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THESIS TITLE: A DUEL OF FATES: THE ROLE OF KETONE BODIES IN HEART FAILURE WITH PRESERVED EJECTION FRACTION

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ABSTRACT

RATIONALE: More than half of patients with heart failure have preserved ejection fraction (HFpEF), a deadly syndrome where pathophysiology is unclear and therapeutic options are limited. Cardiac metabolism derangements and the role of ketone bodies in HFpEF are still under investigation.

OBJECTIVE: To explore, in a mouse model of cardiometabolic HFpEF, the dual fate of β-hydroxybutyrate (BOH) as both an energy substrate and a signaling molecule.

METHODS AND RESULTS: In the "2-hit" HFpEF model, we observed a reduction in both glucose and BOH oxidative pathways. In HFpEF, BOH drove a significant increase in a novel post translational modification (PTM) called lysine (K) β-hydroxybutyrylation (Kbhb). Among the Kbhb targets, we identified 2 components of the malate-aspartate shuttle (MAS), namely the mitochondrial enzymes MDH2 and GOT2. Both MAS and GOT2 activity were increased in HFpEF. Modeling Kbhb in vitro, we described a role for this post translational modification in restoring GOT2 activity under hypoxia, arguably contributing to the modulation of MAS activity in HFpEF.

CONCLUSIONS: In a mouse model of cardiometabolic HFpEF, glucose and ketone bodies oxidation are defective. BOH promotes Kbhb, a new PTM, arguably involved in the increase in MAS activity.

BACKGROUND

Heart failure: an introduction

Definition

Heart failure is a systemic disorder of major clinical and epidemiological relevance. It presents as a clinical syndrome that recognizes different etiologies and pathophysiological mechanisms. Therefore, it is not easy to define the presence of heart failure openly and unequivocally. In fact, the definitions in the literature are manifold and differ depending on the purpose for which they were formulated i.e., educational, clinical or research.

To gain an understanding of the nature of this syndrome, pathophysiological definitions are those most often proposed. Referring to the causal mechanism, heart failure has been defined as a pathological condition in which an abnormality in myocardial function is responsible for an inability of the heart to pump sufficient blood to the metabolic needs of the tissues during ordinary activity[1, 2].

Similarly, heart failure has also been defined as an abnormality of cardiac structure or function that causes an inability of the heart to supply sufficient oxygen for the metabolic needs of tissues.

These definitions focus on the heart's inability to perform its primary function, which is to provide adequate tissue perfusion. However, they do not provide a description of the patient and, therefore, remain an intangible abstraction. To have a clinical framework and facilitate the identification of the syndrome in medical practice, heart failure is most often defined on the basis of the cluster of signs and symptoms that characterize it. Current European guidelines define heart failure as a clinical syndrome characterized by cardinal symptoms (dyspnea, declivous edema, asthenia) that may be accompanied by typical signs (jugular turgor, pulmonary crepitations, peripheral edema)[3]. These clinical manifestations must be caused by a structural and/or functional abnormality of the heart resulting in

elevated intracavitary filling pressures and/or inadequate cardiac output at rest or during exercise[3].

It is clarified that patients with non-cardiovascular disease (such as anemia, pulmonary, renal, thyroid, or liver disease) may have signs and symptoms that are very similar to those of patients with heart failure but cannot be diagnosed with heart failure in the absence of cardiac dysfunction. Such conditions may coexist with heart failure or cause a worsening of the syndrome itself.
Although this definition is generally sufficient in clinical practice it may be imprecise and subject to different interpretations in research.

To address these drawbacks by providing an unambiguous and effective reference in every setting, a universal definition of heart failure has recently been proposed, supported by leading scientific societies around the world. In a landmark position paper[4], heart failure was defined as a clinical syndrome with current or prior symptoms and/or signs (listed in details[4]) caused by a structural and/or functional cardiac abnormality (as determined by EF <50%, abnormal cardiac chamber enlargement, E/E′ >15, moderate/severe ventricular hypertrophy or moderate/severe valvular obstructive or regurgitant lesion) and corroborated by at least one of the following: 1. Elevated natriuretic peptide levels 2. Objective evidence of cardiogenic pulmonary or systemic congestion by diagnostic modalities such as imaging or hemodynamic measurement at rest or with provocative testing[4]. This definition encompasses not only the clinical aspects of the syndrome, but unambiguously defines its pathophysiological substrate so that the definition of a cardiac structural abnormality is not left to the individual judgment of the examining physician or researcher. Furthermore, the presence of congestion requires a finding that is also unbiased, by imaging or blood tests, to confirm what is derived from clinical evidence.

Epidemiology

Heart failure is a disease with high incidence and prevalence in the general population. Currently, the incidence of heart failure in Europe is about 3/1000 subjects per year when considering all age groups and rises to about 5/1000 subjects per year in adults[5, 6]. The estimated prevalence of heart failure in developed

countries is generally between 1 and 2% of the adult population[7]. Although the relative incidence of heart failure is lower in women than in men, women account for at least half of the absolute cases due to their longer life expectancy. In fact, the prevalence of heart failure increases with age, generally doubling each decade of life from 40 to 80. It is less than 1% in those under 40 years of age but exceeds 10% in those over 70 years of age[8-10]. Consistent with this evidence, more than half of patients hospitalized for heart failure are over 75 years old[11].

Although the incidence by age and prevalence of heart failure are decreasing, the total number of heart failure patients is increasing dramatically, due to the increase in the global population and the aging of the population itself[12]. Currently, nearly 40 million people are affected by heart failure worldwide[13].
Improvements in patient clinical management have delayed the development of heart failure in patients with cardiovascular risk factors or overt structural heart disease and prolonged the lives of patients with symptomatic heart failure.

Classification

Considering that most of the treatments prescribed for heart failure have evidence of efficacy only in patients with reduced ejection fraction (FE≤40%), this category is recognized by all international guidelines and grouped under the acronym HFrEF: Heart Failure with Reduced Ejection Fraction. Similarly, patients with preserved ejection fraction (FE ≥50%) are referred to as HFpEF: Heart Failure with preserved Ejection Fraction.
For patients with intermediate values of the ejection fraction, the definition is more controversial. Current European guidelines define patients with an ejection fraction between 41 and 49% as having heart failure with mildly reduced ejection fraction (HFmrEF: Heart Failure with mildly reduced Ejection Fraction)[3].
Previously, the acronym HFmrEF was used as an acronym for the definition Heart Failure with middle-range Ejection Fraction. The shift from middle-range to mildly reduced was intended to reframe these patients: their condition is not simply "intermediate" but is considered a mild form of heart failure with reduced ejection fraction. Therefore, it is likely that the therapeutic strategies used in patients with reduced ejection fraction may also have some effect in patients with ejection fraction between 41 and 49%.

Heart failure has also been classified into 4 stages based on the clinical course of patients according to the American College of Cardiology (ACC)/American Heart Association (AHA) guidelines[14].
This classification is intended to highlight the progression from risk factor to end-stage disease as a process in which different interventions must be taken. From a clinical point of view, symptoms are classified primarily according to the severity of dyspnea. This is summarized in the New York Heart Association (NYHA) classification, a four-stage classification used to describe the severity of the clinical condition[15].

Heart failure with preserved ejection fraction

HFpEF vs HFrEF

Heart failure classification based on ejection fraction remain pivotal in terms of therapeutic strategies to be adopted and basic and clinical research perspectives. Although ejection fraction is not the most accurate parameter to describe global left ventricular pump function, it provides a parameter that is easy to measure and widely used in recent decades in both the development of animal models of heart failure and in clinical trials.

From a pathophysiological perspective, reduced ejection fraction generally corresponds to reduced ventricular contractility and reduced cardiac output both at rest and during exercise. This accounts for inadequate tissue perfusion and congestion in both the pulmonary and the systemic circulation.
When the ejection fraction is preserved, signs and symptoms of heart failure have a different explanation. Here, cardiac output at rest is often normal. However, left ventricular hypertrophy may result in a worsening of ventricular compliance and thus a reduction in left ventricular end-diastolic volume with reduced cardiac output under stress. In addition, the inability to increase cardiac output during exercise may depend on a reduction in basal levels of adenosine triphosphate (ATP) and an inability of the cardiomyocyte to increase ATP synthesis and thus contractility during exercise. HFpEF, in fact, is an ATP-deficient condition with resting myocardial ATP levels being reduced to 20-40% of normal[16-18].

This inability to produce ATP may depend on altered cardiomyocyte mitochondrial function, particularly in cases where HFpEF is associated with metabolic syndrome.

Since many of the HFpEF functional alterations (such as an insufficient increase in cardiac output or congestion of the pulmonary circulation) are only occurring under exercise, the diagnosis is complex as it can require morpho-functional assessments (e.g., echocardiogram) under stress or invasive measurement of hemodynamic parameters[19].

When considering the natural history of the two different classes of heart failure, they generally proceed in parallel as the transition from HFpEF to HFrEF is infrequent[20].

Furthermore, the comorbidities most frequently associated with HFpEF (obesity, hypertension, and atrial fibrillation) correspond only partially to those related to HFrEF, which is more often associated with ischemic heart disease, chronic obstructive pulmonary disease, or dilated cardiomyopathy

These pathophysiological differences between HFrEF and HFpEF have an important clinical correlate, as they affect treatment response.
Several clinical trials designed to demonstrate a reduction in cardiovascular death or heart failure hospitalization using pillars of HFrEF treatment, such as RAS blockers, strikingly failed or nearly missed their primary endpoint when used in HFpEF[21-27]. Intriguingly, a significant improvement in HFpEF outcome came from drugs primarily designed to treat diabetes and metabolic syndrome, such as Sodium-Glucose Transport Protein 2 (SGLT2) Inhibitors[28, 29] and Glucagon-like peptide 1 (GLP-1) receptor agonists[30].

Understanding that the therapeutic arsenal available for HFrEF is almost completely ineffective in HFpEF has pushed the research in this class of heart failure, making HFpEF a major challenge for cardiovascular medicine.

Epidemiology and phenotyping

HFpEF is indeed a syndrome with high prevalence in the population, especially in Western countries. Approximately 5% of patients over the age of 60 years are currently affected by HFpEF[8] and its prevalence is rapidly increasing[31] due to the aging of the population and the increasing prevalence of major risk factors for HFpEF, such as obesity, diabetes mellitus, and hypertension[32-34].

The impact of HFpEF on health and healthcare systems is remarkable. More than half of all HF hospitalizations involve patients with HFpEF[35], with a combined all-cause death and 2-year HF hospitalization rate of 35% and a 2-year mortality rate of 14%[36].

Most epidemiological and clinical intervention studies consider all patients with HFpEF as having the same syndrome. However, this is a generalization that overlooks a key aspect of the disease.
HFpEF is a disorder that encompasses numerous clinical syndromes. Although it is recognized that patients with HFpEF have different clinical presentations, a clear identification of the different phenotypes has not yet been achieved. Many attempts have been made so far, but especially with machine learning approaches, the phenotypes were significantly different[37-39]. The main purpose of phenotyping in HFpEF should be to bring together patients with the same disease, not just the same symptoms. To achieve correct pathophysiological phenotyping, risk factors should play a central role. To date, many risk factors that contribute to the development of different phenotypes of HFpEF have been recognized. None of the canonical risk factors for HFpEF (such as age, obesity, hypertension, etc) is sufficient by itself to determine the HFpEF phenotype. On the contrary, a combination of these factors is likely to be the driving force behind the development of HFpEF.

Among the main phenotypes of HFpEF we certainly recognize the elderly-hypertensive phenotype and the cardiometabolic phenotype. In the former, the combination of long-standing hypertension and aging leads to significant impairment of left ventricular compliance and an increase in left ventricular filling pressure[40]. This results in atrial remodeling with the development of atrial fibrillation and induces the development of pulmonary hypertension. Atrial

fibrillation impairs an already deficient diastolic filling of the left ventricle, promoting reduced cardiac output under stress. Pulmonary hypertension contributes to impaired alveolar-capillary interface function, promoting the development of exertional hypoxemia and thus worsening dyspnea and impaired exercise tolerance.

The cardiometabolic phenotype, on the other hand, is related to risk factors that are becoming more and more frequent in the population of western countries, such as overweight/obesity and metabolic syndrome, and is therefore attracting increasing scientific interest.

Cardiometabolic HFpEF

The cardiometabolic phenotype of HFpEF is closely related to the presence of metabolic syndrome[41], a condition marked by the coexistence of visceral adiposity, dyslipidemia, type II diabetes, and hypertension. About 80% of patients with HFpEF are overweight or obese[42, 43], and visceral adipose tissue plays a major role in the development of HFpEF[44], especially in women[45]. Metabolic comorbidities such as diabetes mellitus and dyslipidemia are more common in patients with HFpEF than in the general population[46-48].

Cardiometabolic HFpEF is among the most prevalent HFpEF phenotypes, affects younger patients than the other subtypes, and is related to worse quality of life and a higher risk of cardiovascular death[49]. As it is mechanistically related to the presence of lipid accumulation (both systemic and tissue) and inflammation, cardiometabolic HFpEF can be considered one of the organ damage of metabolic syndrome, similarly to metabolic associated fatty liver disease (MAFLD) and nonalcoholic steatohepatitis (NASH)[50]. Cardiometabolic HFpEF and MAFLD/NASH not only recognize the same risk factors and similar pathophysiology, but are also often associated from an epidemiological standpoint[51].

Cardiac lipid overload is crucial in the development of cardiometabolic HFpEF. The so-called Western diet, rich in saturated fats and refined carbohydrates, produces a high caloric influx into adipose tissue, resulting in a spill-over of lipids into the bloodstream. These lipid species, in the form of free fatty acids or esterified

lipids, reach target tissues and build up. The heart in adulthood recognizes free fatty acids as a preferred energy substrate. Fatty acid influx, which occurs through the fatty acid translocase (FAT/CD36), plasma membrane fatty acid binding proteins (FABPpm) and tissue specific fatty acid transport proteins (FATP), increases as a function of plasma fatty acid concentration, even outweighing the need for substrates to be oxidized[52]. If the plasma concentration of fatty acids is excessive, this can lead to cardiac fat accumulation, a condition described as myocardial steatosis that has been found both in animal models of HFpEF and in clinical settings. Indeed, lipid accumulation in heart specimens has been described in mouse models of HFpEF[53], and lipid accumulation has been shown to correlate with the development of severe diastolic dysfunction[53].

In a clinical setting, cardiac magnetic resonance imaging investigations have demonstrated the presence of myocardial steatosis in patients with HFpEF[54], especially in women[55] and a correlation between increased myocardial fat and cardiac remodeling (namely, left ventricular hypertrophy, increased atrial volume and diastolic dysfunction-increased E/E' ratio)[54]. In addition, increased TGs content in the myocardium also correlates with lower maximal oxygen consumption (VO2 max) and thus worse exercise capacity in patients with HFpEF[56].

How lipids drive myocardial toxicity is still unclear. One of the hypotheses formulated suggests that some lipid species with intermediate esterification, such as diacylglycerols and ceramides, alter the fluidity and thus the homeostasis of the mitochondrial membranes, causing both altered energy metabolism and oxidative inflammatory damage[57]. Other hypotheses suggest a role for loss of metabolic flexibility in HFpEF generation.

In fact, the normal heart is characterized by a marked ability to select the most suitable energy substrate source depending on existing conditions, switching from one predominant substrate class to another, and is therefore often described as an "omnivore"[58]. Under aerobic conditions, fatty acids are the main fuel for the heart, and mitochondrial fatty acids β oxidation provides 60-90% of the acetyl-CoA required for cardiac contraction and relaxation[59]. When changes in substrate availability or oxygen availability occur, however, a shift to other substrates is necessary. Fatty acid oxidation, in fact, allows the generation of the largest number

of ATP molecules per mole but is also the oxidative process that requires the most oxygen to occur. Under hypoxic conditions, fatty acids are disadvantageous and the heart needs to oxidize mainly carbohydrates. The switch of the heart from fatty acids to glucose is an energetically favorable adaptation, as documented in vivo[60].

In the presence of continuous lipid overload, the heart cannot easily switch to glucose utilization. This depends on several mechanisms, the simplest of which is the competition between fatty acids and glucose that occurs through the Randle cycle, a metabolic process in which acetyl-CoA derived from fatty acid oxidation decreases glucose oxidation, while pyruvate-derived acetyl-CoA inhibits fatty acid oxidation[61]. More complex mechanisms involve post-translational modifications, such as acetylation. In the presence of obesity and overweight, the presence of increased fatty acid oxidation correlates with increased acetyl-CoA pool[62-64] and higher levels of acetylation[65, 66].
Acetylation hinders metabolic flexibility, as it promotes insulin resistance[65] (and thus reduces glucose oxidation capacity) and enhances the activity of enzymes involved in fatty acid oxidation[67, 68].

In summary, in cardiometabolic HFpEF the presence of lipid overload promotes the development of adverse cardiac remodeling. This is seen through changes in both cardiac structure (myocardial hypertrophy, diastolic dysfunction) and cardiac metabolism. It also promotes, through post-translational changes, metabolic inflexibility, making the heart unable to adapt to stressful conditions, such as a reduction in available oxygen flow.

What exactly is the nature of the metabolic remodeling that occurs in HFpEF and what signaling pathways are involved in these changes is still largely unknown.

Metabolic remodeling in HF

In adult hearts, ATP production is based mainly on the oxidation of free fatty acids (40 to 70%). A substantial proportion of energy (about 20 to 30%) comes from the oxidation of glucose and ketone bodies. Under normal conditions, the remaining substrates (such as lactate, amino acids, etc.) assume a secondary and often negligible role. A key feature of healthy heart tissue is the ability to switch between

energy substrates based on nutrient changes, exploiting the so-called metabolic flexibility[69]. Loss of metabolic flexibility is a critical step toward the development of HFrEF[69, 70].

