM vs. HFpEF-DM after treatment, † *P* < 0.05 HFpEF + DM vs. HFpEF + DM after treatment by one-way ANOVA. For analysis of proportions, Fisher's exact test was used. The analysis was performed using GraphPad Prism 10. *P*-values are two-sided and considered statistically significant if *P* < 0.05.

Results

Increased inflammatory signaling pathways and inflammasome activation in HFpEF patients with diabetes mellitus

Inflammation accelerates the deterioration of cardiac structure and function in patients with DM, ultimately leading to heart failure. Figure 1A illustrates the inflammatory signaling pathway that serves a crucial role in linking chronic systemic inflammation and insulin resistance in patients with HFpEF, both diabetic and

Table 4 Corresponding forward and reverse primers list used for qRT-PCR

Target gene	Accession Number	Primer Name	Sequence	Manufacturer
MTOR	NM_004958.4	hsa_MTOR_fw	GAAGCCGCGCGAACCT	Bio-Science Kft
		hsa_MTOR_rev	ATTCGGCTCTTTAGGCCAC	
MAPK1	NM_002745.5	hsa_MAPK1_fw	ATTACGACCCGAGTGACGAG	Bio-Science Kft
		hsa_MAPK1_rev	CTCTGAGCCCTTGCTCTA	
MAPK3	NM_002746.3	hsa_MAPK3_fw	CATGCTGAACCTCCAAGGGCTA	Bio-Science Kft
		hsa_MAPK3_rev	GATCCAGGTAGTGCTTGCCA	
AMPK	NM_006251.6	hsa_AMPK_fw	CGGCAAAGTGAAGTTGGC	Bio-Science Kft
		hsa_AMPK_rev	CCTACCACATCAAGGCTCCG	
NOS3	NM_000603.5	hsa_NOS3_fw	CCGGAACAGCACAAAGAGTT	Bio-Science Kft
		hsa_NOS3_rev	CCCTGCACTGTCTGTGTAC	
PRKG1	NM_006258.4	hsa_PRKG1_fw	AAGAGCCACAGTCCAAGG	Bio-Science Kft
		hsa_PRKG1_rev	ATCTGCGACAGCTCCAAGTT	
STK11	NM_000455.5	hsa_STK11_fw	GAGGCCAGTCACAATGGACA	Bio-Science Kft
		hsa_STK11_rev	CCTGGACACGGGCTGC	

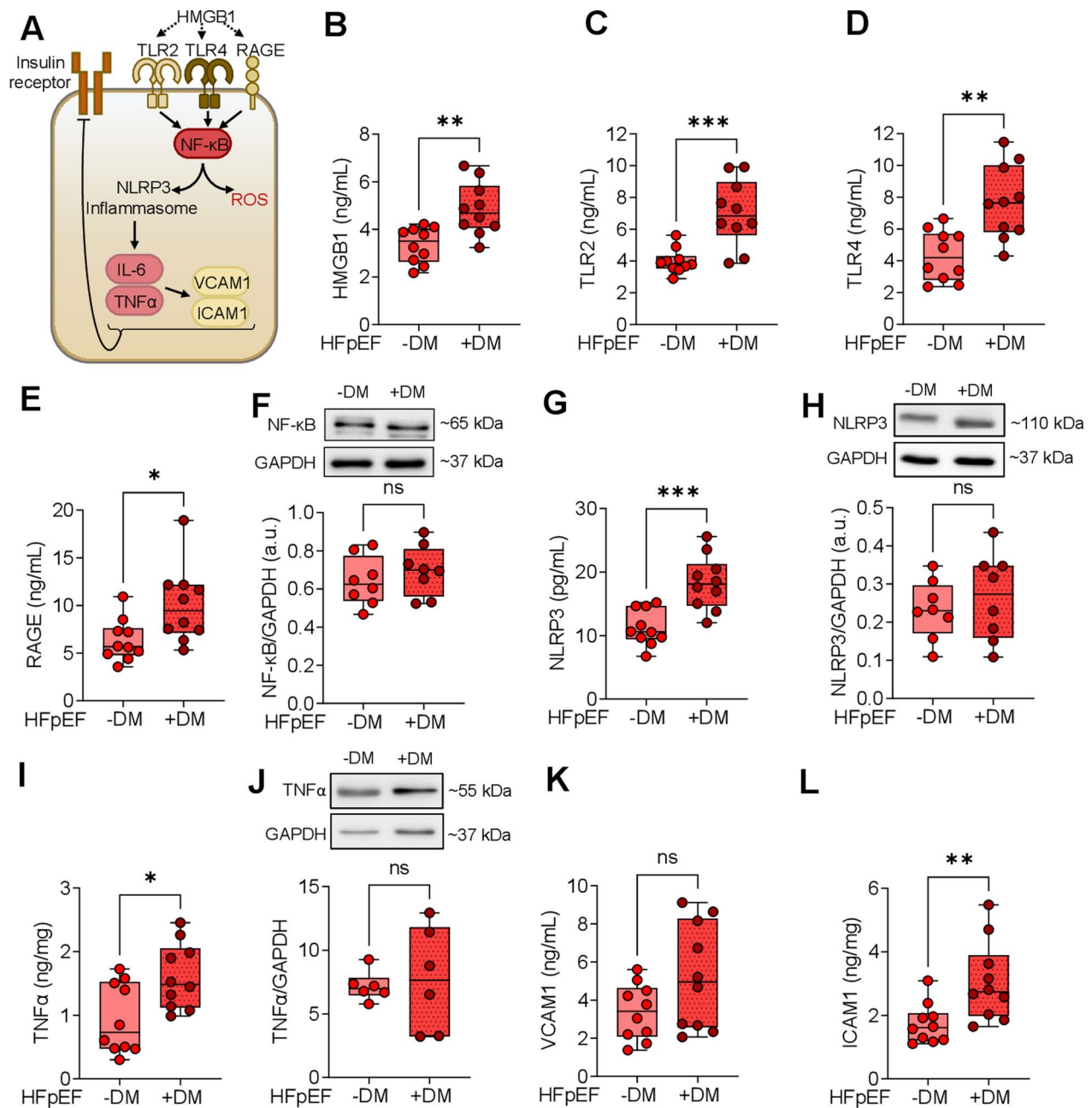


Fig. 1 Signaling Pathways of Inflammation in Left Ventricular Biopsies from Patients with HFpEF, with (+DM) and without Concomitant Diabetes (-DM). **A** Schematic representation of intracellular inflammatory signaling pathways. HMGB1: high mobility group box protein 1; ICAM1: intercellular adhesion molecule; IL-6: interleukin 6; NF- κ B: nuclear factor kappa B; NLRP3: NOD-like receptor protein 3; RAGE: receptor for advanced glycation end products; ROS: reactive oxygen species; TLR: Toll-like receptor; TNF α : tumour necrosis factor α ; VCAM1: vascular cell adhesion protein. **B** HMGB1 levels. **C** TLR2 levels. **D** TLR4 levels. **E** RAGE levels. **F** NF- κ B expression over GAPDH. **G** NLRP3 levels. **H** NLRP3 expression over GAPDH. **I** TNF α levels. **J** TNF α expression over GAPDH. **K** VCAM1 levels. **L** ICAM1 levels. Data are represented as box and whisker plots (median, 25th to 75th percentiles, minimum, and maximum (n=6–10 samples/group)). *P*-values are derived from an unpaired *t*-test; * *P* < 0.05, ** *P* < 0.01, *** *P* < 0.001

non-diabetic. The activation of the inflammasome is initiated by the binding of DAMPs to TLRs and the RAGE, which triggers the NF- κ B signaling pathway and subsequently activates NLRP3 inflammasome [41]. Several stimuli, including oxidative stress, can promote the activation of the NLRP3 inflammasome. NF κ B activation

further induces the release of pro-inflammatory cytokines such as IL-6, TNF α , ICAM-1, and VCAM-1 [42, 43], which ultimately exacerbates insulin resistance in diabetic patients. In myocardial biopsies from HFpEF patients with DM, a significant upregulation of DAMPs such as HMGB1 (Fig. 1B), toll-like receptors including

TLR2 (Fig. 1C) and TLR4 (Fig. 1D), as well as RAGE (Fig. 1E), was observed when compared to HFpEF patients without DM. The increased ligand-receptor binding was accompanied by significantly elevated levels of the NLRP3 inflammasome in HFpEF patients with DM compared to their non-diabetic counterparts (Fig. 1G), although no significant differences were noted in NLRP3 and NF κ B protein expression levels between the two groups (Fig. 1F, H). Pro-inflammatory cytokines such as TNF α exhibited significantly increased concentration levels in the HFpEF DM group, while TNF α protein expression levels were non-significantly elevated in this group compared to the HFpEF non-DM group (Fig. 1I, J). Additionally, the concentration of the pro-inflammatory cytokine ICAM-1 was significantly increased in HFpEF DM patients compared to HFpEF non-DM patients, whereas VCAM-1 levels were non-significantly elevated in HFpEF DM patients (Fig. 1L, K), indicating a pronounced presence of inflammation in HFpEF patients with DM.

Elevated IL6 levels and improvement in cardiomyocyte passive stiffness upon IL6 inhibitor treatment

The concentration and protein expression levels of IL6 were significantly upregulated in HFpEF DM patients compared to HFpEF non-DM patients (Fig. 2A, B) and clearly in line with enhanced fluorescence intensity shown by immunohistochemistry staining in HFpEF DM patients compared to HFpEF non-DM patients (Fig. 2C).

Emerging evidence suggests that chronic systemic inflammation in patients with HFpEF significantly impacts various cardiac structural and functional alterations. These alterations encompass concentric remodeling, increased cardiomyocyte stiffness (F_{passive}), myocardial hypertrophy, and interstitial fibrosis [44]. Consistent with these findings, we observed a significant increase in F_{passive} in isolated skinned cardiomyocytes obtained from HFpEF DM patients compared to HFpEF non-DM patients, particularly at optimal resting sarcomere lengths (SL) ranging from 2.0 to 2.4 μm (Fig. 2D). To investigate whether the observed increase in inflammatory markers correlates with these findings, we administered an IL6 inhibitor to the isolated skinned cardiomyocytes derived from both HFpEF DM and HFpEF non-DM patients. Notably, treatment with the IL6 inhibitor significantly reduced F_{passive} in HFpEF DM cardiomyocytes at SLs of 2.1 to 2.4 μm . Additionally, administration of the IL6 inhibitor resulted in a decrease in F_{passive} among HFpEF non-DM cardiomyocytes, demonstrating levels lower than those observed in HFpEF DM following IL6 inhibitor treatment. IL6 receptor expression, as determined by western blot analysis and immunohistochemistry staining, was slightly elevated in the HFpEF DM group; however, this difference did not reach statistical significance (Fig. 2E, F). Furthermore,

MPO and NE showed higher fluorescence intensity by immunohistochemistry staining in HFpEF DM patients (Fig. 2G, H).

Diminished NO-sGC-cGMP-PKG pathway in HFpEF DM patients

Figure 3A illustrates the eNOS-NO-sGC-cGMP-PKG pathway and its contribution to oxidative stress and insulin resistance in HFpEF patients with and without diabetes (Fig. 3B–H). The expression level of eNOS remained unchanged in HFpEF DM and HFpEF non-DM patients (Fig. 3B). In HFpEF DM patients, NO bioavailability was reduced, though not to a statistically significant level (Fig. 3D), while NOS3 mRNA levels were increased non-significantly in the HFpEF DM group (Fig. 3C). The reduction in NO bioavailability was accompanied by a significant decrease in sGC activity, reduced cGMP levels, and subsequently a significant reduction in PKG activity observed in HFpEF DM patients compared to HFpEF non-DM patients (Fig. 3E, F, G). However, at the mRNA expression level, PKG exhibited no significant differences between HFpEF diabetic and non-diabetic groups (Fig. 3H).

Increased oxidative stress markers and antioxidative treatment mediated reduction in passive stiffness in HFpEF DM and HFpEF non-DM patients

It is established that oxidative stress can impair the insulin signaling pathway and is considered one of the key drivers of insulin resistance in T2DM patients. Consequently, we investigated the presence of oxidative stress by evaluating oxidative stress markers in HFpEF patients with and without DM (Fig. 4A–D). Oxidative stress markers, including the reduced/oxidized glutathione ratio (GSSG/GSH), hydrogen peroxide (H_2O_2), LPO levels were significantly elevated in LV myocardial tissue of HFpEF DM patients compared to HFpEF non-DM patients, confirming high levels of oxidative stress in HFpEF patients with diabetes (Fig. 4B–D). Conversely, the level of 3-nitrotyrosine was reduced in HFpEF DM patients, though not significantly compared to HFpEF non-DM patients (Fig. 4A).

We further explored the impact of the mitochondria-targeted antioxidative agent Mito-TEMPO in regulating cardiomyocyte passive stiffness (F_{passive}) in HFpEF DM and HFpEF non-DM isolated skinned cardiomyocytes at SLs of 1.8–2.4 μm . Administration of Mito-TEMPO significantly restored F_{passive} at SL 2.0 μm in both HFpEF DM and HFpEF non-DM cardiomyocytes, suggesting the antioxidative efficacy of Mito-TEMPO in normalizing cardiomyocyte passive tension.

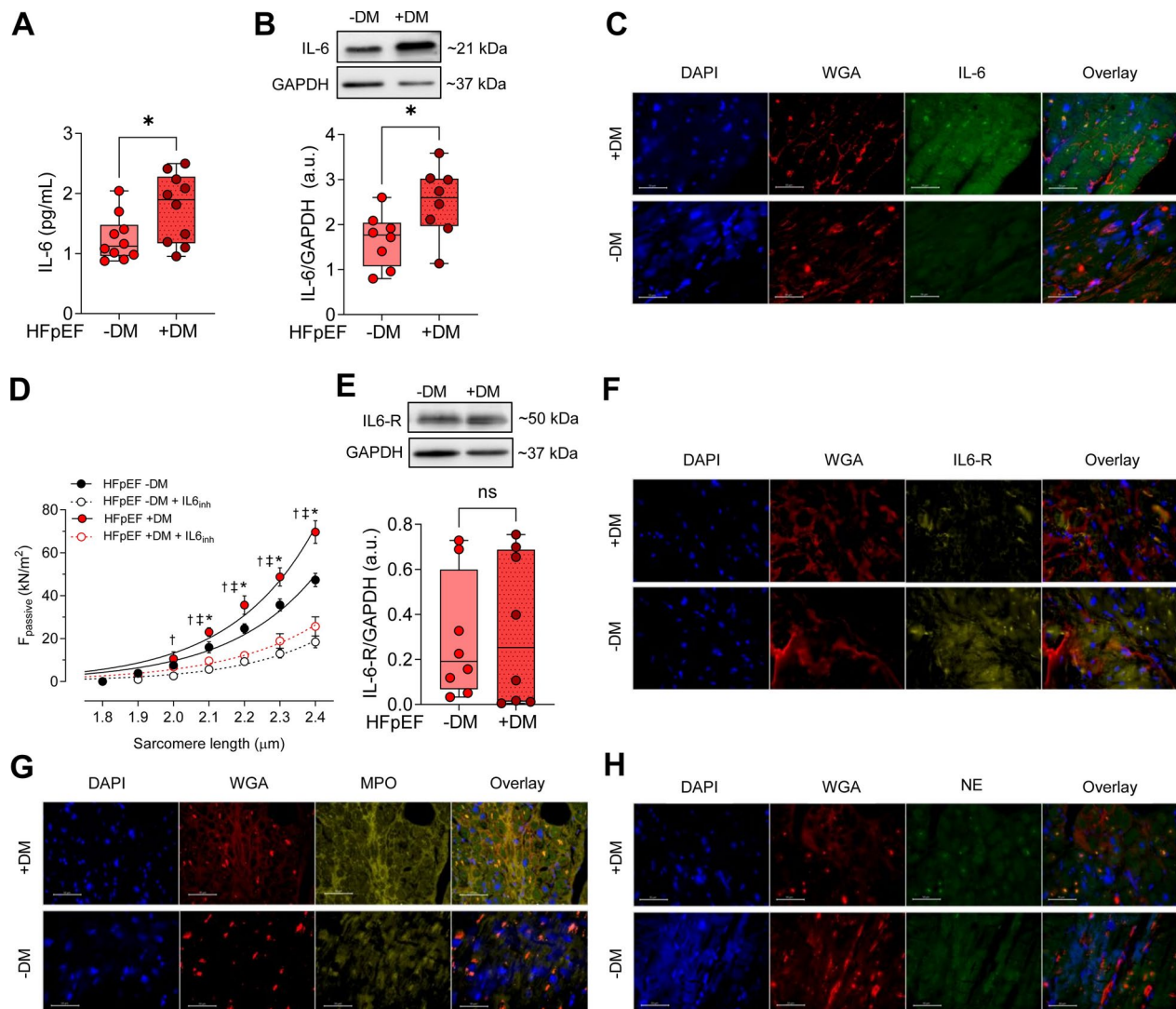


Fig. 2 Inflammatory Markers in Left Ventricular Biopsies from Patients with HFpEF, with (+ DM) and without Concomitant Diabetes (–DM). **A** Interleukin-6 (IL-6) levels. **B** IL-6 expression over GAPDH. **C** Immunofluorescence staining of IL-6. DAPI staining (blue) and WGA (anti-wheat agglutinine 555 conjugate, red) staining are used for nucleic acids and membranes. **D** F_{passive} at sarcomere length 1.8–2.4 μm in the presence or absence of IL-6 inhibitor. Curves are second-order polynomial fits to the means ($\pm$ SEM; $n = 30\text{--}36/5$ cardiomyocytes/heart per group), * $P < 0.05$ HFpEF –DM vs. HFpEF + DM, $^{\dagger}P < 0.05$ HFpEF –DM vs. HFpEF –DM after IL-6_{inh}, $^{\ddagger}P < 0.05$ HFpEF + DM vs. HFpEF + DM after IL-6_{inh} treatment by one-way ANOVA. P -values were corrected for multiple comparisons by the Tukey method. **E** IL-6 receptor (IL6-R) expression over GAPDH. **F** Immunofluorescence staining of IL6-R. **G** Immunofluorescence staining of myeloperoxidase (MPO). **H** Immunofluorescence staining of neutrophil elastase (NE). Panels **A + B + E**. Data are represented as box and whisker plots (median, 25th to 75th percentiles, minimum, and maximum ($n = 6\text{--}10$ samples/group)). P -values are derived from an unpaired t-test

Dysregulated autophagy and metabolic stress regulators in HFpEF DM patients

We evaluated PI3K/Akt/mTOR and AMPK signaling pathways that play a crucial role in metabolic stress, hypertrophy, and autophagy. Figure 5A illustrates insulin-mediated key signaling pathways involved in regulating cellular metabolism, specifically focusing on protein synthesis, cellular growth, and autophagy. Cardiomyocytes abundantly exhibit insulin receptors that facilitate the binding of insulin to the insulin receptors, interact with insulin receptor substrate proteins, and activate downstream signaling pathways such as PI3K/ AKT

[45]. AKT activation can phosphorylate multiple targets, including the mTOR, which plays a crucial role in coordinating cell growth, metabolism, and cell proliferation. Furthermore, AMPK is a key sensor of cellular energy status that is activated under conditions of low intracellular ATP [46]. The serine/threonine kinase STK11 is the major kinase responsible for phosphorylation and activating AMPK under stress conditions [47]. Consequently, AMPK directly phosphorylates the component regulatory protein associated with mTOR, leading to suppression of mTORC1 activity through allosteric inhibition [48].