HFrEF

Fatty acid β-oxidation

When myocardial dysfunction arises and HFrEF develops, fatty acid oxidation is progressively reduced as cardiac function worsens[70, 71].

In animal models, where HFrEF results from hypertensive stress, fatty acid oxidation is significantly reduced[72, 73]. This reduction in fatty acid oxidation proceed hand in hand with the global impairment of mitochondrial oxidative function[74]. In a clinical setting, the use of nuclear imaging techniques has allowed to quantify the reduction in fatty acid oxidation observed in patients with ischemic heart disease[70]. By measurement of metabolite concentration in the coronary circulation, a reduction in fatty acid oxidation was described in patients with idiopathic dilated cardiomyopathy[75].

Mechanistically, the reduction in fatty acid oxidation depends at least in part on a decrease in the transcription of a number of enzymes involved in the fatty acids oxidative pathway[76, 77].

Reduced gene expression of fatty acid oxidative enzymes (medium and long-chain acyl-CoA dehydrogenase - LCAD, MCAD) has been observed in patients with heart failure and during the progression of heart failure in animal models[78]. Also, significant changes in the downstream signaling of the peroxisome proliferator-activated receptors (PPARα - abundant in the heart - , PPARβ/δ, and PPARγ) have been observed in HFrEF[79, 80]. In particular, a key role has been recognized in HFrEF for the inducible PPARγ-1α coactivator (PGC-1α), which is involved in the modulation of the transcriptional activity of the PPAR superfamily[80, 81].

Fatty acids are the endogenous ligand of PPARα and can activate the PPARα/PGC1α pathway for transcriptional regulation. PPARα/PGC1α transcriptional activity has also been shown to regulate pyruvate dehydrogenase

kinase 4 (PDK4), which can reduce glucose oxidation through inactivation of the pyruvate dehydrogenase (PDH) activity[82, 83], but not the glycolysis.
In patients with HFrEF, a reduction in PPARα expression at the cardiac level was observed[84]. Furthermore, it has been described how a subset of genes downstream of PGC-1α are also down-regulated in HFrEF hearts, which correlates with reduced left ventricular ejection fraction[85].

Moving into the analysis of fatty acid oxidation in patients with obesity or diabetes and HFrEF, findings may be conflicting. In the presence of increased visceral adiposity and consequently hyper influx of fatty acids to the heart, fatty acid oxidation at the cardiac level increases[86-88]. Excessive reliance on fatty acid oxidation has been shown to be closely related to worsened cardiac efficiency in both mouse models of obesity[87] and obese patients[86]. In addition, insulin resistance (which is related to the development of metabolic syndrome) inhibits glucose utilization and, consequently, increases fatty acid utilization. Insulin resistance is itself associated with the progression of heart failure[89, 90].

In conclusion, fatty acid oxidation is the main energy source of the healthy heart. In the presence of HFrEF, fatty acid oxidation is reduced. This is particularly evident in HFrEF models that do not include overweight or obesity. The coexistence of obesity and overweight (which promotes fatty acid oxidation) and HFrEF may provide conflicting data on the proportion of fatty acid oxidation in the heart, depending on the stage of heart failure and severity of systolic dysfunction.

Glucose

In HFrEF, the decrease in fatty acid oxidation is mirrored by an increase in glycolysis, with significant alterations in glucose oxidation. A major increase in glycolysis was observed in mice in which HFrEF was induced by hypertensive stress (abdominal aortic constriction - ACC - or transverse aortic constriction, TAC)[91-93].

The development of cardiac dysfunction in HFrEF is also related with a reduction in glucose oxidation, as demonstrated in different animal models[73, 89]. The majority of studies that have examined glucose oxidation rates in the heart, both in animal

models of HFrEF and in clinical settings, have shown that there is an overall reduction in glucose oxidation in the heart and a reduced contribution of glucose oxidation to total ATP production[89, 94-96]. It is not surprising that in HFrEF an increase in glycolysis and a decrease in glucose oxidation are observed simultaneously, as in the heart these two pathways are differentially regulated[97]. Confirming the mismatch between increased glycolysis and decreased glucose oxidation, an increase in lactate and pyruvate was observed in a study of cardiac samples from patients with advanced HFrEF, pointing to a disruption in the entry of pyruvate into the tricarboxylic acid (TCA) cycle[98].

The reduction in glucose oxidation that occurs in the failing heart is due, in part, to a general impairment of mitochondrial oxidative capacity, as well as altered activity of PDH, the rate-limiting enzyme of glucose oxidation[89, 90, 94, 96].

Indeed, it has been shown that mRNA expression of PDH, Monocarboxylate transporter 1 (MCT1), and pyruvate/alanine aminotransferase is decreased in HFrEF, suggesting altered pyruvate metabolism[99]. Furthermore, using hyperpolarized 13C magnetic resonance spectroscopy, the activity of the PDH complex has been shown to be impaired in a rat model of heart failure induced by myocardial infarction[100]. Importantly, the impairment of the PDH complex was progressive and proportional to the degree of cardiac dysfunction.

Furthermore, impaired pyruvate oxidation is associated with the development of left ventricular hypertrophy[100], underscoring the relationship between the maintenance of glucose oxidation and normal cardiac function.

Due to the development of insulin resistance, insulin-induced stimulation of glucose oxidation is attenuated in obesity and diabetes[101, 102], contributing to metabolic inflexibility of the myocardium. Altered insulin signaling and the development of an insulin-resistant myocardium precede cardiac dysfunction in HFrEF and lead to its progression[73, 89, 103]. Overall, insulin resistance participates, at least in part, in the reduction of glucose oxidation in heart failure.

Ketone bodies

Ketone bodies are three small water-soluble molecules, mainly synthesized in the liver from fatty acids and peripherally used as energy sources. The two main ketone bodies are acetoacetate and β-hydroxybutyrate (BOH), while acetone is the least abundant ketone body. During fed state, in healthy subjects the concentration of circulating ketone bodies is low. During fasting or prolonged exercise, the concentration increases to 1-8 mM, as their biological role is to provide an alternative energy source under low-glycemic conditions[104-106].

Liver synthesis of ketone bodies is directly dependent on the amount of circulating fatty acids. Therefore, regulation of lipolysis is a major determinant of ketogenesis. During fasting, reduced blood glucose levels result in reduced insulin and increased glucagon levels. Insulin is the main suppressor of ketogenesis: its inhibitory role on hormone-sensitive lipase prevents the breakdown of triglycerides into glycerol and fatty acids, limiting the availability of fatty acids.

Glucagon, on the other hand, promotes ketogenesis[107]. When the inhibitory role of insulin fades, increased lipolysis promotes the release of fatty acids for hepatic ketogenesis.
Since high levels of fatty acids themselves constitute a stimulus to ketogenesis, any stimulus that increases lipolysis results in increased levels of ketone bodies. For example, catecholamine-induced lipolysis in heart failure can contribute to increased levels of fatty acids and ketone bodies[108, 109]. In addition, high levels of cortisol and growth hormone in heart failure patients stimulate lipolysis and the release of fatty acids for the production of ketone bodies[110]. Interestingly, natriuretic peptides have also recently been found to stimulate lipolysis and increase levels of ketone bodies[111].

The core steps of ketogenesis take place in hepatocyte mitochondria. After synthesis, circulating ketone bodies can be used as an energy substrate in almost all organs. The exception is the liver itself, where the key ketolytic enzyme 3-oxoacid CoA-transferase 1 (OXCT1, also known as succinyl-CoA:3-ketoacid-CoA transferase - SCOT) is not present[112]. Among extra-hepatic tissues, the heart has the highest expression of OXCT1[113, 114] and thus the heart is the organ with the highest baseline capacity for ketone oxidation.

Of the three circulating ketone bodies, only acetoacetate and BOH can be internalized within the cell to produce energy. Acetone is not re-converted to acetyl-CoA and is excreted through urine or exhaled. Acetoacetate and BOH enter the cytosol of extrahepatic cells through MCT1. Once in the cytoplasm, they diffuse down gradient to the mitochondria, where the oxidation process (ketosis) can begin. The mitochondrial enzyme β-hydroxybutyrate dehydrogenase (BDH1) catalyzes the oxidation of BOH to acetoacetate. The ratio of BOH to acetoacetate is determined by the redox state in the mitochondria and is representative of the mitochondrial $NADH/NAD^+$ ratio[115]. BDH1 is critical in the regulation of ketolysis, and modulation of BDH1 activity is therefore crucial for the metabolism and oxidation of ketone bodies. Increased expression of PPAR-γ, for example, has been correlated with increased BDH1 in both a mouse model of obesity-induced cardiac dysfunction and in diabetic mice[116]. In addition, BDH1 has acetylation sites regulated by sirtuin 3 (SIRT3)[117], and in mice with KO of SIRT3, a significant increase in BDH1 expression was observed resulting in increased oxidation of BOH and heart[67].
Acetoacetate is converted to acetoacetyl-CoA by the enzyme SCOT. Acetoacetyl-CoA is then cleaved into two acetyl-CoA molecules that can enter the TCA cycle[113, 118].

In view of their structure, ketone bodies are very energy-efficient, producing more ATP per mole of oxygen consumed than glucose and fatty acids[119-121]; however, the release of ATP per mole of ketone bodies is significantly less than that achieved by fatty acid oxidation. Given their role as an alternative energy substrate, the entry of ketone bodies into the cell is rapid and their transport within the mitochondria occurs by gradient, without any limiting transporters. On this basis, ketone bodies have been proposed as a "superfuel" for the heart in heart failure. Under normal conditions, ketone bodies supply 5 to 15 percent of ATP to the heart[122]. However, the heart becomes, along with the brain, the main peripheral user of ketone bodies when the availability of other substrates is limited[105].

A determinant of ketone oxidation rates in the heart is circulating ketone levels. Several studies have indicated that blood ketone levels are elevated in patients with HFrEF to an extent proportional to the severity of cardiac dysfunction[110, 123].

In a mouse model of pressure overload-induced cardiac hypertrophy, proteomics data demonstrated a 2-3-fold increase in BDH1 expression[124]. Furthermore, the myocardial metabolite profile of heart failure mice was comparable to the profile of mice on a 4-week ketogenic diet. Analysis of heart specimens of patients with advanced HFrEF demonstrated increased levels of BOH and increased expression of BDH1 and SCOT in heart failure patients, indicating increased oxidation of ketone bodies[125].

Increased oxidation of ketone bodies appears to be an adaptive response in HFrEF. In a study with mice with hypertensive HFrEF, heart failure mice with SCOT-specific cardiac knockout showed abnormalities in mitochondrial ultrastructure and accelerated pathological cardiac remodeling[126]. In the same HFrEF model, overexpression of cardiac BDH1 mitigated oxidative stress and attenuated cardiac remodeling[127].

Ketones participate in the substrate competition that has already been described between glucose and fatty acids. Indeed, ketone bodies are able to suppress the oxidation of glucose and vice versa[128], as both compete for available oxygen and as sources of acetyl CoA for the TCA cycle. In an ex vivo model, it was observed that, in the presence of insulin, acetoacetate significantly reduced glucose oxidation[129]. This may be explained by the ability of ketones to increase the mitochondrial acetyl-CoA/CoA ratio and consequently inhibit activity and flux through PDH[130-132]. Data from positron emission tomography (PET) performed in healthy subjects, showed that ketone bodies are able to decrease myocardial glucose uptake and increase myocardial blood flow[133].

HFpEF

To date, knowledge about cardiac metabolism in HFpEF is very limited. The currently available data regarding cardiometabolic HFpEF seem to describe a metabolic scenario quite distinct from HFrEF.

Obesity is a hallmark of this syndrome, and therefore an overload of fatty acids to the heart, and an imbalance between cardiac fatty acid uptake and fatty acid

oxidation is observed[67, 86, 134]. As mentioned, obesity and overweight have been shown to induce increased fatty acid oxidation in the heart. There are insufficient data on what happens to fatty acid oxidation when there is a transition from obesity without overt heart disease to cardiometabolic HFpEF. It is reasonable to assume that, at an early stage, fatty acid oxidation increase persists. Whether at a later stage this trend is still present or instead, a reduction in fatty acid oxidation occurs, as in HFrEF, is still largely unknown.

Regarding glucose metabolism, some evidence supports the presence of marked insulin resistance resulting in reduced glucose uptake[135, 136]. This would appear to be related to reduced glucose uptake and reduced glucose oxidation[90, 95, 137].

A decrease in PDH activity and diastolic function was observed in a streptozotocin-induced diabetic cardiomyopathy model[138]. Interestingly, dichloroacetate (DCA), a PDK inhibitor that increases PDH activity, reversed insulin resistance, increasing PDH flux and improving cardiac function[138]. In the same context, heart-specific PDHA1(-/-) knockout mice showed altered insulin signaling, associated with diastolic dysfunction[139].

Systemic insulin resistance is a feature of the metabolic syndrome and thus correlates with the presence of obesity. The changes observed in diabetic cardiomyopathy could therefore be present in cardiometabolic HFpEF but limited data are available at the moment.

Ketones in HFpEF

As described above, studies in animal models and humans have revealed a reprogramming of myocardial fuel utilization in HFrEF, with a shift away from the main metabolic fuel pathway, namely fatty acid oxidation.

Increasing ketone concentration and ketone oxidation in the heart could improve HFpEF. In mouse models of hypertension and myocardial ischemia, cardiomyocyte-specific BDH1 deletion resulted in worsened cardiac hypertrophy, ventricular remodeling, and contractile dysfunction[124]. In contrast, overexpression

of the enzyme protected against the development of myocardial fibrosis and cardiac dysfunction[127].

Although the circulating levels and content of BOH in the cardiac tissue of HFpEF patients appear to be similar or even higher than those reported in HFrEF[67, 140, 141], ketone bodies oxidation does not appear to be increased but, on the contrary, might be inhibited due to downregulation of BDH1[67].

The role of ketones in HFpEF may not be restricted to their use as an alternative energy substrate.

For example, increasing BOH levels by fasting or exercise has been shown to inhibit NLRP3 (Nod-like receptor family protein 3)-mediated inflammation. In fact, BOH inhibited inflammasome assembly resulting in a reduction of inflammasome-mediated interleukin (IL)-1β and IL-18 production in human monocytes[142]. This inhibition of ketone body-mediated inflammation is independent of oxidative pathways and suggests how under conditions of energy deprivation, such as prolonged fasting or heart failure, BOH can reduce innate immune responses, saving more ATP for the heart[142]. Furthermore, in the clinical setting, the ketogenic diet has been shown to reduce levels of circulating inflammatory cytokines[143]. In addition, in a mouse model of HFpEF, ketosis induced by SGLT2is or ketone ester suppressed NLRP3 inflammasome activity and improved diastolic dysfunction[67].

In addition to their anti-inflammatory role, ketones may also have a signaling role.

BOH, the most abundant of the ketone bodies, has recently been identified as a substrate for a post-translational modification of protein lysine (K), called β-hydroxybutyrylation (Kbhb)[144]. It has been described how Kbhb plays an important role in both epigenetic regulation and modulation of the function of numerous proteins in cells. Histone Kbhb levels are significantly induced under fasting conditions, with a dose-dependent correlation between BOH and Kbhb levels[144]. Histone Kbhb-induced epigenetic regulation influences cellular metabolism, including the PPAR signaling pathways, and oxidative phosphorylation in the liver[144]. Although a complete understanding of the effects of BOH on gene expression has not been achieved, the most recent evidence points to an

antiproliferative effect in high replicative tissues[145]. Importantly, knowledge about the role of Kbhb in cardiac homeostasis and disease is still limited.

Kbhb modification is not limited to histones. The transcription factor p53 is modified by Kbhb[145]; p53-Kbhb results in reduced expression of the transcriptional target genes of p53, p21, and p53 upregulated modulator of apoptosis (PUMA), as well as reduced cell growth arrest and apoptosis[146]. Other nonhistone proteins are affected by Kbhb. A paper examining the role of BOH in an aging model of HFpEF described an increase in the activity of the TCA cycle enzyme citrate synthase[67]. The authors reported that the increase in BOH in an aging model of HFpEF, led to an increase in citrate synthase activity. The authors reported that the higher BOH levels were accompanied by an increase in citrate synthase-Kbhb and a subsequent increase in its enzymatic activity, suggesting that the BOH-induced improvement in mitochondrial function was dependent on citrate synthase-Kbhb[67]. However, how Kbhb affects mitochondrial function and overall cardiac metabolism in HFpEF is still largely unknown. The key regulators of mitochondrial Kbhb have not been fully elucidated, but it appears that the mitochondrial de-acetylase SIRT3 also exerts a de-Kbhb effect[147]. It is not known whether SIRT3 acts as a de-β-hydroxybutyrylation enzyme in the heart.

In summary, Kbhb is a novel post-translational modification involved in transcriptional modulation of cellular metabolism and, potentially, fine-tuning of mitochondrial protein activity. The most recent evidence suggests an involvement of Kbhb in heart disease of metabolic origin, including HFpEF, but it is not known whether Kbhb is adaptive or maladaptive.

Exploring cardiac metabolism in HFpEF

Rewiring of cardiac metabolism is then a key element of HFrEF. Preliminary findings in the literature suggest that substantial changes in energy substrate utilization also occur in the hearts of patients with HFpEF. There is currently a significant gap of knowledge between the current understanding of cardiac metabolism in HFrEF and the limited evidence available in HFpEF. To fill this gap, data from animal models have a major role to play.

Animal models vs clinical approach

The use of animal models allows direct measurement of gene expression and concentration of enzymes and transporters involved in all pathways of cardiac metabolism. Mass spectrometry techniques can allow quantification of key metabolites of the TCA cycle and oxidation pathways of different substrates in the heart. The use of stable isotope tracing allows the anabolic or catabolic fate of exogenously administered substrates to be defined. One can also directly measure the mitochondrial oxidation capacity of different substrates from isolated cardiac mitochondria.