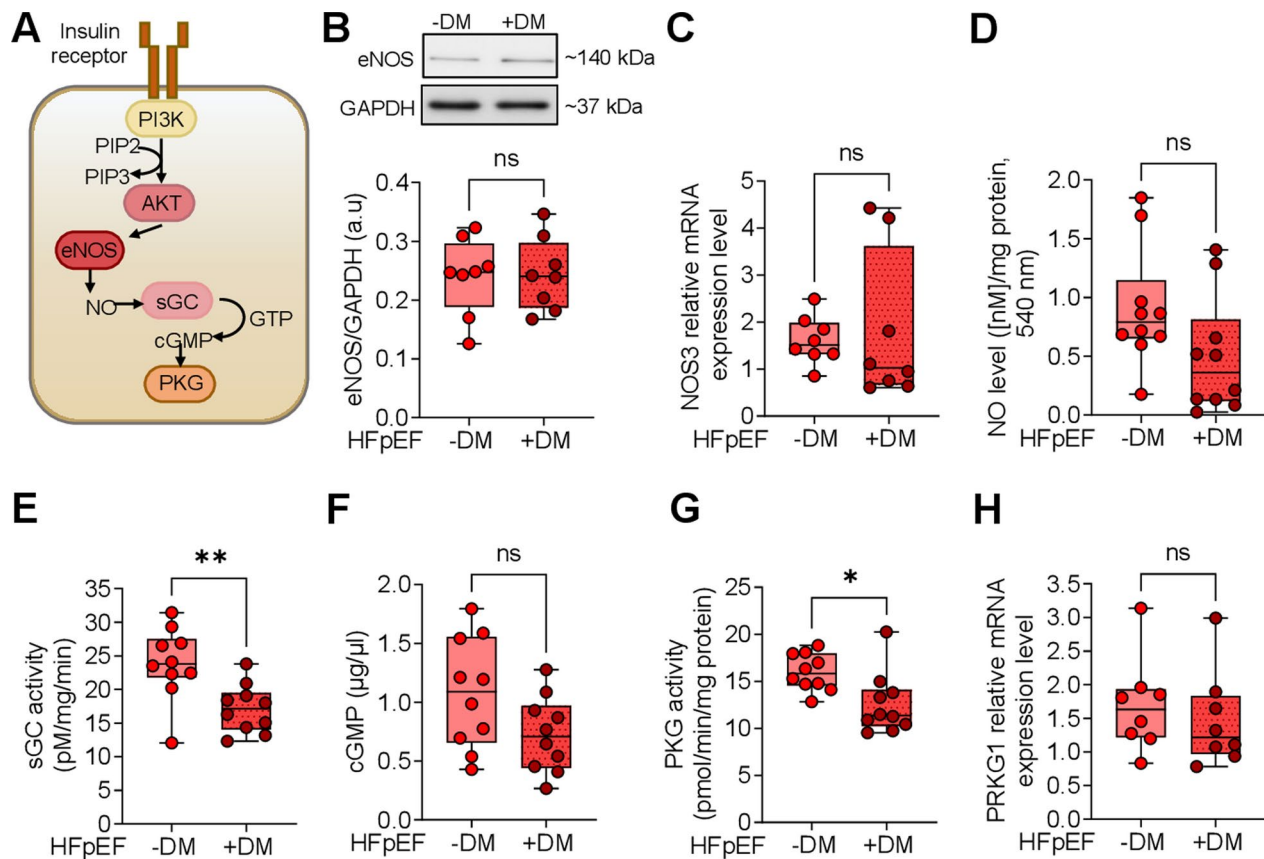


Fig. 3 Protein kinase G Signaling Pathway in Inflammation in Left Ventricular Biopsies from Patients with HFpEF, with (+DM) and without Concomitant Diabetes (-DM). **A** Schematic representation of the PKG signaling pathway. AKT: AKT serine/threonine kinase; cGMP: cyclic guanosine monophosphate; eNOS, endothelial nitric oxide synthase; GTP: guanosine triphosphate; NO: nitric oxide; PI3K: phosphatidylinositol-3-kinase; PIP2: phosphatidylinositol-4,5-bisphosphate; PIP3: phosphatidylinositol-3,4,5-trisphosphate; PKG: protein kinase G; sGC: soluble guanylyl cyclase. **B** Expression of eNOS over GAPDH. **C** NOS3 mRNA level. **D** NO level. **E** Activity of sGC. **F** Myocardial cGMP level. **G** PKG activity. **H** PRKG1 mRNA level. Data are represented as box and whisker plots (median, 25th to 75th percentiles, minimum, and maximum ($n=8-10$ samples/group)). P -values are derived from an unpaired t -test; * $P < 0.05$, ** $P < 0.01$

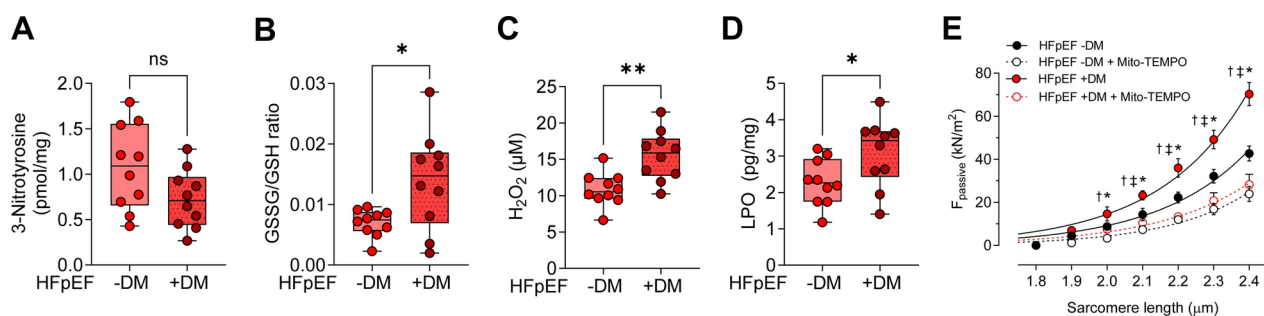


Fig. 4 Oxidative Stress Markers in Inflammation in Left Ventricular Biopsies from Patients with HFpEF, with (+DM) and without Concomitant Diabetes (-DM). **A** 3-Nitrotyrosine levels. **B** Ratio of myocardial oxidized glutathione (GSSG) over reduced glutathione (GSH). **C** Myocardial hydrogen peroxide (H_2O_2) levels. **D** Myocardial lipid oxidation (LPO) levels. Data are represented as box and whisker plots (median, 25th to 75th percentiles, minimum, and maximum ($n=10$ samples/group)). P -values are derived from an unpaired t -test; * $P < 0.05$, ** $P < 0.01$. **E** F_{passive} at sarcomere length 1.8–2.4 μm in the presence or absence of Mito-TEMPO. Curves are second-order polynomial fits to the means ($\pm$ SEM; $n=30-36/5$ cardiomyocytes/heart per group), * $P < 0.05$ HFpEF -DM vs. HFpEF + DM, † $P < 0.05$ HFpEF -DM after Mito-TEMPO, ‡ $P < 0.05$ HFpEF + DM vs. HFpEF + DM after Mito-TEMPO treatment by one-way ANOVA. P -values were corrected for multiple comparisons by the Tukey method