An additional advantage of using animal models is the possibility to develop a HFpEF phenotype by modifying only specific risk factors. The development of HFpEF may depend on numerous risk factors (obesity, hypertension, aging, etc...). Each combination of risk factors results in a syndrome with different characteristics. Potentially, different changes in metabolism can be found for each phenotype. To understand how different triggers affect energy substrate utilization in the heart with HFpEF, it is necessary for the phenotype investigated to be as straightforward and reproducible as possible. This is only feasible using an animal model, with the same genetic background and exposure to risk factors.

Furthermore, understanding how a single change in a metabolic pathway affects the overall picture is only possible through an intervention aimed at a specific target. Intervening in animal models by silencing or over-expression of enzymes, transporters, or other key proteins in cardiac metabolism is a fundamental tool to understand the role of that target in the syndrome.

The uptake and utilization of energetic substrates can also be investigated in a clinical setting (by use of nuclear imaging techniques, stable isotope tracing, endomyocardial biopsies, measurement of metabolite concentrations in the coronary bloodstream, etc...). The main advantage of this approach is that it does not explore a model, which always has limitations in terms of matching the reality of the disease under investigation. The disadvantages are the feasibility of research techniques and the heterogeneity of patients, which in turn implies a large sample size.

In addition, in a clinical setting, metabolic pathways cannot be directly manipulated, for example, by intervening in enzymes involved in the oxidation of different substrates or signaling pathways. This limits the understanding of the exact role of the changes detected and prevents the possibility to establish whether a particular difference from the control subjects represents an adaptive or maladaptive response.

In summary, the use of animal models currently remains a key stepstone in the exploration of cardiac metabolism in heart failure. It is irreplaceable to the end of gaining a more focused exploration of metabolic alterations in a clinical setting and/or in mechanistically confirming the role of a specific alteration found in patients.

Post-translational modifications in cardiac metabolism

A specific aspect of the exploration of cardiac metabolism is the investigation of post-translational modifications of critical proteins in the metabolic machinery. The metabolic flexibility that distinguishes the healthy heart is based on the ability to rapidly adapt pathways according to environmental changes. If, for example, fatty acid availability increases, the oxidative capacity of fatty acids in the heart must increase accordingly. To achieve this goal, a cardiomyocyte can respond by increasing the concentration of transporters and enzymes involved in the pathway. This requires increasing transcription and subsequently translation of the proteins needed. However, this response takes time. A more rapid response may come from post-translational modifications, that is, conditions under which specific molecules bind to proteins, changing their structure and function. Well described post-translational modifications are, for example, phosphorylation or acetylation. Such post-translational modifications occur rapidly and are usually reversible. They constitute the first response in the modulation of a metabolic pathway.

One example is the inhibition of glucose oxidation by phosphorylation of the rate-limiting enzyme of this pathway, namely PDH. A particularly interesting aspect of this regulatory mechanism is that it is often the energy substrates themselves (or their derivatives) that are installed on other molecules as post-translational modifiers. Acetylation, for example, is closely related to acetyl-CoA availability, which depends on fatty acid abundance[148]. Succinylation or malonylation are other

examples of post-translational modifications in which key intermediates of central carbon metabolism are involved in the regulation of metabolism itself[149].
One post-translational modification already mentioned about which very little is known in the heart is lysine (K) β-hydroxybutyrylation (Kbhb). BOH is a substrate that has a great potential as alternative fuel in heart failure. Nevertheless, its role both as an energy substrate and as a promoter of post-translational changes in the heart during HFpEF is almost completely unknown.

HFpEF animal models

Animal models are of paramount importance when investigating cardiac metabolism in HFpEF. However, choosing the appropriate model of HFpEF to use as a platform is not easy.

The first difficulty is to identify a model that not only recapitulates cardiac morpho-functional alterations but also develops a true systemic syndrome.

Indeed, the first thing to evaluate in a model of HFpEF (or HFrEF) is that the model exhibits the key features of the clinical syndrome observed in heart failure. Clinically, heart failure is identified by the development of symptomatic dyspnea or fatigue, which is then confirmed by signs of volume overload, elevated filling pressures, impaired exercise capacity, and impaired tissue perfusion from reduced cardiac output. Assimilating the finding of isolated diastolic dysfunction, in the absence of volume overload or reduced exercise capacity, to diastolic heart failure or HFpEF is an error too frequently found in the published literature.

Assessing whether an animal model accurately reproduces the clinical syndrome of HFpEF is undeniably complicated. The inability of animals to express the cardinal symptoms of heart failure makes modeling heart failure in animals a major challenge.

Although symptoms cannot be described by an animal, lung congestion and exercise capacity can be measured objectively and should be among the first phenotypic aspects to be assessed.

Congestion can be assessed directly by lung weight (wet weight or wet weight/dry weight ratio) or invasive hemodynamics.

In addition, circulating BNP (B-type natriuretic peptide) can be measured in serum or plasma of mice and has been associated with pathological cardiac remodeling and dysfunction in this setting[150].
Similarly, we recommend careful assessment of exercise capacity in each animal model of HFpEF.

Once the heart failure phenotype is confirmed through congestion and exercise intolerance (preferably both), it is necessary for the model to recapitulate alterations in cardiac contractile performance.
In generating models of HFpEF, robust validation of preserved left ventricular ejection fraction is critical, as this is a defining feature of the syndrome.

Although left ventricular ejection fraction is preserved in HFpEF, it is important to note that cardiac contractile performance is not perfectly normal. The ability of the heart to increase its output under physiological or pathological stress is markedly impaired in HFpEF due to deficiencies in cardiac reserve, which depends on altered inotropic, chronotropic, and diastolic release capacity[151, 152].

It is critical that the left ventricular ejection fraction in the model remains normal, even after long exposure to the identified risk factor: models marked by transition to HFrEF and termed temporary HFpEF are not considered reliable because transition from HFrEF to HFpEF is rare in the general population[20].

Different phenotypes, different models

As mentioned, HFpEF is a syndrome that can manifest with different phenotypes. Therefore, the first element to consider is which phenotype is reproduced in the model that is chosen. A single model of HFpEF that reproduces all phenotypes does not exist and is not even desirable, as it would not allow to distinguish the contribution of individual hits to the syndrome. Rather than expecting a single animal model to reproduce all aspects of the different clinical manifestations of HFpEF syndrome, it is important that there be multiple models, each capturing the different features of individual phenotypes. This allows not only for the alignment

of preclinical research with the heterogeneity of the clinical syndrome, but also for the identification of different treatments suitable for personalizing therapeutic approaches in HFpEF.

Elderly HFpEF

Normal aging, in itself, leads to many of the alterations observed in HFpEF. Exercise capacity progressively decreases by ≈10% for each decade of life, and this deterioration accelerates further beyond 70 years of age[153]. Similarly to HFpEF, exercise intolerance in older adults results largely from reductions in cardiac reserve, but also from impairments in peripheral oxygen extraction[154-156]. In addition, progressive age-related arterial stiffening contributes to the development of the cardiac hypertrophy, adverse myocardial remodeling, and subclinical systolic and diastolic dysfunction observed in the elderly[154, 155].

Several animal models recapitulate the cardiac and systemic changes observed in elderly HFpEF.

The aged C57BL/6 mouse (generally >24 months) is by far the most widely used rodent model in cardiac aging studies. Extensive cardiovascular phenotyping of the aged C57BL/6 mouse has consistently shown that these animals exhibit many of the hallmark features of HFpEF, including exercise intolerance, congestion, preserved LVEF, impaired systolic strain, decreased cardiac reserve, diastolic dysfunction, pathologic LV remodeling, and arterial stiffness[157-159]. Importantly, HFpEF phenotype in this model develop in the absence of overt hypertension, pulmonary vascular disease, glucose intolerance, or obesity, making this model particularly suitable for studying age-specific mechanisms that do not necessarily overlap with other phenogroups of HFpEF. However, there is no agreement on whether the aged C57BL/6 mouse truly represents a model of HFpEF or whether it is simply representative of what physiological cardiovascular aging is.

Like the aged C57BL/6 mouse, the aged Fisher 344 (F344) rat recapitulates many of the phenotypes observed in human aging and HFpEF. These changes develop progressively with age and begin to manifest around 24-30 months of age, becoming more apparent at 36 months of age. At these latter times, animals show

signs of exercise intolerance, pathological cardiac hypertrophy and diastolic dysfunction[160-164].

However, studies on this model have shown that F344 rats are prone to develop systolic dysfunction as well, which has diminished enthusiasm for the use of this model[161-163].

Overall, these models may be useful in identifying the contribution of aging to HFpEF. However, it is difficult to distinguish the effect of physiological aging from the actual HFpEF syndrome (which does not develop in all elderly subjects). In addition, although the shorter average lifespan of rodents (approximately 2-3 years) has made them a powerful tool for studying the mechanisms of cardiovascular aging and age-related HFpEF, the considerable time required to acquire clinically relevant phenotypes in these animals still poses a major challenge in their use in preclinical research.

The role of hypertension: cardiorenal HFpEF

Hypertension remains the most common comorbidity in patients with HFpEF[43, 165], presenting alone or associated with other comorbidities[166]. Overall, the pathophysiology of HFpEF in hypertensive patients is complex and multifactorial. The classic paradigm predicts that poorly controlled hypertension causes left ventricular hypertrophy, left atrial remodeling, diastolic dysfunction, and finally heart failure[40].

HFpEF associated with chronic hypertension is often accompanied by renal dysfunction which has led to define the combination of these two organ damages as cardiorenal HFpEF[167, 168].

The effects of increased afterload on cardiac remodeling and function have been studied extensively over the years, and several animal models of hypertension-associated HFpEF ranging from malignant hypertension to mild-to-moderate hypertension have been reported. The latter is the most clinically relevant condition, as it is the most commonly observed phenotype in patients with HFpEF. It is often accompanied by left ventricular hypertrophy and concentric remodeling, as well as diastolic dysfunction, both of which should be rigorously evaluated in models of this phenotype. Furthermore, in an attempt to faithfully reproduce the clinical syndrome of HFpEF, left ventricle ejection fraction must remain preserved. In

many models of malignant hypertension, left ventricle ejection fraction tend to decrease after long exposure to the extreme increase of the afterload and this is why these models should be excluded.

Chronic stimulation with angiotensin II (in a dose-dependent manner) in mice leads to cardiac hypertrophy and pathological myocardial remodeling, both in the presence[169-171] and absence[172, 173] of hypertension. This phenomenon is paralleled by the development of diastolic dysfunction, including worsening of the left ventricular isovolumetric relaxation time and increased left ventricular end-diastolic pressure[170, 171, 173, 174]. However, with chronic angiotensin II stimulation, both preserved and reduced[174] left ventricular ejection fraction have been documented and as such this stimulus can induce both HFpEF and HFrEF, depending on the dose and duration of the treatment. This is a considerable disadvantage of the model, as it does not allow a clear distinction between HFrEF and HFpEF.

Cardiorenal HFpEF can also develop in rats by deoxycorticosterone acetate treatment combined with unilateral nephrectomy and administration of water with increased salt content[175, 176]. This model develops hypertension with exercise intolerance but not pulmonary congestion[177, 178]. In another model, called SAUNA, the combination of salty drinking water, unilateral nephrectomy, and chronic aldosterone results in inducing HFpEF in both rats and mice[179, 180]. Both exercise intolerance and pulmonary congestion are achieved in this model.
Other proposed models of hypertension-associated HFpEF described in the literature include Dahl's salt-sensitive rat, the spontaneously hypertensive rat, the mouse model of pressure overload induced by transverse aortic constriction[181], or transverse aortic constriction in the cat[182]. The first two models develop severe or malignant hypertension, and all four demonstrate progression to eccentric/dilated left ventricular hypertrophy and ultimately a reduction in left ventricular ejection fraction[183].
As mentioned, such progression from HFpEF to HFrEF is rarely observed in patients with HFpEF[20], which would suggest that these patterns differ from the clinical syndrome and are therefore unreliable.

Models of cardiometabolic HFpEF

The world is experiencing a global increase in obesity and associated metabolic stress. The World Health Organization reports that in 2016 more than 1.9 billion adults worldwide were overweight and, of these, over 650 million were obese (13% of the world's adult population) Moreover, 80% of HFpEF patients in the USA are overweight or obese[42] with an average BMI exceeding 35 kg/m^2 [42, 184].

Overall, the phenotype of obese HFpEF patients with hypertension is progressively increasing and has made this subgroup of patients probably the most prevalent in the general population. Animal models that are able to recapitulate this phenotype, defined cardiometabolic HFpEF, are therefore of particular interest. One of the models that has emerged is the obese diabetic rat ZSF1 (hybrid F1 Zucker spontaneously hypertensive fat). The animal phenotype is quite consistent with that of HFpEF in the obese patient[185-189]. By 10-20 weeks, obese ZSF1 rats show concentric left ventricular hypertrophy with preserved ejection fraction, diastolic dysfunction, and increased filling pressures. Pulmonary congestion and exercise intolerance are also observed[186, 189].

The myocardium of these animals also shows the features of adverse remodeling commonly observed in patients with HFpEF, including cardiomyocyte hypertrophy, interstitial fibrosis, and microvascular fibrotic changes[190]. Equally important is the remarkable recapitulation of extracardiac HFpEF phenotypes observed in this model. Consistent with cardiometabolic stress, insulin resistance and obesity are present, but also modest hypertension, increased vascular stiffness, renal dysfunction, and adverse skeletal muscle remodeling associated with altered perfusion and function[188, 190, 191].

However, this model has the limitation of being an exclusively genetic model. This is, first of all, a fundamental difference from patients with HFpEF, in whom obesity is predominantly determined by diet. The mechanisms that distinguish diet-derived obesity from genetic obesity are multifaceted, and the contribution of genetic background in obesity can have an extremely relevant effect on metabolism[192].

Using a model in which obesity is genetically determined to obtain information on the cardiac metabolism of patients with diet-induced obesity may therefore be a relevant bias. Furthermore, the onset of the different hits (e.g., hypertension, insulin resistance) in the ZSF1 rat occurs simultaneously, making it impossible to

distinguish the contribution of individual factors to the syndrome. Finally, the progression is unstoppable, so it is not possible to assess the effects of, for example, dietary changes or discontinuation of the hypertensive stimulus.

A nongenetic model, based on a multi-hit approach, impressively recapitulated the phenotype of cardiometabolic HFpEF in mouse[193]. In this model, animals are exposed to two factors: a high lipid diet that triggers obesity and metabolic syndrome and L-NAME (L-NG-Nitro arginine methyl ester), a nitric oxide synthase inhibitor that causes a modest increase in blood pressure (≈40 mmHg). The rationale for this model comes from the convergence of the 2 main comorbidities found in patients with HFpEF: mechanical load (hypertension) and metabolic/inflammatory stress (obesity, metabolic syndrome, diabetes). None of the 2 hits is sufficient, alone, to drive the syndrome. It is the convergence of the 2 factors that generates a phenotype consistent with the cardiometabolic HFpEF. These animals manifest diastolic dysfunction, preserved left ventricular ejection fraction, impaired left ventricular strain, exercise intolerance, elevated left ventricular filling pressures, microvascular dysfunction, modest cardiomyocyte hypertrophy, capillary rarefaction, susceptibility to atrial fibrillation, and modest myocardial fibrosis[193]. Importantly, these animals do not develop HFrEF over time.

In addition to the consequences of the inflammatory state, cardiomyocytes exhibit significant steatosis (lipid accumulation) resulting in lipotoxicity, resulting from the myocardium's altered utilization of lipids as an energy source[53]. These events occur in concert with alterations in mitochondrial fatty acid oxidation and altered myocardial energy[194].

In this model, circulating lipids also act as inflammatory molecules and promote recruitment of immune cells to the myocardium, contributing to altered cardiomyocyte metabolism[53].

In a few years, the “2-hit” murine model of HFpEF has been successfully adopted by a large number of investigators worldwide[195-197], becoming a powerful platform to systematically define and manipulate mechanisms underlying HFpEF.

AIM OF THE STUDY

Millions of people around the world suffer from HFpEF, a grievous disorder characterized by substantial morbidity, premature mortality, and high health care cost. The search for treatments specifically designed to treat HFpEF has stalled for more than 2 decades, without significant progress. Meanwhile, the syndrome continues to expand in prevalence, affecting an increasing percentage of the population.

To find new therapeutic strategies, it is critical to gain a deeper understanding of the pathophysiology of HFpEF, especially for what concerns cardiac metabolism in this syndrome. To this purpose, an animal model of the syndrome is needed as it is an irreplaceable platform to be used for mechanistic assessments. HFpEF is an heterogeneous syndrome. It may be driven by a combination of multiple risk factors (hypertension, ageing, obesity, metabolic syndrome, etc…). Arguably, one of the most common phenotype of HFpEF arises from the combination of obesity and hypertension. Considering the skyrocketing progression in prevalence of obesity and the burden of hypertension in the general population of Western countries, expanding the knowledge on the so-called cardiometabolic HFpEF appears to be of paramount importance.

The "2-hit" mouse model of cardiometabolic HFpEF has contributed to the understanding of the molecular mechanisms underlying this syndrome, unveiling the critical role of metabolic stress in HFpEF cardiomyocytes. However, many fundamental questions in the pathophysiology of cardiometabolic HFpEF remain unresolved.

The present research project has the aim to provide a deeper understanding of cardiac metabolism in cardiometabolic HFpEF. In particular, a largely unexplored area of HFpEF biology will be explored: the mechanistic role of ketones in cardiac metabolism in HFpEF. Ketone bodies will be evaluated as alternative fuels in HFpEF. Moreover, their effect as protein modifiers through lysine β-hydroxybutyrylation, thereby regulating metabolic signaling in cardiomyocytes, will be explored.