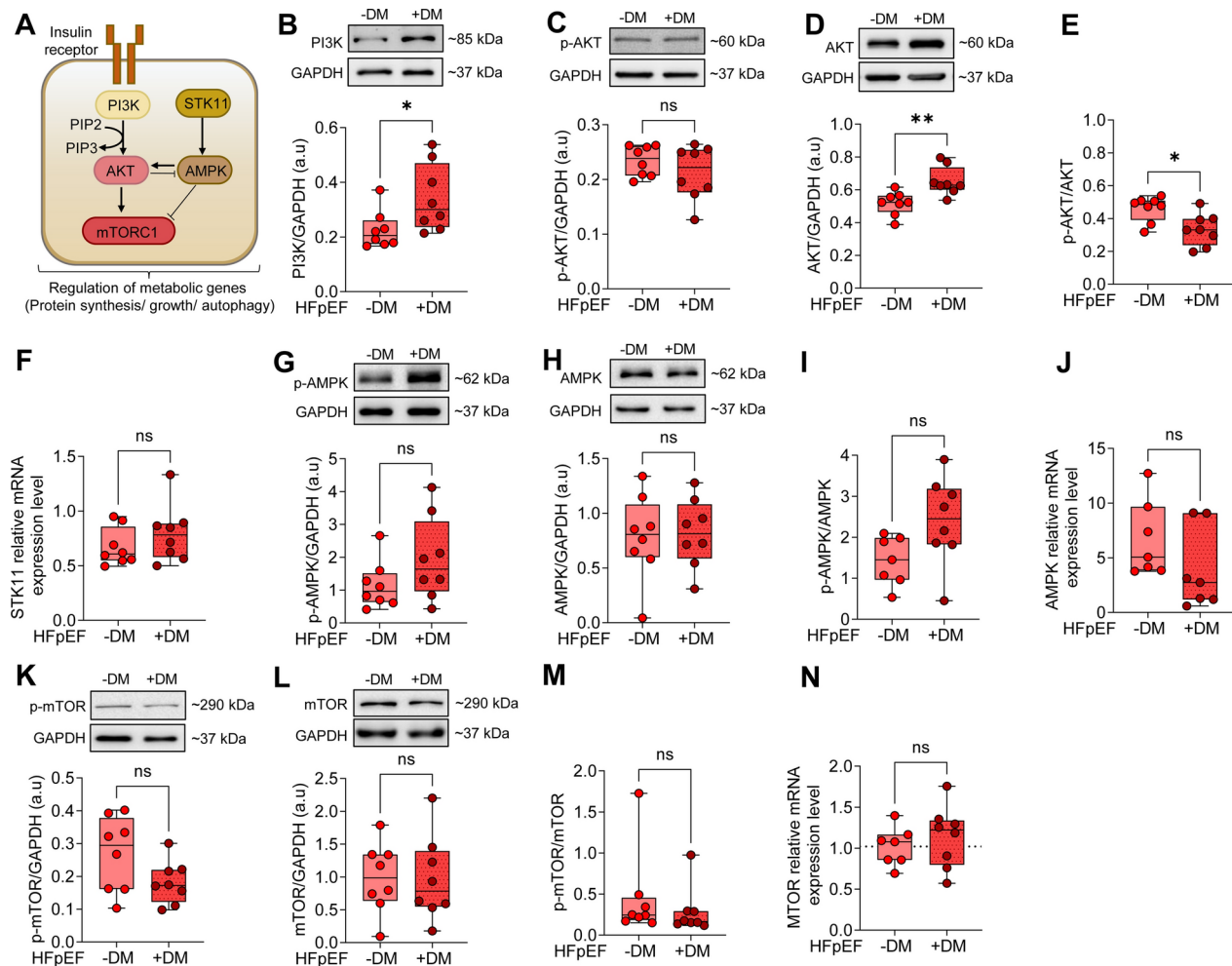


Fig. 5 The PI3K/AKT/AMPK/mTOR Signaling Pathway Left Ventricular Biopsies from Patients with HFpEF, with (+ DM) and without Concomitant Diabetes (-DM). **A** Schematic representation of the PI3K/AKT/AMPK/mTOR signaling pathway. AMPK: AMP-activated protein kinase; AKT: AKT serine/threonine kinase; mTORC1: mammalian target of rapamycin complex 1; PI3K: phosphatidylinositol-3-kinase; PIP2: phosphatidylinositol-4,5-bisphosphate; PIP3: phosphatidylinositol-3,4,5-trisphosphate; STK11: serine/threonine kinase 11. **B** Expression of PI3K over GAPDH. **C** Phosphorylation of AKT over GAPDH. **D** Expression of AKT over GAPDH. **E** Ratio of phosphorylated over total AKT. **F** STK11 mRNA level. **G** Phosphorylation of AMPK over GAPDH. **H** Expression of AMPK over GAPDH. **I** Ratio of phosphorylated over total AMPK. **J** AMPK mRNA level. **K** Phosphorylation of mTOR over GAPDH. **L** Expression of mTOR over GAPDH. **M** Ratio of phosphorylated over total mTOR. **N** mTOR mRNA level. Data are represented as box and whisker plots (median, 25th to 75th percentiles, minimum, and maximum (n = 7–10 samples/group). *P*-values are derived from an unpaired *t*-test; * *P* < 0.05, ** *P* < 0.01

In our study, PI3K expression demonstrated an increased trend in myocardial tissue of HFpEF DM patients compared to HFpEF non-DM patients (Fig. 5B). It was accompanied by the significantly heightened expression of AKT in HFpEF DM patients (Fig. 5D). However, no significant differences were observed in the phosphorylated status of AKT in HFpEF DM and HFpEF non-DM groups (Fig. 5C), while the activation of AKT was significantly reduced in the HFpEF DM group denoted by the p-AKT/total AKT ratio (Fig. 5E). Furthermore, the mRNA level of STK11 remained unchanged in both groups (Fig. 5F). Despite the unchanged STK11 level, the phosphorylation status of AMPK was upregulated (Fig. 5G), and the ratio of phospho/total AMPK showed an increased tendency but was non-significant

in HFpEF DM patients compared to HFpEF non-DM patients (Fig. 5I), indicating the increased activation of AMPK in HFpEF diabetic hearts. Nevertheless, no alteration was observed in the AMPK protein expression level between both groups (Fig. 5H), and AMPK mRNA expression level was non-significantly reduced in the HFpEF DM group (Fig. 5J).

Additionally, we evaluated the downstream target of AKT, including the protein expression, mRNA expression, and phosphorylation levels of mTOR in HFpEF patients with and without diabetes (Fig. 5K–N). The phosphorylation status of mTOR non-significantly exhibited a downward trend in HFpEF DM patients (Fig. 5K). The protein expression level of mTOR detected by western blots was similar in both groups (Fig. 5L), while the

mRNA expression level was slightly increased non-significantly in the HFpEF DM group (Fig. 5N). In alignment with the phosphorylation status of mTOR, the p-mTOR/total mTOR ratio was non-significantly reduced in HFpEF DM patients compared to HFpEF non-DM patients (Fig. 5M).

Dysregulated MAPK signaling pathways in HFpEF DM and HFpEF non-DM hearts

We investigated an additional downstream pathway of insulin signaling, specifically the MAPKs pathway. Figure 6A illustrates the activation of the MAPKs pathway, which is associated with impaired insulin signaling

leading to insulin resistance in the diabetic heart. The activation of the mitogen-activated protein kinase kinase 1/2 (MEK 1/2)-extracellular signal-regulated kinases 1/2 (ERK 1/2) (MEK/ERK pathway) and p38 MAPK pathways elevates the expression of GLUT1 while reducing the expression of GLUT4, resulting in increased basal glucose uptake and impaired insulin-sensitive uptake. Furthermore, the AKT activation pathway, stimulated by insulin binding, represents the primary regulatory pathway that phosphorylates glycogen synthase kinase-3 (GSK3) and inhibits its activity [49]. MAPKs can also phosphorylate and inhibit GSK3, which is associated with cardiac energy metabolism, inflammation, and fibrosis

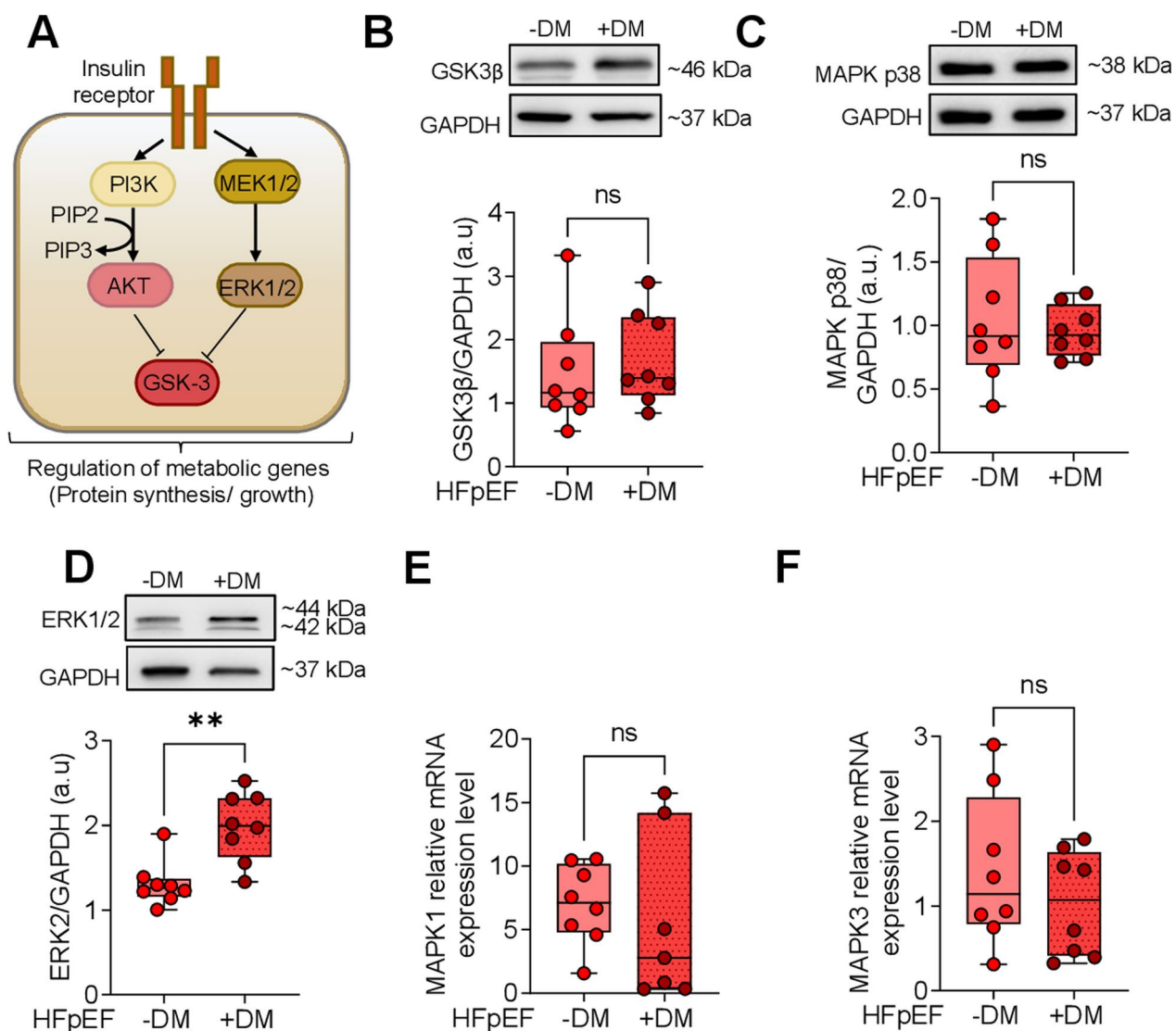


Fig. 6 MAPKs and GSK3β in Left Ventricular Biopsies from Patients with HFpEF, with (+DM) and without Concomitant Diabetes (-DM). **A** Schematic representation of the PI3K/AKT/ERK signaling pathway. AKT: AKT serine/threonine kinase; ERK1/2: extracellular-signal regulated kinases; GSK-3: glycogen synthase kinase 3; MEK: mitogen-activated protein kinase; PI3K: phosphatidylinositol-3-kinase; PIP2: phosphatidylinositol-4,5-bisphosphate; PIP3: phosphatidylinositol-3,4,5-trisphosphate; **B** Expression of GSK3β over GAPDH. **C** Expression of MAPK p38 over GAPDH. **D** Expression of ERK2 over GAPDH. **E** MAPK1 mRNA level. **F** MAPK3 mRNA level. Data are represented as box and whisker plots (median, 25th to 75th percentiles, minimum, and maximum (n=6–10 samples/group). P-values are derived from an unpaired t-test; ** P<0.01

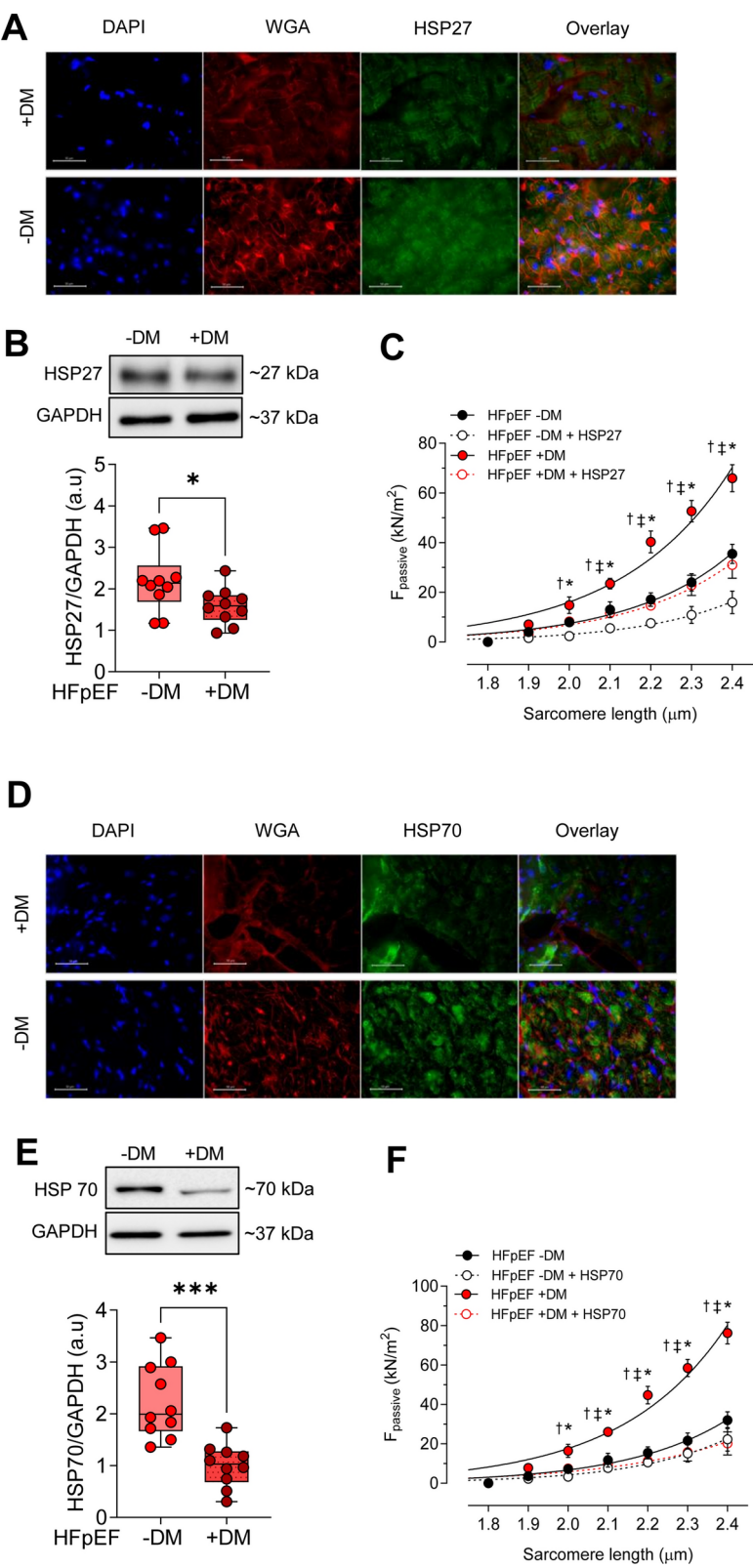


Fig. 7 (See legend on next page.)

(See figure on previous page.)

Fig. 7 Heat-Shock Proteins (HSPs) and Cardiomyocyte Passive Stiffness in Left Ventricular Biopsies from Patients with HFpEF, with (+DM) and without Concomitant Diabetes (-DM). **A** Immunofluorescence staining of HSP27. DAPI staining (blue) and WGA (anti-wheat agglutinine 555 conjugate, red) staining are used for nucleic acids and membranes. **B** Expression of HSP27 over GAPDH. **C** F_{passive} at sarcomere length 1.8–2.4 μm in the presence or absence of HSP27. **D** Immunofluorescence staining of HSP70. **E** Expression of HSP70 over GAPDH. **F** F_{passive} at sarcomere length 1.8–2.4 μm in the presence or absence of HSP70. Panels **B+E**: Data are represented as box and whisker plots (median, 25th to 75th percentiles, minimum, and maximum ($n=6-10$ samples/group)). P -values are derived from an unpaired t -test; * $P<0.05$, *** $P<0.001$. Panels **C+F**: Curves are second-order polynomial fits to the means ($\pm$ SEM; $n=30-36/5$ cardiomyocytes/heart per group). * $P<0.05$ HFpEF -DM vs. HFpEF +DM, † $P<0.05$ 0.05 HFpEF -DM vs. HFpEF -DM after HSP27 or HSP70 treatment, ‡ $P<0.05$ HFpEF +DM vs. HFpEF +DM after HSP27 or HSP70 treatment by one-way ANOVA. P -values were corrected for multiple comparisons by the Tukey method

[50]. In our study, the expression level of GSK3 β protein was slightly upregulated in myocardial tissue from HFpEF and DM compared to those with HFpEF and non-DM (Fig. 6B). The protein expression level of p38 MAPK was non-significantly reduced in HFpEF DM patients compared to HFpEF non-DM patients (Fig. 6C). Conversely, ERK 1/2 protein expression demonstrated a significant increase in HFpEF DM patients (Fig. 6D). The mRNA expression level of MAPK1 was markedly increased in the HFpEF DM group, while the mRNA level of MAPK3 was decreased in the HFpEF DM group compared to the HFpEF non-DM group; however, these observations did not reach statistical significance (Fig. 6E, F).

Alterations of HSPs and HSPs mediated improvement in passive stiffness in HFpEF DM and HFpEF non-DM patients
HSPs and their co-chaperons are well-defined as crucial players in PQS due to their pivotal roles in correcting misfolded proteins, directing the aberrant proteins to degradation, and inhibiting apoptosis. Therefore, we examined the alteration of heat shock proteins in HFpEF patients in both diabetic and non-diabetic groups. A significant decrease in the protein expression level of heat shock proteins, including HSP27 and HSP70, was observed in HFpEF DM samples compared to HFpEF non-DM samples (Fig. 7B, E). Reduced HSP27 and HSP70 expression is also supported by a clear reduction of the fluorescence intensity shown by immunohistochemistry staining in HFpEF DM group compared to HFpEF non-DM group (Fig. 7A, D).

To investigate whether alterations of HSPs are associated with the cardiomyocyte dysfunction, we tested the ability of HSP27, and HSP70 to reverse the elevated F_{passive} in HFpEF DM and non-DM patients. Figure 7C and F illustrate a significantly elevated F_{passive} in cardiomyocytes from HFpEF DM isolated skinned cardiomyocytes when compared to those from HFpEF patients without DM, particularly at optimal resting SL ranging from 1.8 to 2.4 μm . Administration of HSP70 corrected the increased F_{passive} at SL 2.0 μm in HFpEF DM cardiomyocyte, while no significant effects on F_{passive} was detected following HSP70 administration in HFpEF non-DM cardiomyocyte (Fig. 7F). Furthermore, a significant decrease in F_{passive} was observed in HFpEF DM cardiomyocytes following HSP27 administration, but it could

not restore the F_{passive} and the reduced F_{passive} was similar to the F_{passive} of HFpEF non-DM cardiomyocyte without HSP27 treatment (Fig. 7C). In contrast, HSP27 administration restored the F_{passive} among HFpEF non-DM cardiomyocytes, exhibiting reduced levels compared to those in HFpEF DM post-treatment with HSP27 (Fig. 7C).

Discussion

This study provides evidence of increased myocardial oxidative stress and diminished PQC, as manifested by altered autophagy and HSPs. Additionally, it highlights increased apoptotic processes and dysregulated insulin signaling pathways, which are implicated in glucose uptake, metabolic stress, and post-translational modifications in HFpEF-DM compared to non-DM, all of which contribute to increased F_{passive} .