In summary, cardiac metabolism and in particular ketone body metabolism in HFpEF represents an important gap of knowledge. The aim of this project is to fill this gap by understanding the role of ketone metabolism and ketone-driven post-translational modifications in cardiomyocyte homeostasis in HFpEF.

METHODS

Experimental animals

C57BL/6 N mice were purchased from Charles River Laboratories. Only male adult (8–10-week-old) mice were used in the experiments as our recent work demonstrated that female mice are less susceptible to HFD+L-NAME treatment[198]. Mice were maintained on a 12-hour light/dark cycle from 6 am to 6 pm and had unrestricted access to food and water. To establish the HFpEF phenotype, 10-week-old C57BL/6N mice were subjected to a combination of high-fat diet (HFD; #D12492, Research Diet Inc.) and N[w]-nitro-l-arginine methyl ester (L-NAME; Sigma-Aldrich) supplied in drinking water for 15 weeks as previously described[193]. Chow-fed mice were used as control animals. Pregnant Sprague-Dawley rats were purchased (Charles River Laboratories) and were used for the sole purpose of harvesting primary cardiomyocytes from 1–2-day old rat pups.

Isolation of Cardiac Mitochondria

Immediately following euthanasia, hearts were excised and homogenized in ice-cold isolation buffer (10 mmol/L MOPS [3-(N-morpholino) propanesulfonic acid], 1.0 mmol/L ethylenediaminetetraacetic acid (EDTA), 210 mmol/L mannitol, and 70 mmol/L sucrose, pH 7.4) using a Dounce Homogenizer. The homogenate was centrifuged at 2000 rpm (5 minutes, 4°C), and the supernatant was filtered through cheesecloth. The mitochondrial pellet was obtained by centrifugation of the supernatant at 10 000 rpm for 10 minutes (4 °C)[199]. Mitochondria were resuspended in homogenization buffer to the final concentration needed for the subsequent experiments. Protein determinations were made using the bicinchoninic acid method (BCA - Pierce) with bovine serum albumin (BSA) as standard.

Analysis of Mitochondrial Respiratory Function

Oxygen consumption rate (OCR) was measured using a fluorescence- based oxygen sensor (NeoFox, Ocean Optic) connected to a phase measurement system from the same company. The sensor was calibrated according to the manufacturer's

instructions as previously described[200]. Mitochondria were diluted to 0.25 mg/mL in 10 mmol/L MOPS, 210 mmol/L mannitol, 70 mmol/L sucrose, and 5.0 mmol/L K2HPO4 at pH 7.4 containing respiratory substrates (25 μM palmitoylcarnitine or 0.1 mmol/L pyruvate or 10 mmol/L glutamate, 1 mmol/L malate, 10 mmol/L β-Hydroxybutyrate) as indicated. State 3 respiration was initiated by addition of ADP at a final concentration of 0.25 mmol/L, and state 4 was measured after ADP exhaustion.

Evaluation of PDH Activity

Mitochondria were diluted to 0.05 mg/mL in buffer containing 25 mmol/L MOPS and 0.05% Triton X-100 at pH 7.4. Solubilization of mitochondria with 0.05% Triton X-100 inhibits complex I of the respiratory chain preventing consumption of NADH. PDH activity was measured spectrophotometrically as the rate of NAD+reduction to NADH (340nm) upon addition of 2.5 mmol/L pyruvate, 0.1 mmol/L CoASH, 0.2 mmol/L thiamin pyrophosphate, 1.0 mmol/L NAD+, and 5.0 mmol/L MgCl2 at pH 7.4[199].

Evaluation of MDH2 Activity

Mitochondria were diluted to 0.05 mg/mL in a Triton X-100 buffer containing (final concentration): MgCl2 5 mmol/L, Citrate synthase 1 unit/ml, Acetyl-CoA 0.3 mM, NAD+ 10 mM, Malate 25 mM. MDH2 activity was measured spectrophotometrically as the rate of NAD^+ reduction to NADH (340nm).

Evaluation of BDH1 activity

BDH1 activity was determined in isolated mitochondria homogenates using an adapted method[201]. Mitochondrial homogenates were preincubated in assay buffer (50mM Tris-HCl (pH 8.0), 2.0 mM NAD+, 0.3 μg of rotenone, 0.4mg of BSA) for 10 min at 30°C. The reaction was initiated by the 50ul addition of 0.2M β-hydroxybutyrate. The rate of NAD+ reduction was calculated from the increase in 340nm for 2 minutes. Enzyme activity is expressed as nmol of NAD+ reduced/min/mg of protein.

GOT2 activity

GOT2 activity was measured using a commercial AST activity assay (Merck, MAK055) according to manufacturer's instruction. In brief, in this kit, the transfer of an amino group from aspartate to α-keto-glutarate results in the generation of glutamate, resulting in the production of a colorimetric (450 nm) product proportional to the AST enzymatic activity present. For mice, 10.5 ug of mitochondrial protein were diluted in the recommended master reaction mix and absorbance at 450 nm was measured at 37° every 5 minutes. For cells, the same amount of protein lysates was used. For each sample, measurement was performed in technical duplicates. The slope of the curve withing the linear range of increase were recorded for each sample, at the same time point, and used for comparisons.

MAS activity

Cardiac mitochondria were prepared as above. The MAS assay was adapted from Wang et al[202] and Cao et al[203], with some modifications. In brief, after BCA quantification, 150 μg of mitochondria were resuspended in 100 μl of a buffer containing the following reagents (final concentrations): Mannitol 300 mM, K2HPO4 10 mM, Tris pH 7.4 10 mM, KCl 10 mM, MgCl2 5 mM, Aspartate 2 mM, ADP 2 mM, NADH 140 μM, AST 2 U/ml, MDH 3 U/L. After resuspension, the solution was loaded in technical duplicates in a 96-well plate. Baseline oxidation of NADH was monitored at 340 nm at a constant temperature of 37°C for 4 min. MAS activity was initiated with the addition of 4 mM malate and 4 mM glutamate (final concentrations), and the oxidation of NADH was monitored at 340 nm at a constant temperature of 37°C. The difference between the rate of change of absorbance with and without added substrates was normalized to added mitochondrial protein to determine the shuttle capacity.

Cell culture and transfection

HEK293T and AC16 cells, a proliferating human cardiac myocyte line derived from fusion of primary adult ventricular myocytes with SV40 fibroblasts[204], were purchased from Millipore. NRVMs were isolated from 1 to 2-day-old Sprague-

Dawley rats[193]. HEK293T were cultured in Dulbecco's modified eagle medium (DMEM) complete media (Gibco, USA) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich, USA) and penicillin/streptomycin (100 U/mL, 100 U/mL, PAA) at 37°C in 5% CO2. AC16 cardiomyocytes were cultured in DMEM/F12 complete media (Gibco, USA) supplemented with 12.5% FBS (Sigma-Aldrich, USA), penicillin/streptomycin (100 U/mL, 100 U/mL, PAA) and Amphotericin B (0.25 µg/mL, Invitrogen) at 37°C in 5% CO2. NRVMs were cultured in DMEM medium/M199 (3:1) containing 3% FBS and antibiotics before performing the experiments. HEK293T, AC16 and NRVMs were treated with Sodium 3-hydroxybutyrate (Sigma) diluted in Phosphate-buffered saline (PBS) at final concentrations of 2 to 10 mM or Aminooxyacetic Acid (AOA, Cayman Chemicals) at final concentration of 400 uM for 24 hours when appropriate. In silico BOH treatment was modified from Wagner et al[205]. In brief, after protein extraction by lysis of AC16 cells in ice-cold modified RIPA buffer (150 mmol/L NaCl, 50 mmol/L Tris HCl pH 7.4, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 5 mmol/L EGTA, 2 mmol/L EDTA), total protein lysates were separated in 2 small tubes. Half of the proteins were denatured with a 5 minutes 95° heat treatment; the other half was stored in ice. Denatured and normal proteins were then treated in different tubes with increasing concentration of BOH. Total reaction volumes of 45 µl were incubated for 6 h at 37 °C at 400 rpm in an Eppendorf Thermomixer. After BCA protein quantification, samples were analyzed by SDS-PAGE.

For hypoxia experiments, cells were incubated at 1% hypoxia for 24 hours prior GOT2 assay. PBS-treated cells were used as controls. Plasmid transfections were performed using SIRT3 (13814), SIRT5 (13816), GCN5L1 (164625) purchased from Addgene. Custom vector for GOT2 WT and K396R were designed and constructed by VectorBuilder. Transfection was performed for 24 hours using plasmids at 1 ug/uL and Lipofectamine (Life Technologies) 4% diluted in a Opti-MEM™ Reduced Serum Medium (Thermo Fisher) solution. For gene silencing, predesigned siRNAs for GCN5L1 (BLOC1S1, SASI_Hs02_00331970), SIRT3 (EHU093591) and SIRT5 (SASI_Hs02_00358645) were purchased from Sigma. Cells treated with MISSION® siRNA Universal Negative Control (Sigma) were used as scramble (Scr) controls. Silencing was performed for 6 hours using siRNAs at 50 to 100 nM concentration and siLentFect lipid reagent (Biorad) 2% diluted in Opti-MEM™.

Immunoprecipitation

Protein extracts from HEK293T cells, transfected with flag-tagged GOT2 plasmids, were prepared by lysis in ice-cold modified RIPA buffer (150 mmol/L NaCl, 50 mmol/L Tris HCl pH 7.4, 1% Triton X-100, 0.5% sodium deoxycholate, 5 mmol/L EGTA, 2 mmol/L EDTA) containing protease and phosphatase inhibitors. Protein determinations were made using the BCA method (Pierce) with BSA as standard. Immunoprecipitation was carried out using 500 µg labelled protein diluted in 200 µL of modified RIPA buffer, incubated with Flag antibody (1:50, 4µL) overnight, 4 °C. Afterwards, 25 µl Dynabeads protein G (Thermo Fisher) were added for a 2 hours incubation time at room temperature. Beads were magnetically separated and washed four times with immunoprecipitation buffer and then eluted using a 2x Laemmli buffer (5 minutes, 95 °C). Immunoprecipitation for normal rabbit IgG antibody (Cell Signaling, 2729S) and total protein fraction (input) were used as controls. Samples were analyzed by SDS-PAGE.

RNA Isolation and Quantitative PCR

Total RNA was extracted from murine hearts, HEK293T and AC16 cells using the Quick-RNA™ MicroPrep kit (Zymo Research). A total of 500 ng RNA was used for reverse transcription using iScript reagent (Bio-Rad). qPCR reactions were performed in duplicate with SYBR master mix (Bio-Rad). The $2^{-\Delta\Delta CT}$ relative quantification method, using GAPDH for normalization, was used to estimate the amount of target mRNA in samples, and fold ratios were calculated relative to mRNA expression levels from control samples. The following PCR primer sequences were used (forward, reverse):

GAPDH-human (TCAACGACCACTTTGTCAAGCTCA, GCTGGTGGTCCAGGGGTCTTACT)

GAPDH-mouse (CGCTAACATCAAATGGGGTG, TTGCTGACAATCTTGAGGGAG)

PDK4-mouse (GTCGAGCATCAAGAAAACCGTCC, GCGGTCAGTAATCCTCAGAGGA)

GCN5L1-human (AAGAGGAGGCGAGAGGCTAT, GGACCTGTAGGGTCTTCACC)

Immunoblot Analysis

Protein extracts from frozen mouse hearts, AC16, HEK293T, and NRVMs were prepared by lysis in ice-cold modified RIPA buffer (150 mmol/L NaCl, 50 mmol/L Tris HCl pH 7.4, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 5 mmol/L EGTA, 2 mmol/L EDTA) containing protease and phosphatase inhibitors. Protein determinations were made using the BCA method (Pierce) with BSA as standard. Proteins were separated by SDS-PAGE on 4% to 20% gradient gels (Bio-Rad) and transferred to a nitrocellulose membrane. An Odyssey scanner (LI-COR version 3.0) was used as detection system. Protein expression was quantified using LI-COR ImageStudioLite software.
Proteins were detected with the following primary antibodies:

GAPDH (Merk Millipore, CB1001), PDHA1 (Thermo Fisher, PA5-21536), Phospho-PDH (Sigma, 31866), BDH1 (Proteintech, 15417-1-AP), OXCT1 (Proteintech, 12175-1-AP), ACAT1 (Abcam ab168342), GOT2 (Abcam, ab171739), MDH2 (Fisher Scientific, 16824654), Anti-β-hydroxybutyryllysine (PTM Bio, PTM-1201RM), SIRT3 (Cell signaling, 5490S), SIRT5 (Cell signaling, 8782S), Flag (Sigma, F1804)

Mass spectrometry

For proteomic analysis, snap-frozen left ventricle samples were homogenized and subjected to tryptic digest [28472372]. Tissue samples were homogenized on a cryo PREP system, followed by lysis in 150 µL Urea lysis buffer ((8 M urea; 75 mM NaCl, 50 mM Tris HCl pH 8.0, 1 mM EDTA, 2 µg/mL aprotinin (Sigma), 10 µg/mL leupeptin (Roche), 1 mM PMSF (Sigma), 10 mM NaF, 5 mM sodium butyrate, 5 mM iodoacetamide (Sigma), Phosphatase Inhibitor Cocktail 2 (1:100, Sigma), Phosphatase Inhibitor Cocktail 3 (1:100, Sigma). After a 20 min rest on ice, the samples were centrifuged at 14 000g for 10 min and 4°C to remove debris. Protein disulfide bonds of the combined lysates were reduced for 45 min with 5 mM dithiothreitol (Thermo Scientific) and alkylated for 45 min with 10 mM

iodoacetamide. Samples were then diluted with 50 mM Tris HCl, pH 8.0, to reduce the urea concentration to 2 M. Lysates were digested overnight at room temperature with trypsin/LysC mixture on a shaker. The protein digests were desalted on a SepPak as described below and a BCA was done to determine the peptide concentration. The samples were lyophilized and stored at -80°C until further processing.

Immunoaffinity purification (IAP) using anti-Kbhb-Ab beads

For the mass spectrometry analysis of Kbhb peptides, an immunoaffinity purification (IAP) using anti-Kbhb-Ab beads (Anti-β-hydroxybutyryllysine Antibody Conjugated Agarose Beads, PTM 1204, PTM Biolabs) was performed. In brief, Anti-β-hydroxybutyryllysine beads were washed 3 times with ice-cold PBS to clean them and then diluted in a 40 µL solution of IP buffer (100 mM NaCl, 1 mM EDTA, 20 mM Tris-HCl, 0.5% IGEPAL CA-630, pH 8.0). Dry peptides were then dissolved in IP buffer in a 10 mg/ml solution and sonicated. 1 mg peptide per sample was taken for the enrichment. Sonicated peptides and Anti-β-hydroxybutyryllysine beads were incubated overnight at 4°C with gentle shaking. The flow-throughs of the enrichment were collected for global proteome analysis. Beads were then washed as per manufacturer's protocol. The enriched peptides were eluted off the beads using 100 µl of elution buffer (0.1% Trifluoroacetic acid - TFA). The eluates were then spun down and desalted on stage-tips before LC-MS (Liquid Chromatography - Mass Spectrometry) measurement.

Desalting of Flow throughs for further analysis

For the global proteome analysis, flow-throughs of Kbhb-enrichments were desalted on Sep-Pak tC18 cartridges (Waters). The cartridges were conditioned with 3 ml acetonitrile (ACN) and 3 ml 50% ACN/0.1% FA (formic acid) followed by an equilibration using 4 x 3 ml of 0.1% TFA. Flow-throughs were loaded on the material and washed using 3 x 3 ml of 0.1% TFA and 1 x 3 ml of 1% FA before elution with 2 x 1.5 ml of 50% ACN/0.1% FA. The Peptide concentrations of the eluates were determined using the Pierce™ BCA Protein Assay (Thermo Scientific) and the samples were dried in a speedvac.

TMT-labelling

To enable multiplexing, the desalted samples underwent an isobaric labelling approach. The samples were labeled with 6 TMT (tandem mass tags) plexes per model organism from TMT 11plex reagent (Thermo Fisher Scientific). A total of 500 µg peptide/sample was assigned to each channel.

The peptides were reconstituted in 50 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), pH 8.5 to a peptide concentration of 5µg/µl, 800 µg TMT reagent was added to each corresponding sample and the reaction was incubated at room temperature with shaking (at 1,000 rpm) for 1 hour.

As a quality control the labelling reaction was quenched in a small aliquot (~1 µg) of each sample followed by stage tip desalting and loading the samples on a QE HF-X LC-MS system (DDA mode at 45,000 MS2 resolution, 1h gradient). The labeling efficiency was confirmed to be >98% and the relative abundance deviation of <25% of average TMT intensities across all channels was confirmed. After this quality control, all labelling reactions were quenched by the addition of 10 µl 5% hydroxylamine at room temperature for 15 minutes with shaking and the samples were pooled (separated by murine model) and dried in a speedvac. The samples were then reconstituted in 1 ml of 3% ACN/0.1% FA, the pH was adjusted to 3 with 98% FA and cleaned on SepPak cartridges as described before.

Offline high pH reverse phase fractionation

Pooled plexes were subjected to offline high pH reverse phase fractionation on a 1290 Infinity II LC system to generate a better protein coverage. 4.5 mM ammonium formate (pH 10) in 2% (vol/vol) ACN was used as mobile phase A and 4.5 mM ammonium formate (pH 10) in 90% (vol/vol) acetonitrile as mobile phase B. After equilibration of the system, the samples were separated over a 96 min gradient with mobile phase B concentration ranging from 0 to 60%.

The samples were initially separated into 96 fractions of which the first two were discarded. Fractions 3-9 and 94-96 were combined into one fraction each and for the remaining samples, that contained most peptides, every 28th fraction was combined. 5 µg peptides were taken from each of the 30 resulting fractions for global proteome analysis, dried on a speedvac and stored at -20°C until measurement.