Myocardial inflammation induced by metabolic abnormalities in diabetes-heart

Diabetes-associated metabolic dysregulations trigger cytokine release and expression in cardiomyocytes [51]. Hyperglycemia, a hallmark of diabetes, was shown to upregulate HMGB1 activity in HFpEF-DM patients compared to the non-DM group. The interaction between HMGB1 and its receptors (TLR4, TLR2, and RAGE) predominantly activates NF- κ B, a classic inflammatory mediator [52]. According to Mariappan et al., NF- κ B activation is a critical first step in cardiac damage [53]. Romeo et al. demonstrated that in diabetic individuals, compounds that deactivate NF- κ B may prove to be a potent and successful strategy for preventing cardiac apoptosis [54]. Additionally, curcumin has been shown to inhibit hyperglycemia-induced inflammation in cardiac cells in vitro and in diabetic hearts through NF- κ B inactivation [55].

We observed that in the HFpEF-DM group, overexpression of NF- κ B amplifies the biosynthesis and activation of multiple inflammatory cytokines (TNF- α , IL-6, ICAM, VCAM, and NLRP3 inflammasomes), which play a crucial role in pathologic inflammatory responses. Moreover, diabetes is associated with increased NET formation, which is linked to slower wound healing [56]. Our data indicate that overproduction of HMGB1 and upregulation of IL-6 promote the formation of NETs, exacerbating HFpEF in T2DM patients. We also show

that myocardial F_{passive} is significantly elevated in the HFpEF-DM group due to increased inflammation, which correlates with greater symptom severity. Notably, F_{passive} was restored with IL-6 inhibition, offering a promising therapeutic strategy for HFpEF-DM.

Dysregulated signaling pathways contributing to diabetic HFpEF progression

Impaired myocardial insulin signaling leads to excessive free fatty acid (FFA) consumption, which is associated with ROS overproduction [57]. Under these conditions, the heart fails to reduce ROS, decreasing NO bioavailability and endothelial dysfunction [58]. Endothelial dysfunction worsens outcomes in HFpEF patients, contributing to increased cardiomyocyte stiffness and hypertrophy [39]. Our study confirms that NO bioavailability is significantly reduced in HFpEF-DM patients, likely due to post-translational modifications or altered cofactor availability affecting eNOS function.

Furthermore, F_{passive} was significantly increased in HFpEF-DM, correlating with insulin resistance, impaired mitochondrial function, and oxidative stress. However, treatment with mito-TEMPO effectively restored F_{passive} in both groups, consistent with findings from Ni et al., who demonstrated the therapeutic potential of mito-TEMPO in reducing diabetic cardiomyopathy via mitochondrial ROS inhibition [59].

AMPK signaling plays a pivotal role in protecting cardiomyocytes during metabolic disorders like diabetes and obesity. Under metabolic stress, AMPK activity declines, exacerbating cardiac dysfunction. However, AMPK activation mitigates metabolic stress, maintains mitochondrial homeostasis, and stimulates cardiac autophagy [60]. Our study showed elevated p-AMPK in HFpEF-DM, potentially due to anti-diabetic medication effects targeting AMPK phosphorylation, balancing autophagy and cell survival. Metformin administration has been shown to increase AMPK phosphorylation, enhancing eNOS activity and NO bioavailability [61].

Additionally, the interplay between Insulin/PI3K/AKT and AMPK signaling regulates mTOR activity. Insulin-activated PI3K/AKT signaling phosphorylates mTOR, regulating autophagy and cell proliferation [62]. Conversely, AMPK suppresses mTOR during metabolic stress [63]. Our study found that the p-AKT/AKT ratio was significantly lower in HFpEF-DM, consistent with reports linking diminished AKT activation to worsening cardiomyopathy [64]. Additionally, mTOR phosphorylation was downregulated in HFpEF-DM, likely due to anti-diabetic drug-mediated AMPK/mTOR targeting, restoring cardiac function. Although most HFpEF patients in our cohort were receiving statin therapy, which is known to modulate PI3K/AKT/eNOS signaling, the uniform use of statins across both diabetic and non-diabetic groups

minimizes the likelihood of a confounding effect on our findings.

HSP dysregulation and cardiac dysfunction in diabetic hearts

Under physiological conditions, HSPs facilitate protein folding and cardioprotection. However, in failing hearts, inadequate PQC is reflected by decreased HSP levels [65]. Overexpression of HSP70 enhances protein stability, reduces apoptosis, and improves cardiac function under ischemic stress [66]. Shepherd et al. demonstrated that HSP70 expression is reduced in T2DM human hearts, suggesting increased misfolded protein accumulation and ER stress [67].

In agreement with these findings, our study demonstrated a significant reduction in HSP27 and HSP70 expression in HFpEF-DM. This decrease may be attributed to oxidative stress-induced HSP modification, leading to loss of cardioprotective function. Notably, administration of HSP27 and HSP70 restored F_{passive} in HFpEF-DM, aligning with our previous study on α B-crystallin restoring elevated F_{passive} in hypertrophic cardiomyopathy (HCM) [15].

Conclusion

PQC systems function as an integrated stress response, interacting at both the protein and transcriptional levels. Adequate PQC regulation is critical for diabetic hearts, as imbalanced PQC contributes to HFpEF development via oxidative stress, proteotoxicity, and apoptosis. Targeting PQC mechanisms at different levels could offer a novel therapeutic strategy for preventing HFpEF progression in diabetes, warranting further investigation.

Abbreviations

T2DM	Type 2 diabetes mellitus
CVD	Cardiovascular disease
CAD	Coronary artery disease
HFpEF	Heart failure with preserved ejection fraction
HFpEF	Heart failure with reduced ejection fraction
FAs	Fatty acids
ROS	Reactive oxygen species
NO	Nitric oxide
PQC	Protein quality control
UPRER	Unfolded protein response in the endoplasmic reticulum
HSPs	Heat shock proteins
HSP70	Heat shock protein70
HSP27	Heat shock proteins27
α B-crystallin	Alpha B-crystallin
AMPK	AMP-activated protein kinase:
mTOR	Mammalian target of rapamycin
NF- κ B	Nuclear factor kappa-light-chain-enhancer of activated B-cells
IL-6	Interleukin 6
TNF- α	Tumor necrosis factor
UPS	Ubiquitin-proteasome system
LV	Left ventricle
HMGB1	High mobility group box 1
TLR2	Toll-like receptor 2
TLR4	Toll-like receptor
RAGE	Receptor for advanced glycation end-products
ICAM-1	Intercellular adhesion molecule-1

VCAM-1	Vascular cell adhesion molecule-1
NLRP3	NOD-like receptor protein-3
NET	Neutrophil extracellular traps
PI3K	Phosphoinositide 3-kinase
AKT	Protein kinase B
GSK3B	Glycogen synthase kinase-3 beta
HCM	Hypertrophic cardiomyopathy
IL-6R	Interleukin-6 receptor
ERK 1/2	Extracellular signal-regulated kinases 1/2
MAPK	Mitogen-activated protein kinase
STK11	Serine/threonine kinase 11
NE	Neutrophil elastase
MPO	Myeloperoxidase
eNOS	Endothelial nitric oxide synthase
NOS3	Nitric oxide synthase 3 (endothelial NOS)
sGC	Soluble guanylate cyclase
cGMP	Cyclic guanosine monophosphate
PKG	Protein kinase G
PRKG1	Protein kinase, cGMP-dependent type 1
LPO	Lipid peroxidation
H ₂ O ₂	Hydrogen peroxide
qRT-PCR	Quantitative real-time reverse-transcription PCR

Author contributions

NH conceptualized the study, funded the project, supervised the project/all experiments, carried out experiments, analysed the data, wrote the manuscript with edits from other authors. SD, IS, SZ, HO, MS, SZ, HB, MH, AK, MK, EG, NB, GS, BS, VET, ZVV, and JW performed the laboratory experiments, contributed to data collection, and analysed the data. SD, IS wrote the manuscript. LV, MK, BS, JW, PH, AM, KJ, PF, ZVV, DM provided materials and assisted in data interpretation and critical revisions and contributed to manuscript preparation. All authors reviewed, edited, and approved the final version of the manuscript.

Funding

Open Access funding enabled and organized by Projekt DEAL.

The research was funded by EU's Horizon 2020 research and innovation program under grant agreement No. 739593 to N.H. and Z.V.V.; DFG (Deutsche Forschungsgemeinschaft) HA 7512/2-4 and HA 7512/2-1 to N.H.; a grant from the Innovation Forum program of the Medical Faculty, RUB to I. El-B. and N.H. No. IF-023-22 and No. IF-034-22; and the Hector-Stiftung (No.; MED1814, M2401) to N.H., I. El-B., and I.A. and the German Heart foundation (F/17/23) to I. El-B.; funding from Else-Kröner-Fresenius foundation to I. El-B. Project no. RRF-2.3.1-21-2022-00003 has been implemented with the support provided by the European Union. The project was supported by grants from the National Research, Development and Innovation Office (NKFIH) of Hungary (FK134751 to Z.V.V.). TKP2021-EGA-23 has been implemented with the support provided by the Ministry of Innovation and Technology of Hungary from the National Research, Development and Innovation Fund, financed under the TKP2021-EGA funding scheme.