LC-MS analysis

For global proteome analysis, dried peptides were reconstituted in 10 µL MS sample buffer (3% ACN/0.1% FA) and 2 µL were injected into the LC-MS/MS system consisting of an Easy-nLCTM 1200 coupled to an Orbitrap ExplorisTM 480 mass spectrometer (Thermo Fisher Scientific). Samples were separated on a 20 cm reverse-phase column packed in house with 3 µm C18-Reprosil beads (inner diameter 75µm) with a gradient of sample buffer and buffer B (90% ACN/0.1% FA). Buffer B was increased from 4% to 30% in the first 88 min, followed by an increase to 60% B in 10 min and a plateau of 90% B for 5 min. MS data was acquired in data dependent acquisition mode (DDA) with a top 20 method. MS1 spectra were acquired at a resolution of 60000 in the scan range from 375 to 1500 m/z, automated gain control (AGC) target was set to 300% and maximum injection time to 50 ms. MS/MS spectra were acquired at a resolution of 45000, AGC target of 100% and maximum injection time of 86 ms. Ions were isolated with a 0.4 m/z isolation window and normalized collision energy (NCE) was set to 35. For measurement of Kbhb-enriched peptides, a high field asymmetric ion mobility spectrometry approach was applied to increase protein coverage, due to the low amount of material expected after the enrichment.

Proteomic data analysis

For database search, MaxQuant version 1.6.10.43 was used. For global proteome analysis TMT 11-plex reporter ion quantitation was enabled with a PIF setting of 0.5. For PTM analysis Kbhb was added as a variable modification. Downstream analysis was done with Perseus, Protigy and R. For quantitation, a minimum of 3 valid intensities in either Control or HFpEF group was required. The remaining missing values were imputed using Gaussian distribution with downshift (width = 3, shift = 1.8). Corrected reporter ion intensities were scaled using median-median absolute deviation (median-MAD) normalization. For significance calling 2-sample moderated, 2-tailed Student's t testing was applied. P values were adjusted using the Benjamini-Hochberg method.

Statistical analysis

Data are presented as median ± interquartile range. For normally distributed data, the Student t test (unpaired 2-tailed) was used for 2-group analysis. Ordinary 1-way

ANOVA with Tukey multiple comparisons was used for multiple-group analysis, and 2-way ANOVA with Sidak multiple comparisons test was used for analysis of experiments with 2 independent variables. For non-normally distributed data, the Mann-Whitney test was used for 2-group analysis, and the Kruskal-Wallis test with Dunn multiple comparisons test was used for multiple-group analysis. A $P<0.05$ was considered statistically significant. No additional corrections for multiple testing were made across experiments. No statistical analyses were used to predetermine sample sizes; estimates were made based on our previous experience, experimental approach, and availability and feasibility required to obtain statistically significant results. Experimental animals were randomly assigned to each experimental/control group. All experiments were performed with at least three biological replicates. When representative images are shown, the selected images are those that most accurately represent the average data obtained in all the samples. Statistical analyses were conducted using GraphPad Prism software 9.0.

RESULTS

Glucose oxidation is defective in HFpEF

Consistent with our previous report[193], after 15 weeks of exposure to HFD plus L-NAME, mice developed the hallmark features of metabolic syndrome (obesity, glucose intolerance, hypertension) and HFpEF (left ventricular hypertrophy, diastolic dysfunction, exercise intolerance and lung congestion), whereas left ventricular ejection fraction remained preserved.

In previous reports, in HFpEF mice showed significant changes in mitochondrial structure (mitochondria in HFpEF myocardium were misaligned and segmented and mitochondrial cristae appeared swollen). Morphological changes were paralleled by altered function[194].

To better elucidate fuel preferences in HFpEF hearts, we tested mitochondrial OCR in response to different substrates in freshly isolated mitochondria. Despite absence of significant changes in succinate and glutamate-initiated respiration, we confirmed that pyruvate-initiated respiration was reduced in mitochondria isolated from HFpEF mouse hearts, as compared with control groups (***Figure 1***). A non-significant increase in State 3 was observed in palmitate OCR

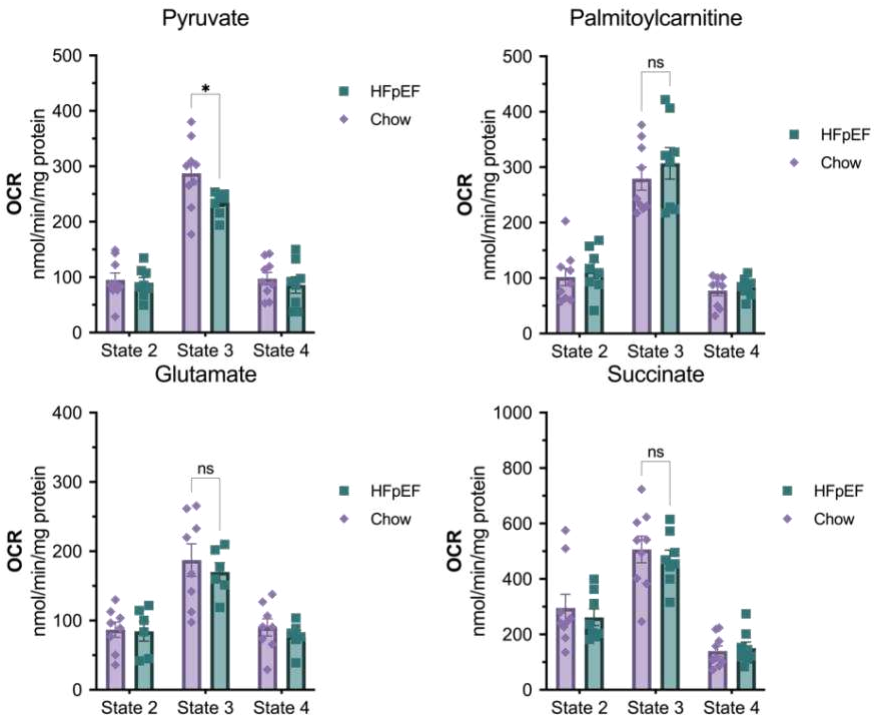

Figure 1 Oxygen consumption rates (OCRs) of different energy substrates in HFpEF vs control mice mitochondria

To further investigate glucose oxidation in HFpEF hearts, we evaluated the activity of the glucose oxidation rate limiting enzyme, namely PDH. PDH is a critical component of a complex of three enzymes that converts pyruvate into acetyl-CoA. This complex, therefore links glycolysis to the citric acid cycle. PDH activity is inhibited when its own specific PDK increases specific serine residues phosphorylation (***Figure 2***). PDK4 is the main PDH kinase in the heart[206].

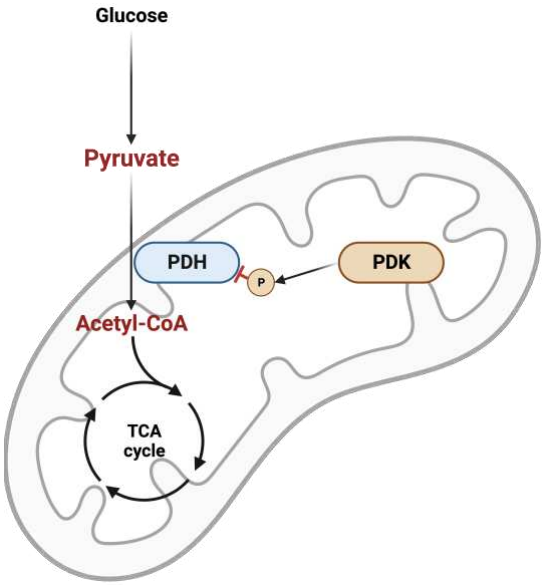

Figure 2 Central role of PDH in glucose oxidation pathway

In line with our previous findings[194], we observed that PDK4 was significantly upregulated in HFpEF mice (***Figure 3A***). This was coupled with increased PDH phosphorylation and a reduction in PDH activity (***Figure 3B and 3C***). These data are consistent with the lower pyruvate-initiated oxidation measured by OCR.

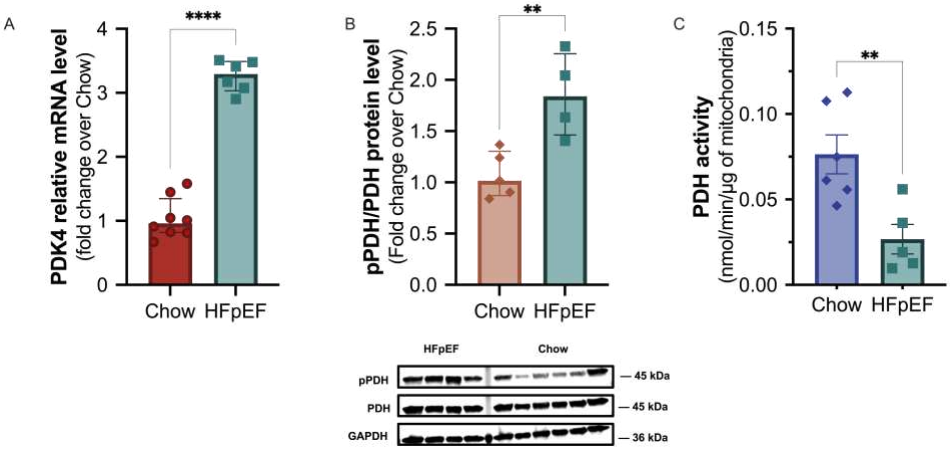

Figure 3 PDK4 relative expression (A), PDH phosphorilation (B), PDH activity (C)

Impaired ketone bodies oxidation in HFpEF model

Another substrate of great interest in heart failure are ketone bodies. In HFrEF, an increase in ketone bodies oxidation is reported[125-127] and therefore ketone bodies have been suggested as an alternative fuel also in HFpEF[207]. BOH is the most abundant ketone body in mammals and therefore we measured the oxidation of this substrate in freshly isolated mitochondria. Intriguingly, OCR in response to BOH was reduced in HFpEF hearts compared with chow (***Figure 4***).

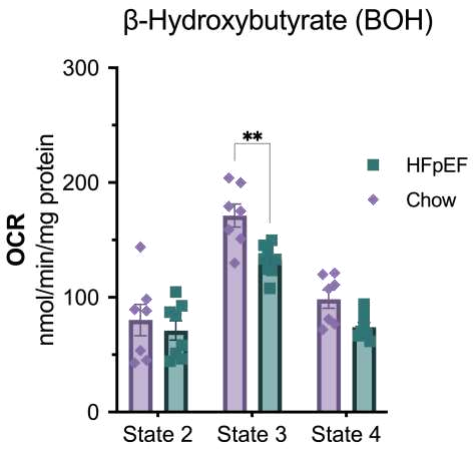

Figure 4 BOH oxygen consumption rate in HFpEF vs control mice mitochondria

The ketone bodies oxidation pathway starts from the oxidation of BOH into acetoacetate catalyzed by BDH1. Acetoacetate is then converted into acetoacetyl-

CoA by OXCT1. The final step is operated by acetyl-CoA acetyltransferase 1 (ACAT1), and consist in the transformation of acetoacetyl-CoA into 2 molecules of acetyl-CoA (***Figure 5***).

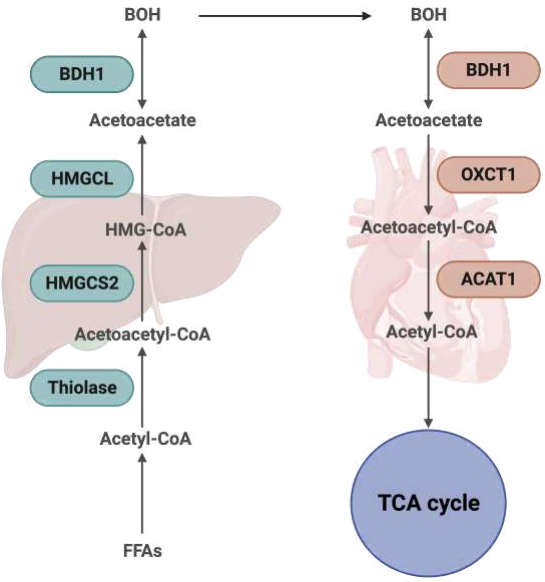

Figure 5 Ketone bodies metabolism

To elucidate the regulation of the whole pathway of ketone bodies oxidation in HFpEF, we measured the levels of all the involved enzymes. BDH1 and ACAT1 protein levels were significantly lower in HFpEF hearts compared with chow, while OXCT1 was not significantly reduced (***Figure 6A, B, and C***).

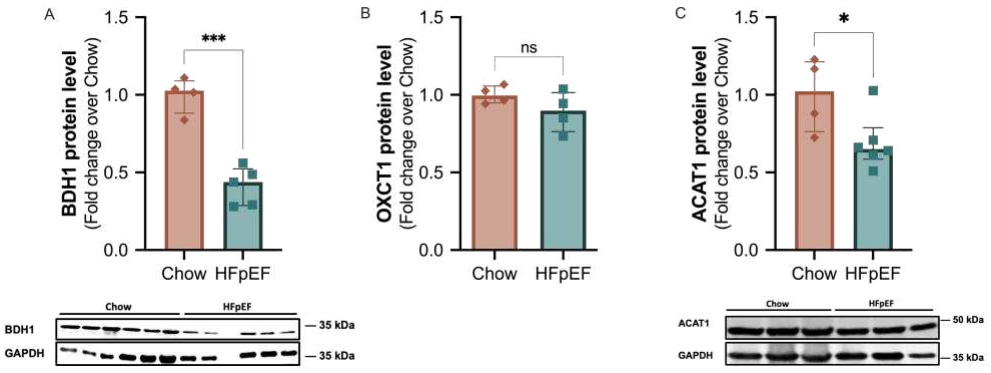

Figure 6 BDH1 (A), OXCT1 (B), and ACAT1 (C) protein levels

Moreover, BDH1 enzyme activity was significantly hampered (***Figure 7***).

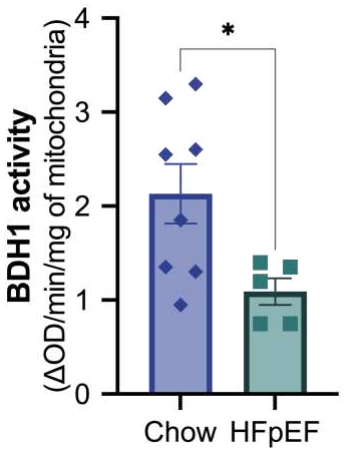

Figure 7 BDH1 activity

These findings expand and confirm previous reports suggesting a reduction in ketone bodies oxidation in another mouse model of HFpEF[67].

β-Hydroxybutyrate as a signaling molecule in HFpEF

BOH is not only a fuel but is also a substrate for a post-translational modification called lysine (K) β-hydroxybutyrylation (Kbhb)[144].

Considering the reduction in BOH oxidation rate, we aimed to investigate the alternative fate of this molecule and in particular, it's potential role as substrate for Kbhb in HFpEF hearts.

Interestingly, an increase in Kbhb was observed in protein lysates from HFpEF hearts when compared to Chow (***Figure 8***).

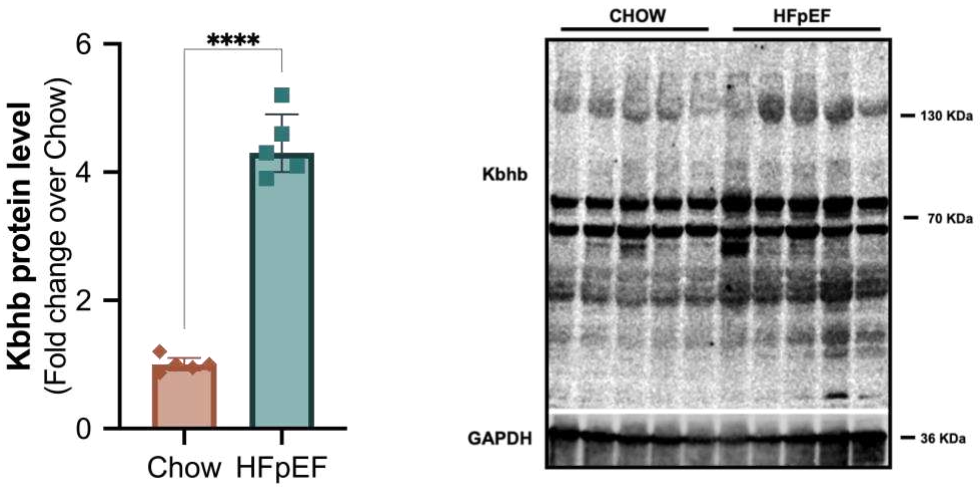

Figure 8 Kbhb levels in HFpEF vs control mice (left ventricles total proteins)

A mass-spectrometry analysis on left ventricles specimens confirmed the overall increase in Kbhb in HFpEF hearts and showed 44 targets with significant changes in Kbhb between HFpEF and Chow. The subcellular distribution of the differentially Kbhb proteins revealed that 84.4% of Kbhb targets are located in mitochondria. In most of the peptides, Kbhb was significantly upregulated in HFpEF (***Figure 9***).

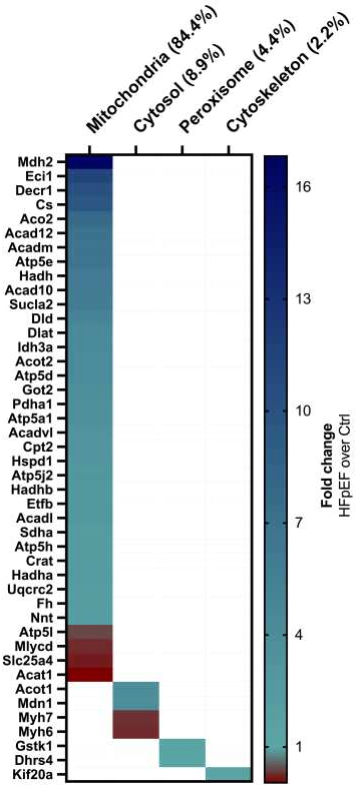

Figure 9 Heat map of Kbhb targets: fold change (HFpEF over controls) and locations

The results were supported by the Gene Ontology analyses of proteins exhibiting significant differences in Kbhb. The analyses uncovered that Kbhb proteins are involved in key pathways of cardiomyocyte metabolism (fatty acid oxidation, TCA cycle) and morphology (cardiac muscle hypertrophy) (***Figure 10***).

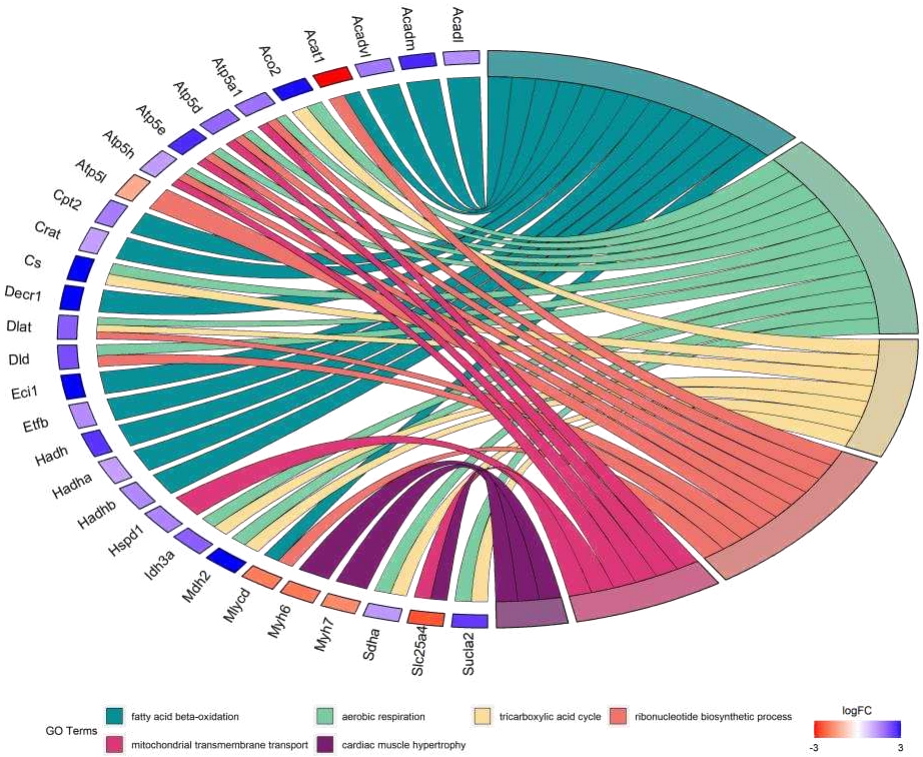

Figure 10 Gene Ontology of Kbhb targets (HFpEF over controls)

β-hydroxybutyrylation and malate-aspartate shuttle (MAS)

Among the several Kbhb proteins, considering the rank of their relative Kbhb fold change and their functional affinity, we focused on the evaluation of malate dehydrogenase 2 (MDH2) and glutamate oxaloacetate transaminase 2 (GOT2) (***Figure 11***).

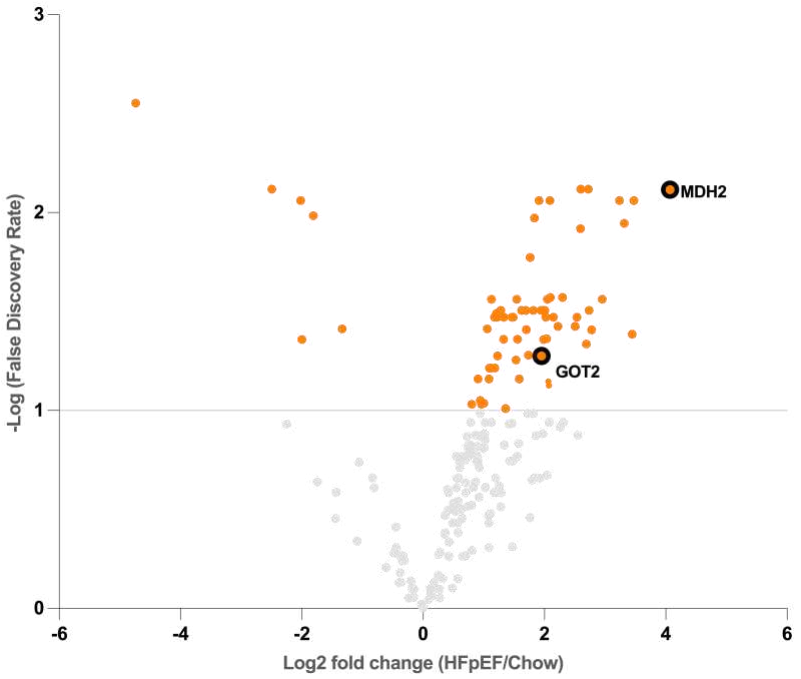

Figure 11 Volcano plot of Kbhb targets: fold change and False Discovery Rate of GOT2 and MDH2

These 2 proteins are the mitochondrial components of the malate-aspartate shuttle (MAS), a critical pathway in cardiomyocyte metabolism (***Figure 12***).

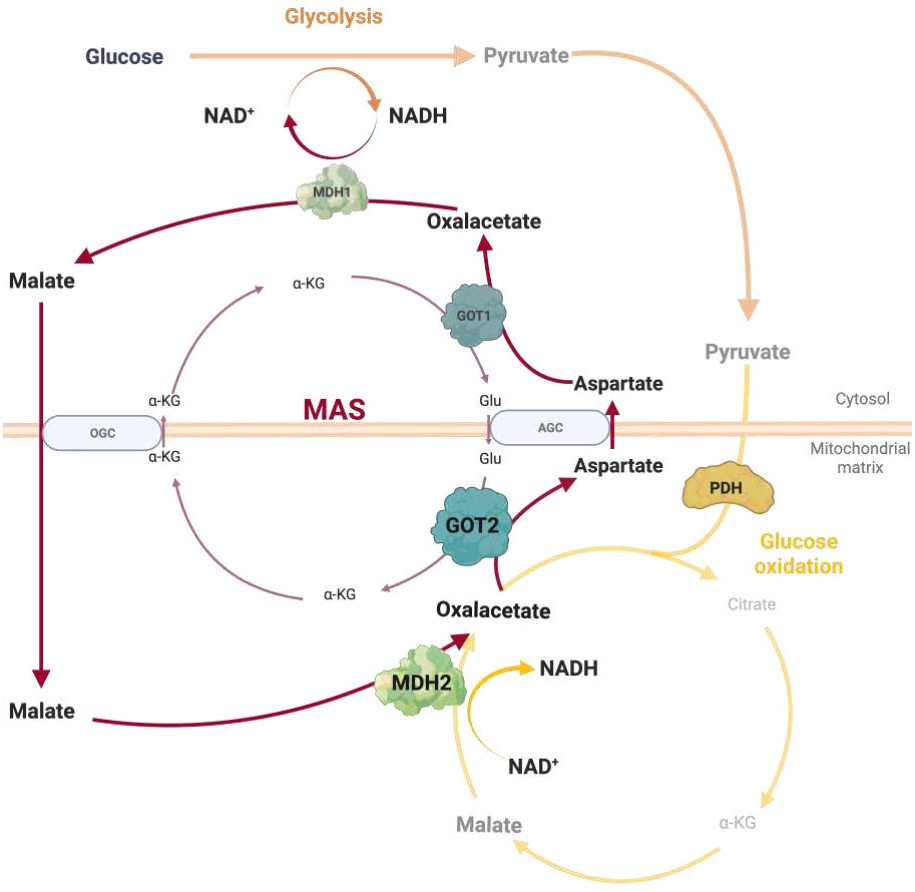

Figure 12 The malate-aspartate shuttle

During glycolysis, NAD^+ is reduced to NADH in the cytosol. The reducing cytosolic equivalents generated in this process must be transferred across the inner mitochondrial membrane. In this way, reducing equivalents can reach the mitochondrial matrix and NAD^+ is regenerated. This transfer, however, requires specific shuttles because the inner mitochondrial membrane is impermeable to NADH. In mammals, reducing equivalents transfer rely on the α-glycerophosphate shuttle and the MAS. MAS is the main shuttle in the heart[208, 209] and is operated by two pairs of enzymes, namely mitochondrial and cytoplasmic glutamate oxaloacetate transaminases (GOT) and malate dehydrogenases (MDH) (***Figure 12***).

In consideration of the importance of the MAS in heart metabolism, we investigated its role in HFpEF. Surprisingly, MAS activity was increased in HFpEF left ventricle mitochondria (***Figure 13***).

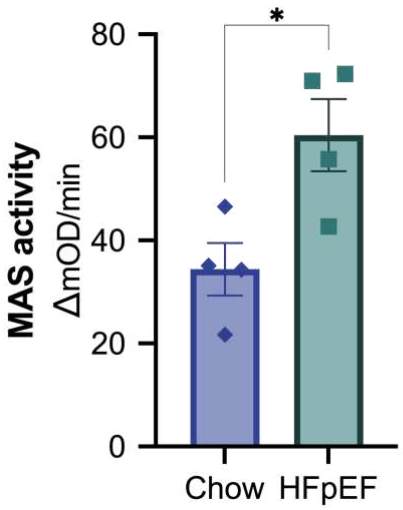

Figure 13 MAS activity in HFpEF vs control mice

We measured the activity of Kbhb MAS enzymes and, while MDH2 activity was unchanged, a significant increase in GOT2 activity was observed (***Figure 14 A and B***).

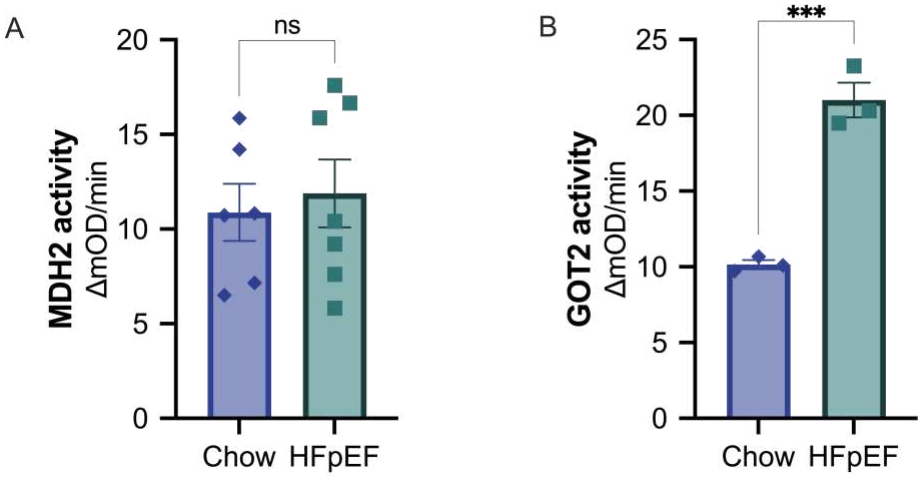

Figure 14 MDH2 and GOT2 activity in HFpEF vs control mice

This change did not depend on increased concentration of GOT2, as levels of this enzyme were comparable between HFpEF and Chow left ventricles (***Figure 15***).

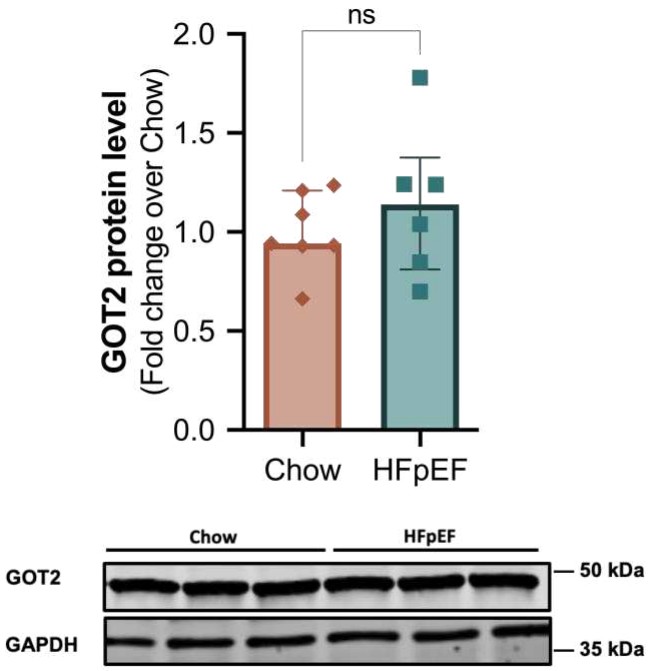

Figure 15 GOT2 levels in HFpEF vs control mice

Moreover, a global proteome evaluation didn't show higher levels of any of the other components of the MAS (***Figure 16***).

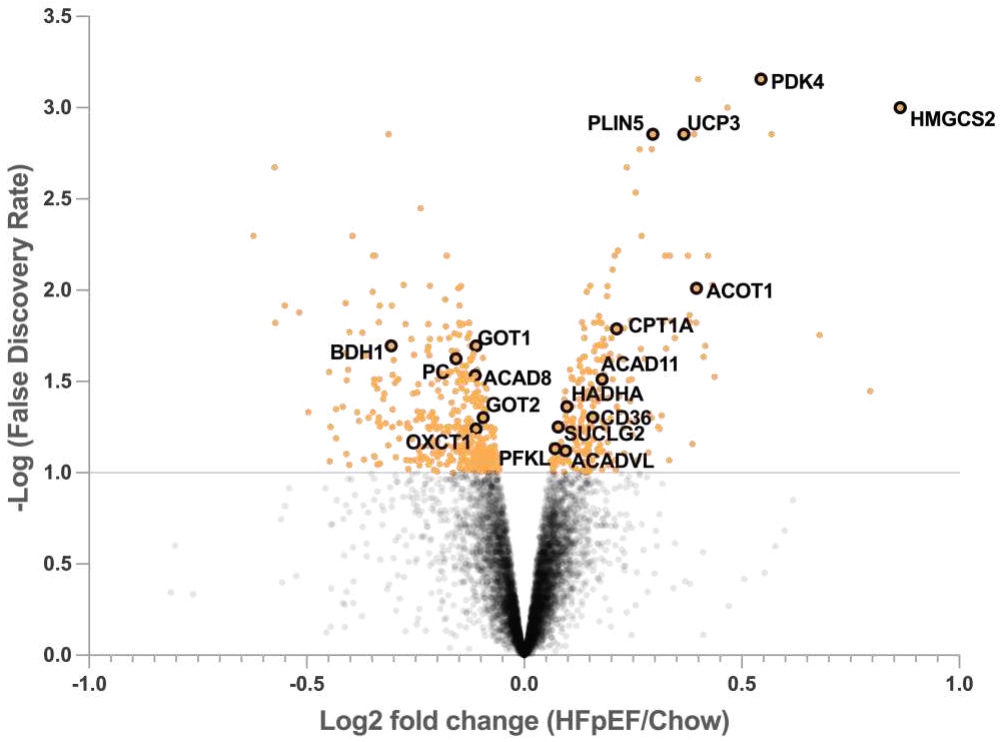

Figure 4 Volcano plot of global proteome (HFpEF vs control mice)

Modelling β-hydroxybutyrylation in cardiomyocytes

We then hypothesized that MAS and GOT2 increased activity in HFpEF depends on GOT2 Kbhb. To test this hypothesis, we aimed at modelling Kbhb in cell culture. Treatment with BOH at different concentrations induced a consensual increase in Kbhb in immortalized human cardiomyocytes (AC16 cells, ***Figure 17***) and primary cell culture of Neonatal Rat Ventricular Myocytes (NRVMs) (***Figure 18***).