Availability of data and materials

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

All samples were obtained with informed consent and approval from the local Ethics Committee. All procedures were performed according to the Declaration of Helsinki and were approved by the local ethics committee. Biopsies were obtained for the primary purpose of diagnosis following Ethics Committee approval numbers WO18.026, 20-6976 BR, 20-6976-1- BR and informed consent.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

Author details

¹Medical Faculty, Department of Cellular and Translational Physiology, Institute of Physiology, Molecular and Experimental Cardiology, Institut Für Forschung und Lehre (IFL), Ruhr University Bochum, 44801 Bochum, Germany

²Division of Clinical Physiology, Department of Cardiology, Faculty of Medicine, University of Debrecen, Debrecen 4032, Hungary

³Medical Department II, Marien Hospital Herne, Ruhr University Bochum, Bochum, Germany

⁴Department of Cardiothoracic Surgery, University Hospital Bergmannsheil Bochum, Bochum, Germany

⁵Department of Medicine I, St. Josef Hospital, UK RUB, Ruhr University Bochum, 44801 Bochum, Germany

⁶Department of Internal Medicine I— General Internal Medicine, Endocrinology and Diabetology, Gastroenterology and Hepatology, BG University Hospital Bergmannsheil, Bochum, Germany

⁷Department of Cardiology, OLVG, 1091 AC Amsterdam, The Netherlands

⁸Department of Biophysics and Radiation Biology, Semmelweis University, Tüzoltó utca 37-47, 1094, Budapest 1085, Hungary

⁹In Vivo Imaging Advanced Core Facility, Hungarian Centre of Excellence for Molecular Medicine, Budapest Tüzoltó utca 37-47, 1094, Hungary

¹⁰HCEMM-SU Cardiometabolic Immunology Research Group, Department of Pharmacology and pharmacotherapy, Semmelweis University, Budapest 1089, Hungary

¹¹Center for Pharmacology and Drug Research & Development, Department of Pharmacology and Pharmacotherapy, Semmelweis University, Budapest 1089, Hungary

¹²Department of Physiology, Cardiovascular Research Institute, Maastricht University, Maastricht, The Netherlands

¹³Medical Faculty, Department Cellular and Translational Physiology, Institute of Physiology, Ruhr University Bochum, MA 2/156, 44780 Bochum, Germany

¹⁴Institut Für Forschung und Lehre (IFL), Molecular and Experimental Cardiology, Gudrunstraße 56, 44791 Bochum, Germany

¹⁵Department of Cardiology, St. Josef-Hospital, UK RUB, Ruhr University Bochum, Bochum, Germany

Received: 19 February 2025 / Accepted: 7 April 2025

Published online: 14 May 2025

References

1. Ren J, Pulakat L, Whaley-Connell A, Sowers JR. Mitochondrial biogenesis in the metabolic syndrome and cardiovascular disease. *J Mol Med*. 2010;88(10):993–1001. <https://doi.org/10.1007/s00109-010-0663-9>.
2. Sasso FC, et al. Role of tight glycemic control during acute coronary syndrome on CV outcome in type 2 diabetes. *J Diabetes Res*. 2018;2018:1–8. <https://doi.org/10.1155/2018/3106056>.
3. IDF Diabetes Atlas, 9th edn. Federation ID, editor. Brussels: International Diabetes Federation (2019). 2019.
4. Jia G, Hill MA, Sowers JR. Diabetic cardiomyopathy. *Circ Res*. 2018;122(4):624–38. <https://doi.org/10.1161/CIRCRESAHA.117.311586>.
5. Jankauskas SS, et al. Heart failure in diabetes. *Metabolism*. 2021;125:154910. <https://doi.org/10.1016/j.metabol.2021.154910>.
6. Ren J, Wu NN, Wang S, Sowers JR, Zhang Y. Obesity cardiomyopathy: evidence, mechanisms, and therapeutic implications. *Physiol Rev*. 2021;101(4):1745–807. <https://doi.org/10.1152/physrev.00030.2020>.
7. Kannel WB, Hjortland M, Castelli WP. Role of diabetes in congestive heart failure: the Framingham study. *Am J Cardiol*. 1974;34(1):29–34. [https://doi.org/10.1016/0002-9149\(74\)90089-7](https://doi.org/10.1016/0002-9149(74)90089-7).
8. Marinescu KK, Uriel N, Mann DL, Burkhoff D. Left ventricular assist device-induced reverse remodeling: it's not just about myocardial recovery. *Expert Rev Med Devices*. 2017;14(1):15–26. <https://doi.org/10.1080/17434440.2017.1262762>.
9. Aronow WS. Current treatment of heart failure with reduction of left ventricular ejection fraction. *Expert Rev Clin Pharmacol*. 2016;9(12):1619–31. <https://doi.org/10.1080/17512433.2016.1242067>.
10. Alsamara M, Alharethi R. Heart failure with preserved ejection fraction. *Expert Rev Cardiovasc Ther*. 2014;12(6):743–50. <https://doi.org/10.1586/14779072.2014.911086>.