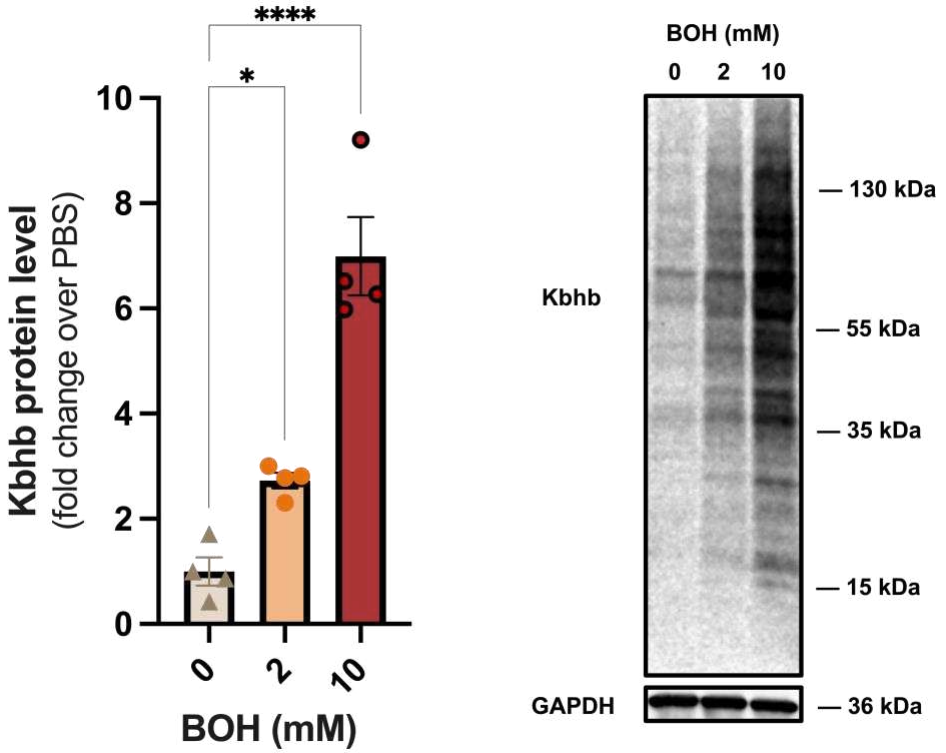

Figure 17 Kbhb levels in AC16 cells after BOH treatment

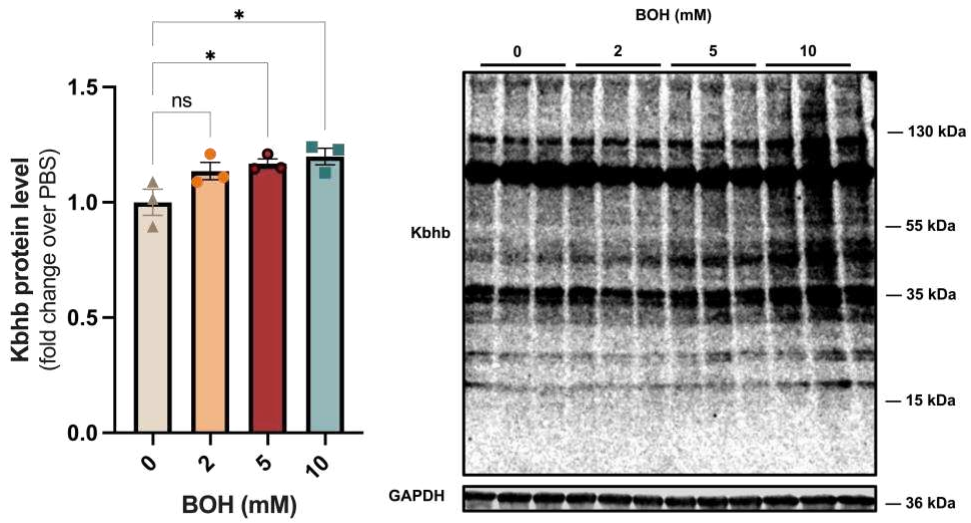

Figure 18 Kbhb levels in NRVMs after BOH treatment

Whether Kbhb is an enzymatic or non-enzymatic post-translational modification is unclear. Sirtuins, which are established de-acetylases, have been suggested as potential Kbhb removing enzymes. Indeed, a role for SIRT3 has been proposed as de-Kbhb enzyme for histones[147]. If SIRT3 acts as a de-Kbhb enzyme for proteins other than histones or whether SIRT3 acts as de-Kbhb enzyme also in cardiomyocytes is unknown.

To answer to these questions, we first tested the effect of BOH in silico, on isolated protein lysates. A 6 hours incubation of AC16 protein lysates with BOH produced a significant increase in Kbhb (***Figure 19***). Intriguingly, if the proteins were exposed to a 5-minute heat denaturation cycle, treatment with increasing concentration of BOH didn't result in a consensual increase in Kbhb (***Figure 19***). This experiment remarks the involvement of enzymatic reactions in Kbhb.

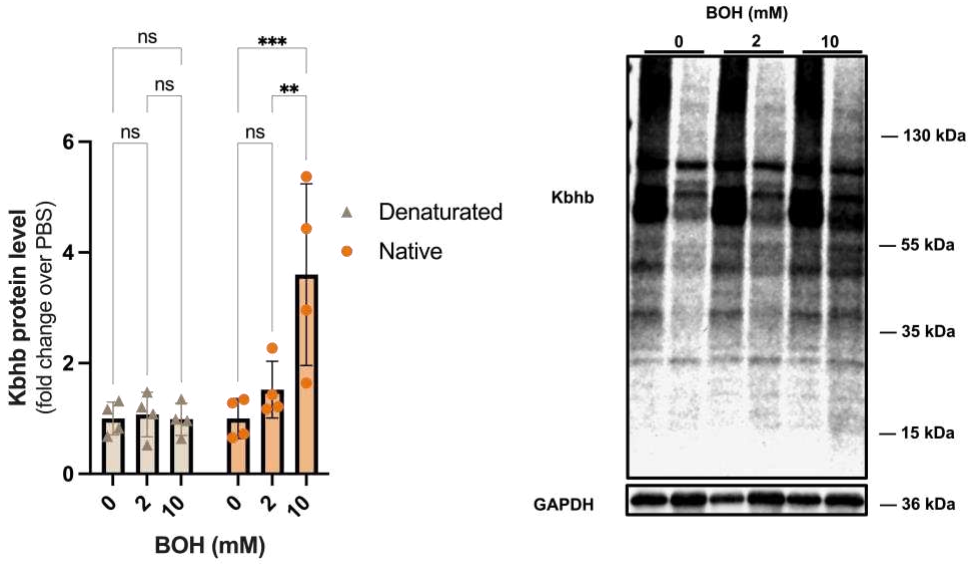

Figure 19 Kbhb levels in isolated protein incubated with BOH

Installing and removing Kbhb enzymes

We then started to test for potential enzymes involved in this process. Since Kbhb was mainly a mitochondrial post-translational modification in HFpEF hearts, to explore the role of Kbhb installing enzymes we focused on mitochondrial acetylases. A qPCR-confirmed silencing of general control of amino acid synthesis 5 like 1 (GCN5L1) enzyme reduced the overall Kbhb signal in AC16 (***Figure 20***).

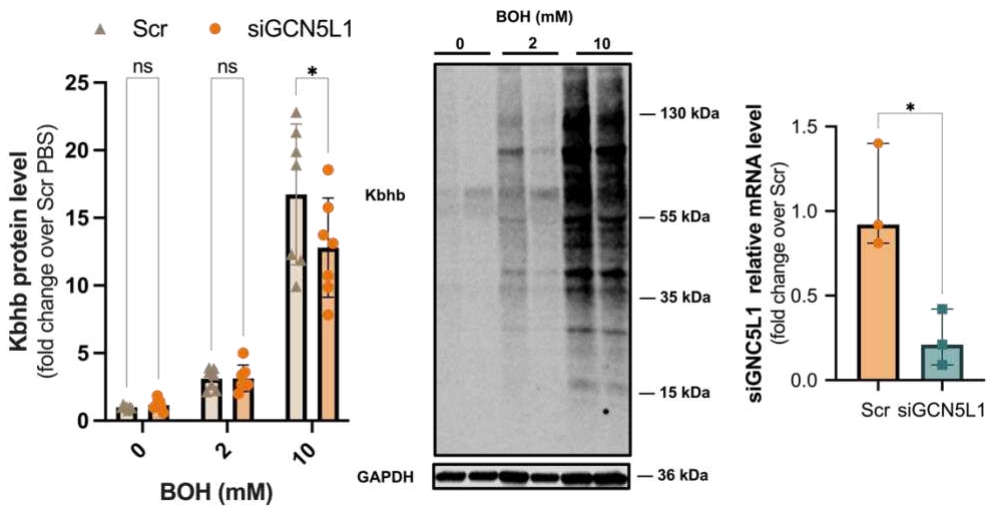

Figure 20 Kbhb levels in AC16 cells after BOH treatment, with and without GCN5L1 silencing

We moved then to potential de-Kbhb enzymes. SIRT3 confirmed its role as de-Kbhb, with a reduction of Kbhb signal after SIRT3 transfection (***Figure 21***) and higher Kbhb after SIRT3 silencing (***Figure 22***).

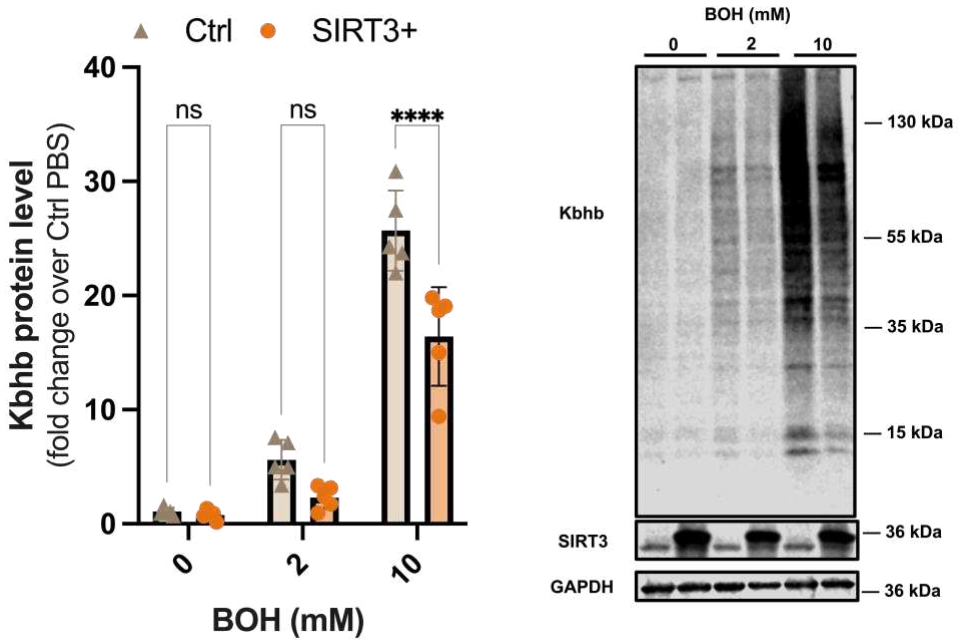

Figure 21 Kbhb levels in AC16 cells after BOH treatment and SIRT3 transfection

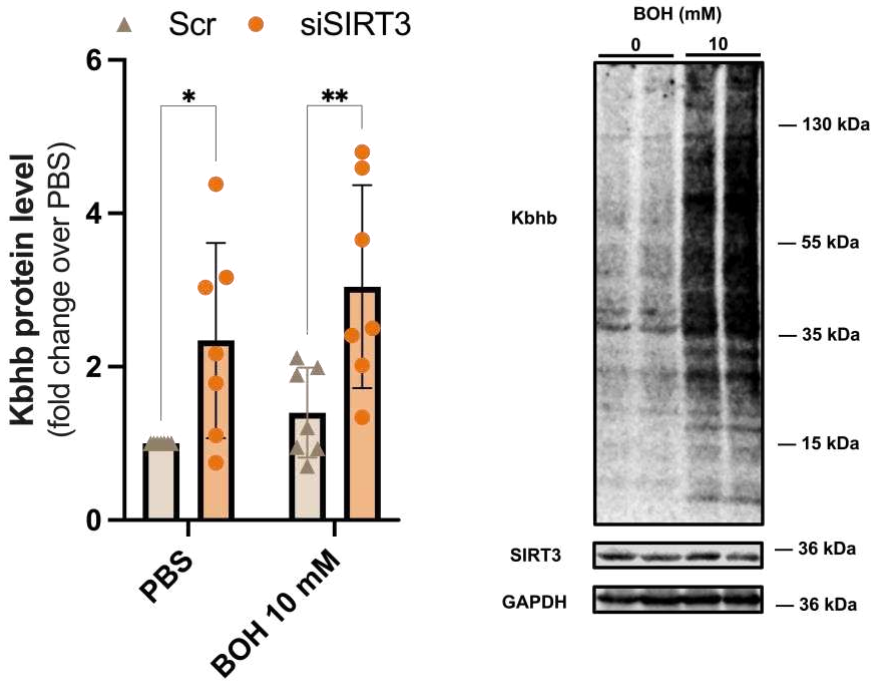

Figure 22 Kbhb levels in AC16 cells after BOH treatment and SIRT3 silencing

Considering the role of SIRT5 in several mitochondrial post-translational modifications, especially those involved in metabolic pathways[210], we tested for a potential role of this enzyme also in Kbhb modulation.

Interestingly, overexpression of SIRT5 resulted in a reduction in Kbhb levels in cardiomyocytes (***Figure 23***)

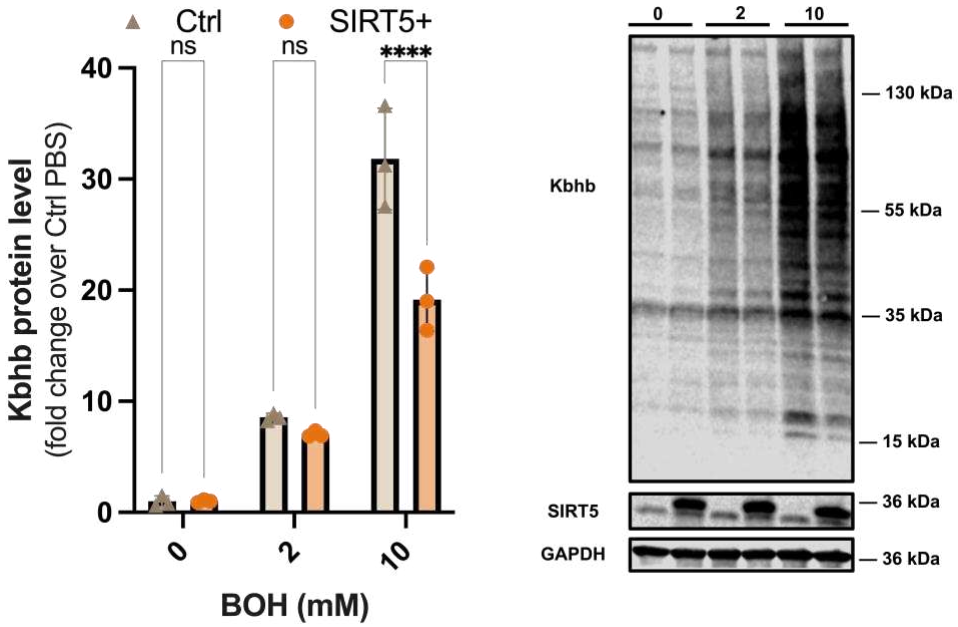

Figure 23 Kbhb levels in AC16 cells after BOH treatment and SIRT5 transfection

BOH induced Kbhb of GOT2 increases enzyme activity

To further clarify the role of Kbhb on GOT2 activity, we evaluated all the Kbhb residues on the protein. Among the Kbhb sites, the K396 was the only with a significant increase between HFpEF and Chow. We therefore generated a flag-tagged GOT2 plasmid were the K396 was mutated to arginine (R). HEK293T cells were transfected with the GOT2 K396R mutant as well as with a wild-type (WT) isoform of the enzyme and subsequently treated with BOH. Immunoprecipitation of protein lysates for flag Ab and immunoblotting for Kbhb confirmed the increased Kbhb of WT GOT2 in BOH treated cells. Kbhb in the K396R mutant was also increased in BOH treated cells, but the difference with PBS-treated cells was not significant (***Figure 24***).

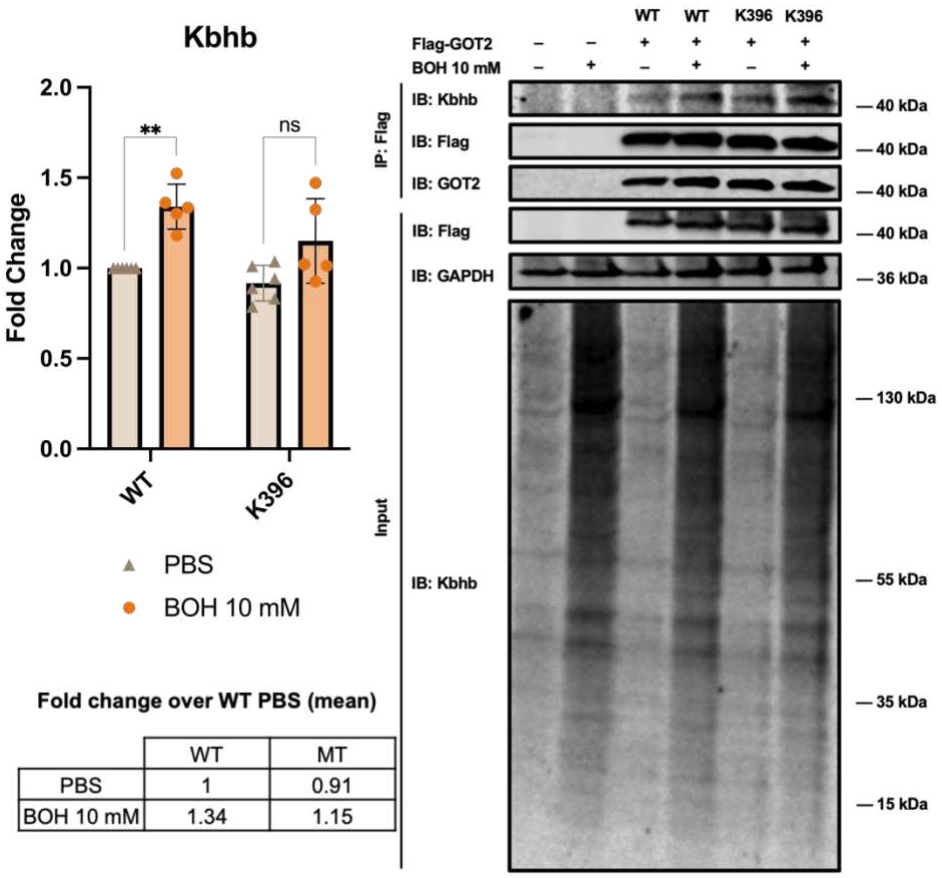

Figure 24 Kbhb levels in WT vs K396T isoform of GOT2 enzyme after BOH treatment

As expected, in WT and K396R transfected HEK293T cells, overall GOT2 activity was increased compared with not-transfected cells, while treatment with the amino

acid transaminase inhibitor aminooxyacetate (AOA) lowered enzyme activity (***Figure 25***).