11. Rogers FJ, Gundala T, Ramos JE, Serajian A. Heart failure with preserved ejection fraction. *J Am Osteopath Assoc*. 2015;115(7):432–42. <https://doi.org/10.7556/jaoa.2015.089>.
12. Abudureyimu M, et al. Heart failure with preserved ejection fraction (HFpEF) in type 2 diabetes mellitus: from pathophysiology to therapeutics. *J Mol Cell Biol*. 2022;14(5):mjac028. <https://doi.org/10.1093/jmcb/mjac028>.
13. Wang ZV, Hill JA. Protein quality control and metabolism: bidirectional control in the heart. *Cell Metab*. 2015;21(2):215–26. <https://doi.org/10.1016/j.cmet.2015.01.016>.
14. Yang L, Zhao D, Ren J, Yang J. Endoplasmic reticulum stress and protein quality control in diabetic cardiomyopathy. *Biochim Biophys Acta (BBA) Mol Basis Dis*. 2015;1852(2):209–18. <https://doi.org/10.1016/j.bbdis.2014.05.006>.
15. Hassoun R, et al. Stress activated signalling impaired protein quality control pathways in human hypertrophic cardiomyopathy. *Int J Cardiol*. 2021;344:160–9. <https://doi.org/10.1016/j.ijcard.2021.09.009>.
16. Kaur N, Raja R, Ruiz-Velasco A, Liu W. Cellular protein quality control in diabetic cardiomyopathy: from bench to bedside. *Front Cardiovasc Med*. 2020;7:585309. <https://doi.org/10.3389/fcvm.2020.585309>.
17. Mayer MP, Bukau B. Hsp70 chaperones: cellular functions and molecular mechanism. *Cell Mol Life Sci*. 2005;62(6):670. <https://doi.org/10.1007/s00018-004-4464-6>.
18. Benjamin IJ, McMillan DR. Stress (Heat Shock) proteins. *Circ Res*. 1998;83(2):117–32. <https://doi.org/10.1161/01.RES.83.2.117>.
19. Kalmar B, Greensmith L. Induction of heat shock proteins for protection against oxidative stress. *Adv Drug Deliv Rev*. 2009;61(4):310–8. <https://doi.org/10.1016/j.addr.2009.02.003>.
20. Arrigo, A-P. The cellular 'networking' of mammalian Hsp27 and its functions in the control of protein folding, redox state and apoptosis. In *Molecular aspects of the stress response: chaperones, membranes and networks*. New York: Springer New York; pp. 14–26. https://doi.org/10.1007/978-0-387-39975-1_2.
21. Wang RC, Levine B. Autophagy in cellular growth control. *FEBS Lett*. 2010;584(7):1417–26. <https://doi.org/10.1016/j.febslet.2010.01.009>.
22. Gurusamy N, Das D. Is autophagy a double-edged sword for the heart? *Acta Physiol Hung*. 2009;96(3):267–76. <https://doi.org/10.1556/APhysiol.96.2009.3.2>.
23. Hotamisligil GS. Endoplasmic reticulum stress and the inflammatory basis of metabolic disease. *Cell*. 2010;140(6):900–17. <https://doi.org/10.1016/j.cell.2010.02.034>.
24. Chan YK, Sung HK, Jahng JWS, Kim GHE, Han M, Sweeney G. Lipocalin-2 inhibits autophagy and induces insulin resistance in H9c2 cells. *Mol Cell Endocrinol*. 2016;430:68–76. <https://doi.org/10.1016/j.mce.2016.04.006>.
25. Jung CH, Ro S-H, Cao J, Otto NM, Kim D-H. mTOR regulation of autophagy. *FEBS Lett*. 2010;584(7):1287–95. <https://doi.org/10.1016/j.febslet.2010.01.017>.
26. Wang S, Li H, Yuan M, Fan H, Cai Z. Role of AMPK in autophagy. *Front Physiol*. 2022;13:1015500. <https://doi.org/10.3389/fphys.2022.1015500>.
27. Haye A, Ansari MA, Rahman SO, Shamsi Y, Ahmed D, Sharma M. Role of AMP-activated protein kinase on cardio-metabolic abnormalities in the development of diabetic cardiomyopathy: a molecular landscape. *Eur J Pharmacol*. 2020;888:173376. <https://doi.org/10.1016/j.ejphar.2020.173376>.
28. Sciarretta S, Volpe M, Sadoshima J. Mammalian target of rapamycin signaling in cardiac physiology and disease. *Circ Res*. 2014;114(3):549–64. <https://doi.org/10.1161/CIRCRESAHA.114.302022>.
29. Guo R, Zhang Y, Turdi S, Ren J. Adiponectin knockout accentuates high fat diet-induced obesity and cardiac dysfunction: role of autophagy. *Biochim Biophys Acta (BBA) Mol Basis Dis*. 2013;1832(8):1136–48. <https://doi.org/10.1016/j.bbdis.2013.03.013>.
30. Hu N, Zhang Y. TLR4 knockout attenuated high fat diet-induced cardiac dysfunction via NF- κ B/JNK-dependent activation of autophagy. *Biochim Biophys Acta (BBA) Mol Basis Dis*. 2017;1863(8):2001–11. <https://doi.org/10.1016/j.bbdis.2017.01.010>.
31. Kandadi MR, Panzhinskiy E, Roe ND, Nair S, Hu D, Sun A. Deletion of protein tyrosine phosphatase 1B rescues against myocardial anomalies in high fat diet-induced obesity: role of AMPK-dependent autophagy. *Biochim Biophys Acta (BBA) Mol Basis Dis*. 2015;1852(2):299–309. <https://doi.org/10.1016/j.bbdis.2014.07.004>.
32. Peh ZH, et al. Inflammation as a therapeutic target in heart failure with preserved ejection fraction. *Front Cardiovasc Med*. 2023;10:1125687. <https://doi.org/10.3389/fcvm.2023.1125687>.
33. Graham B, Gibson SB. The two faces of NF κ B in cell survival responses. *Cell Cycle*. 2005;4(10):1342–5. <https://doi.org/10.4161/cc.4.10.2047>.
34. Hamdani N, Bishu KG, von Frieling-Salewsky M, Redfield MM, Linke WA. Deranged myofilament phosphorylation and function in experimental heart failure with preserved ejection fraction. *Cardiovasc Res*. 2013;97(3):464–71. <https://doi.org/10.1093/cvr/cvs353>.
35. Hamdani N, et al. Crucial role for Ca²⁺/calmodulin-dependent protein kinase-II delta in regulating diastolic stress of normal and failing hearts via titin phosphorylation. *Biophys J*. 2013;104(2):18a. <https://doi.org/10.1016/j.bpj.2012.11.129>.
36. Kolijn D, et al. Empagliflozin improves endothelial and cardiomyocyte function in human heart failure with preserved ejection fraction via reduced pro-inflammatory-oxidative pathways and protein kinase G α oxidation. *Cardiovasc Res*. 2021;117(2):495–507. <https://doi.org/10.1093/cvr/cvaa123>.
37. Herwig M, et al. Protein kinase D plays a crucial role in maintaining cardiac homeostasis by regulating post-translational modifications of myofilament proteins. *Int J Mol Sci*. 2024;25(5):2790. <https://doi.org/10.3390/ijms25052790>.
38. Kolijn D, et al. Enhanced cardiomyocyte function in hypertensive rats with diastolic dysfunction and human heart failure patients after acute treatment with soluble guanylyl cyclase (sGC) activator. *Front Physiol*. 2020;11:35. <https://doi.org/10.3389/fphys.2020.00345>.
39. Franssen C, et al. Myocardial microvascular inflammatory endothelial activation in heart failure with preserved ejection fraction. *JACC Heart Fail*. 2016;4(4):312–24. <https://doi.org/10.1016/j.jchf.2015.10.007>.
40. van Heerebeek L, et al. Low myocardial protein kinase G activity in heart failure with preserved ejection fraction. *Circulation*. 2012;126(7):830–9. <https://doi.org/10.1161/CIRCULATIONAHA.111.076075>.
41. Satheesan A, Kumar J, Leela KV, Murugesan R, Chaitanya V, Angelin M. Review on the role of nucleotide-binding oligomerization domain-like receptor protein 3 (NLRP3) inflammasome pathway in diabetes: mechanistic insights and therapeutic implications. *Inflammopharmacology*. 2024;32(5):2753–79. <https://doi.org/10.1007/s10787-024-01556-2>.
42. Liu T, Zhang L, Joo D, Sun S-C. NF- κ B signaling in inflammation. *Signal Transduct Target Ther*. 2017;2(1):17023. <https://doi.org/10.1038/sigtrans.2017.23>.
43. Kasner M, et al. Diastolic tissue Doppler indexes correlate with the degree of collagen expression and cross-linking in heart failure and normal ejection fraction. *J Am Coll Cardiol*. 2011;57(8):977–85. <https://doi.org/10.1016/j.jacc.2010.10.024>.
44. Mesquita T, Lin Y, Ibrahim A. Chronic low-grade inflammation in heart failure with preserved ejection fraction. *Aging Cell*. 2021;20(9):e13453. <https://doi.org/10.1111/ace1.13453>.
45. Abel ED. Insulin signaling in the heart. *Am J Physiol Endocrinol Metab*. 2021;321(1):E130–45. <https://doi.org/10.1152/ajpendo.00158.2021>.
46. Kahn BB, Alquier T, Carling D, Hardie DG. AMP-activated protein kinase: ancient energy gauge provides clues to modern understanding of metabolism. *Cell Metab*. 2005;1(1):15–25. <https://doi.org/10.1016/j.cmet.2004.12.003>.
47. Hardie DG. AMP-activated/SNF1 protein kinases: conserved guardians of cellular energy. *Nat Rev Mol Cell Biol*. 2007;8(10):774–85. <https://doi.org/10.1038/nrm2249>.
48. Suhara T, Baba Y, Shimada BK, Higa JK, Matsui T. The mTOR signaling pathway in myocardial dysfunction in type 2 diabetes mellitus. *Curr Diabets Rep*. 2017;17(6):38. <https://doi.org/10.1007/s11892-017-0865-4>.
49. Cohen P. The twentieth century struggle to decipher insulin signalling. *Nat Rev Mol Cell Biol*. 2006;7(11):867–73. <https://doi.org/10.1038/nrm2043>.
50. Sugden PH, Fuller SJ, Weiss SC, Clerk A. Glycogen synthase kinase 3 (GSK3) in the heart: a point of integration in hypertrophic signalling and a therapeutic target? A critical analysis. *Br J Pharmacol*. 2008;153(5):S137–53. <https://doi.org/10.1038/sj.bjp.0707659>.
51. Frati G, et al. An overview of the inflammatory signalling mechanisms in the myocardium underlying the development of diabetic cardiomyopathy. *Cardiovasc Res*. 2017;113(4):378–88. <https://doi.org/10.1093/cvr/cvx011>.
52. Oeckinghaus A, Hayden MS, Ghosh S. Crosstalk in NF- κ B signaling pathways. *Nat Immunol*. 2011;12(8):695–708. <https://doi.org/10.1038/ni.2065>.
53. Mariappan N, et al. NF- κ B-induced oxidative stress contributes to mitochondrial and cardiac dysfunction in type II diabetes. *Cardiovasc Res*. 2010;85(3):473–83. <https://doi.org/10.1093/cvr/cvp305>.
54. Romeo G, Liu W-H, Asnaghi V, Kern TS, Lorenzi M. Activation of nuclear factor- κ B induced by diabetes and high glucose regulates a proapoptotic program in retinal pericytes. *Diabetes*. 2002;51(7):2241–8. <https://doi.org/10.2337/diabetes.51.7.2241>.
55. Pan Y, et al. Inhibition of JNK phosphorylation by a novel curcumin analog prevents high glucose-induced inflammation and apoptosis in cardiomyocytes and the development of diabetic cardiomyopathy. *Diabetes*. 2014;63(10):3497–511. <https://doi.org/10.2337/db13-1577>.

56. Wong SL, et al. Diabetes primes neutrophils to undergo NETosis, which impairs wound healing. *Nat Med*. 2015;21(7):815–9. <https://doi.org/10.1038/nm.3887>.
57. Guo CA, Guo S. Insulin receptor substrate signaling controls cardiac energy metabolism and heart failure. *J Endocrinol*. 2017;233(3):R131–43. <https://doi.org/10.1530/JOE-16-0679>.
58. Marso SP, et al. Semaglutide and cardiovascular outcomes in patients with type 2 diabetes. *N Engl J Med*. 2016;375(19):1834–44. <https://doi.org/10.1056/NEJMoa1607141>.
59. Ni R, et al. Therapeutic inhibition of mitochondrial reactive oxygen species with mito-TEMPO reduces diabetic cardiomyopathy. *Free Radic Biol Med*. 2016;90:12–23. <https://doi.org/10.1016/j.freeradbiomed.2015.11.013>.
60. Qi D, Young LH. AMPK: energy sensor and survival mechanism in the ischemic heart. *Trends Endocrinol Metab*. 2015;26(8):422–9. <https://doi.org/10.1016/j.tem.2015.05.010>.
61. Calvert JW, et al. Acute metformin therapy confers cardioprotection against myocardial infarction via AMPK-eNOS-mediated signaling. *Diabetes*. 2008;57(3):696–705. <https://doi.org/10.2337/db07-1098>.
62. Laplante M, Sabatini DM. mTOR signaling in growth control and disease. *Cell*. 2012;149(2):274–93. <https://doi.org/10.1016/j.cell.2012.03.017>.
63. Kim YC, Guan K-L. mTOR: a pharmacologic target for autophagy regulation. *J Clin Investig*. 2015;125(1):25–32. <https://doi.org/10.1172/JCI73939>.
64. VanderHaar E, Lee S, Bandhakavi S, Griffin TJ, Kim D-H. Insulin signaling to mTOR mediated by the Akt/PKB substrate PRAS40. *Nat Cell Biol*. 2007;9(3):316–23. <https://doi.org/10.1038/ncb1547>.
65. Wang X, Su H, Ranek MJ. Protein quality control and degradation in cardiomyocytes. *J Mol Cell Cardiol*. 2008;45(1):11–27. <https://doi.org/10.1016/j.yjmc.2008.03.025>.
66. Williamson CL, Dabkowski ER, Dillmann WH, Hollander JM. Mitochondria protection from hypoxia/reoxygenation injury with mitochondria heat shock protein 70 overexpression. *Am J Physiol Heart Circ Physiol*. 2008;294(1):H249–56. <https://doi.org/10.1152/ajpheart.00775.2007>.
67. Shepherd DL, et al. Mitochondrial proteome disruption in the diabetic heart through targeted epigenetic regulation at the mitochondrial heat shock protein 70 (mtHsp70) nuclear locus. *J Mol Cell Cardiol*. 2018;119:104–15. <https://doi.org/10.1016/j.yjmcc.2018.04.016>.

Publisher's Note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.