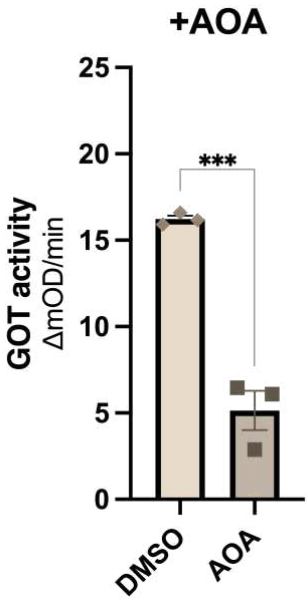

Figure 25 GOT activity after AOA treatment

Treatment with BOH in normal cell culture resulted in a nonsignificant increase in GOT2 activity in control HEK293T cells as well as in WT transfected cells. We registered no changes in GOT2 activity between PBS and BOH treated cells transfected with GOT2 K396R (***Figure 26***).

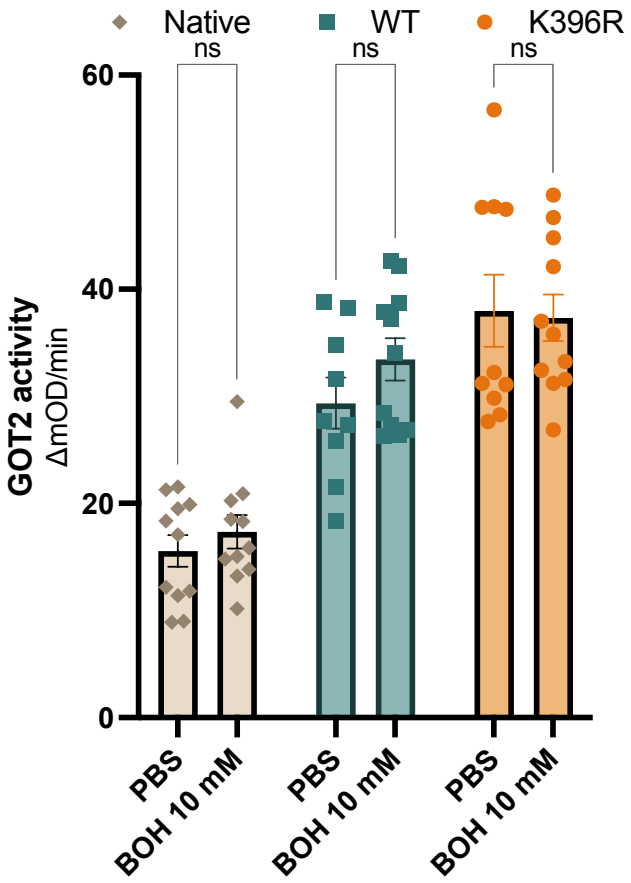

Figure 26 GOT2 activity in control cells GOT2 WT and K396T transfected cells, with and without BOH

To test if Kbhb effect on GOT2 could be related to other coexisting conditions characterizing HFpEF hearts, we explored a potential role of hypoxia in GOT2 activity modulation.

Microvascular dysfunction and subsequent cardiomyocytes hypoxia have a role in HFpEF pathogenesis[211]. Consistently, hypoxia-inducible factors - HIFs (and in particular HIF 1-alpha – HIF1α) have been proposed as key factors in HFpEF, especially when the syndrome is related to obesity and metabolic syndrome[212]. Under hypoxic conditions, HIF1α promotes the switch toward an increase in glycolysis in cardiac metabolism[213, 214].

At the same time, HIF1α inhibits GOT1 and GOT2 activity[215] and arguably MAS activity, potentially affecting the shuttling of glycolysis derived NADH.

As expected, under hypoxic conditions, we observed a reduction of GOT2 activity in not transfected cells, as well as in WT and K396R transfected ones (***Figure 27***).

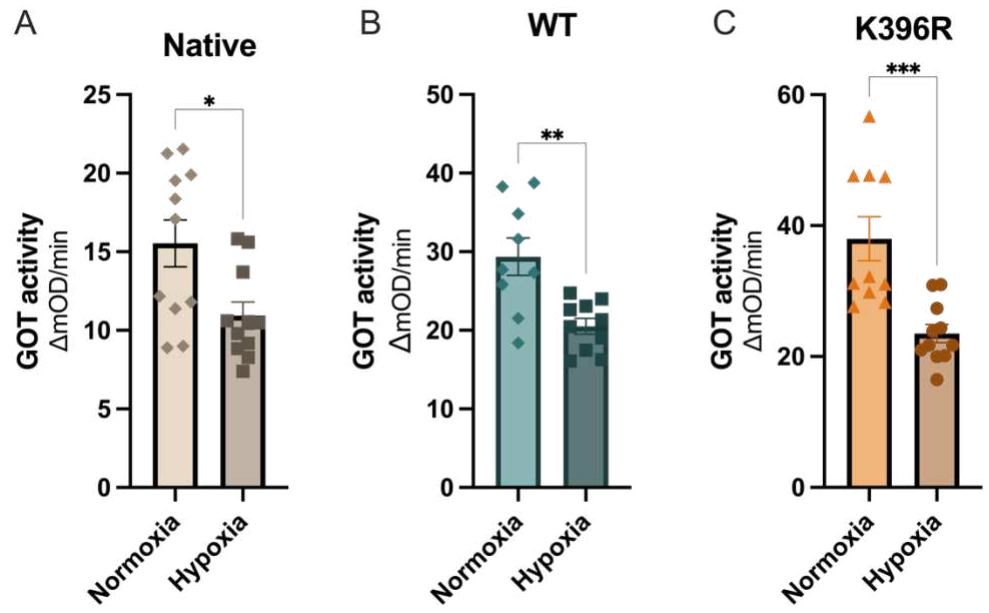

Figure 27 GOT levels in control cells, WT GOT2 and K396R GOT2 transfected cells with and without hypoxia

Interestingly, BOH treatment under hypoxia drove an increase in GOT2 activity only in WT transfected cells, while GOT2 activity in K396R cells was unaffected (***Figure 28***).

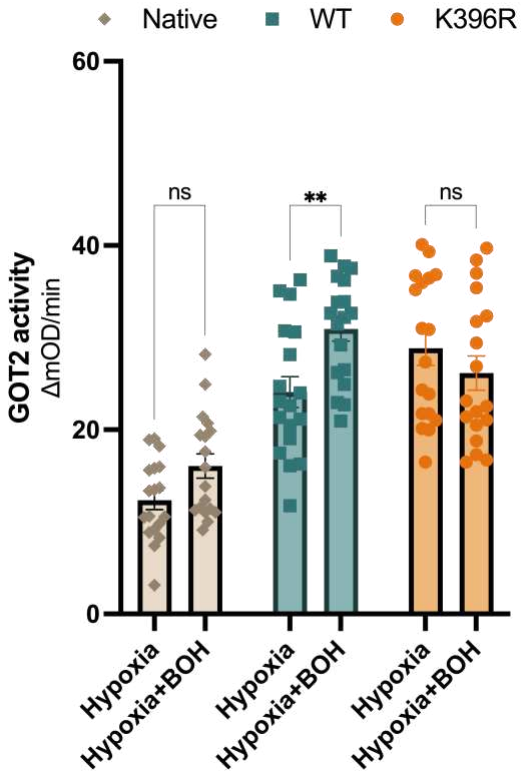

Figure 28 GOT2 activity in control cells GOT2 WT and K396T transfected cells, under hypoxia, with and without BOH

DISCUSSION

HFpEF is a systemic disorder affecting an ever-growing proportion of the general population. Only a few treatments have been approved in this syndrome and there is an urgent need for more therapeutic strategies. In most of HFpEF patients, the clinical syndrome is driven by the co-existence of obesity, hypertension and systemic derangements related to the development of metabolic syndrome. A better understanding of this specific HFpEF phenotype, known as cardiometabolic HFpEF, is a needed stepstone to reach the goal of a significant improvement in patient quality of life and life expectancy. Using a validated mouse model of cardiometabolic HFpEF, we explored cardiac metabolism changes and the dual of ketone bodies, as both energy substrates and signaling molecules, in this syndrome.

Our main findings are: 1. In cardiometabolic HFpEF, BOH oxidative pathway is compromised and BOH oxidation is blunted 2. BOH drives a post translational modification called Kbhb, affecting multiple metabolic pathways 3. Kbhb is an enzymatic post translational modification modulated by several enzymes 4. In HFpEF, MAS activity and GOT2 activity are increased 5. Under hypoxic condition, Kbhb enhance GOT2 enzyme activity.

Heart failure is a complex syndrome, with an heterogeneous pathogenesis and pathophysiology. In HFrEF, the reduction in cardiac output is manifest and drives both an insufficient perfusion of peripheral organs and a symptomatic congestion of systemic and pulmonary circulation. The whole-body response to these derangements is well known and include neuro-hormonal changes, cardiac and vascular remodeling and multi-organ dysfunction[216]. Grounded on this vast pathophysiological knowledge, counter-acting therapeutic strategies have been developed over the years[217], each aiming at different targets: Renin-Angiotensin-Aldosterone-System (RAAS) blockers, including angiotensin-converting enzyme (ACE) inhibitors, angiotensin receptor blockers (ARB) and Mineralocorticoid receptor antagonists (MRAs) significantly prevent the detrimental consequences of pressure and volume overload. Beta-blockers modulate the maladaptive sympathetic response. Angiotensin receptor neprilysin inhibitor (ARNI) enhances the beneficial effect of natriuretic peptides in modulating diuresis, improving

vasodilatation and reducing filling pressures. More recently, SGLT2i have proven to be beneficial in HFrEF but the mechanisms behind this serendipitous discovery are multiple and are still under investigation[218].

On the other hand, the mechanisms underlying HFpEF are more elusive. The ability of the left ventricle to pump an adequate amount of blood seems to be unaffected in baseline imaging evaluations. Nevertheless, patients develop a full clinical syndrome, resembling the one observed in those with an obvious reduction of the ejection fraction. Furthermore, a significant reduction in exercise capacity, quality of life and life expectancy is observed in HFpEF as well as in HFrEF[219, 220]. Translating the HFrEF designed therapeutic strategies to HFpEF patients, didn't significantly improved patients' outcomes[21-27]. SGLT2i have proven to be effective also in HFpEF[28, 29], proposing a role for whole-body metabolic drugs in heart failure treatment. Another class of primarily anti-diabetic drugs, GLP1 agonists, drove a significant improvement in symptoms and functional limitation of obese HFpEF patients[30].

Collectively, these evidences suggest that exploiting HFpEF pathophysiology is imperative for the development of disease-modifying drugs. HFrEF-designed drugs are ineffective in HFpEF, while metabolic drugs are beneficial, especially in patients with overweight/obesity.

To expand our knowledge of HFpEF molecular pathophysiology, reliable animal models are fundamental. A recently developed animal model of HFpEF accurately recapitulate the clinical and molecular features of the cardiometabolic HFpEF[193]. In this "2-hits" model, a combination of high fat diet and drug-induced mild hypertension results in both cardiac derangements (diastolic dysfunction with preserved left ventricular ejection fraction, impaired left ventricular strain, elevated left ventricular filling pressures, microvascular dysfunction) and symptoms surrogates (exercise intolerance, lung congestion)[193]. We used this model to explore one of the many unknown features of HFpEF, namely what are the changes in cardiac metabolism in this syndrome.

Our first finding is that glucose oxidation is significantly decreased in cardiometabolic HFpEF. This is consistent with our previous studies on HFpEF mice, but involving animals treated for a shorter period of time (5 weeks vs 15 weeks)[194]. On the other hand, the observed reduction in fatty acid oxidation[194] is not visible in our evaluation of mitochondrial oxygen consumption rates. These finding shades a new light on the critical balance between glucose oxidation and

fatty acid oxidation in HFpEF. Instead of further affect fatty acid oxidation, a longer exposure to high fat diet, even in the context of heart failure, resulted in a relative increase in the contribution of fatty acids oxidation to mitochondrial metabolism. A reduction in fatty acid oxidation is observed in HFrEF[72, 73]. On the other hand, an increase in fatty acid oxidation is expected in cardiac metabolism in obesity and diabetes[62] and a protective effect has been proposed for increased fatty acid oxidation in high fat diet induced cardiomyopathy[221]. Collectively, fatty acid oxidation in cardiometabolic HFpEF seems to be differentially modulated compared to HFrEF, with an absence of fatty acid oxidation impairment after long time exposure to high fat diet.

We then moved to explore the role of ketone bodies, a class of energy substrates of growing interest in heart failure research. In HFrEF a significant increase in ketone bodies oxidation has been described both in preclinical models[124, 126, 127] and in a clinical setting[125]. This is considered an adaptive response, since in mouse models of both hypertension and myocardial ischemia, cardiomyocyte-specific deletion of BDH1 resulted in worsening of cardiac function[126] while BDH1 overexpression protected against the development of myocardial fibrosis and cardiac dysfunction[127]. In addition, in hypertensive and ischemic cardiomyopathy, BOH infusion/supplementation has proven to be protective in both mouse models[124 222] and HFrEF patients[223].

Our findings depict a completely different scenario in HFpEF. Measuring BOH OCR we found a significant reduction in ketone body oxidation. Moreover, BDH1 protein levels were significantly lower and BDH1 activity was blunted in HFpEF. Therefore, ketone bodies don't appear to play a role as rescue fuel in the context of cardiometabolic HFpEF. A reduction of BDH1 was already described in HFpEF[67], but here for the first time BOH OCR was tested and found defective. Moreover, the whole ketone bodies oxidation pathway has been described, collectively depicting a blunted oxidative capacity for ketone bodies in HFpEF.

Our next step was exploring a different biological significance for ketone bodies in HFpEF hearts. Ketone bodies, and BOH in particular, promote a post translational modification defined Kbhb[144]. Kbhb has been first described in histones and considered as a component of the cell response to starvation[144]. Recent findings highlight how Kbhb is not limited to histones[145, 146]. Importantly, the knowledge about the role of Kbhb in cardiac homeostasis and disease is still limited. A work examining the role of BOH in an aging model of HFpEF, described an increase in

the activity of the TCA cycle enzyme citrate synthase (CS)[67]. The authors reported that the increase in BOH levels was accompanied by an increase in CS Kbhb and, at the same time, an increase in its enzymatic activity, suggesting that the BOH-induced improvement in mitochondrial function was dependent on CS Kbhb[67]. However, how Kbhb impacts on mitochondrial function and overall cardiac metabolism in HFpEF is still largely unknown.

Using a Kbhb-specific antibody, for the first we investigated the presence of this post translational modification in the proteome of HFpEF left ventricles. In HFpEF, Kbhb is significantly over represented with a number of proteins affected. The subcellular distribution of Kbhb proteins revealed that most of Kbhb targets are located in the mitochondria, suggesting a role for Kbhb in cell metabolism. Moreover, the gene ontology revealed a potential role for Kbhb in the regulation of key mitochondrial metabolic pathways, such as TCA cycle and fatty acid oxidation. This finding proposes Kbhb as a new player in the landscape of post translational regulation of metabolic function in the heart. Other post translational modifications have already been described as having this role. Acetylation is implicated in the regulation of cardiac metabolism in both HFrEF and HFpEF[193, 224, 225]. The role of acetylation depends on the molecular function of acetyl-CoA, the substrate for acetylation itself. Acetyl-CoA is the end-product of intermediate metabolism and is therefore a sentinel of cellular metabolic state[226]. Therefore, changes in energy substrate availability and nutritional status have significant effects on the level of acetylation of enzymes involved in substrate utilization[148, 227]. Similarly, ketone bodies levels represent a signal of an unusual energy state. Their concentration usually increase during starvation as their biological significance is to provide an alternative source of fuel during low blood glucose conditions[104]. However, since elevated levels of free fatty acids constitute a stimulus to ketogenesis, a significant increase in the circulating levels of fatty acids is sufficient to drive ketone bodies synthesis. Arguably, the increase in fatty acids pool observed after a prolonged high fat diet is implicated in the increase in cardiac Kbhb observed in cardiometabolic HFpEF.

Among the top Kbhb targets, the highest fold change increase was registered for MDH2, a TCA cycle enzyme. MDH2 is also a key component of the MAS, a shuttle of paramount importance in cardiomyocyte metabolism. Intriguingly, among the 44 peptides with a significant change of Kbhb in HFpEF hearts, there was another component of the MAS, namely GOT2. The co-existence of 2 targets belonging to

the MAS in the Kbhb targets moved us to investigate the role of this metabolic pathway in cardiometabolic HFpEF and a potential involvement of Kbhb in MAS modulation.

The MAS is the main shuttle allowing the transfer of the glycolysis derived NADH into the mitochondrial matrix in cardiomyocytes. Despite the relevance of this pathway in cardiac metabolism, data about MAS activity in heart failure are scant. In a model of pressure overload induced HFrEF, MAS activity was blunted and reduced activity of the shuttle was attributed to hyperacetylation of mitochondrial MAS proteins[224]. In our work, for the first time MAS activity has been evaluated in a mouse model of HFpEF. Surprisingly, our findings suggest an higher activity of the shuttle in cardiometabolic HFpEF. An higher MAS activity may be an adaptive response to NAD^+ deficiency and to the reduction in glucose oxidative phosphorylation. Cardiometabolic HFpEF is a NAD^+ deficient condition[194] and one of the MAS purposes is to reconstitute the cytosolic NAD^+ pool. Moreover, our data suggest a reduction in glucose oxidation, suggesting glycolysis as a potential rescue metabolic pathway for NADH generation. Whether MAS activity increase is related to an higher glycolytic rate in HFpEF is still speculative and need further investigation.

To better understand what determined MAS activity increase, we measured MDH2 and GOT2 activities. While MDH2 activity was unchanged, we detected a 2-fold increase in GOT2 activity in HFpEF hearts. GOT2 protein levels, however, were not significantly altered between control and HFpEF mice. We therefore hypothesized a role for GOT2 Kbhb in GOT2 activity modulation. To test this hypothesis, we modeled Kbhb in cell culture.

Treating several cell lines, i.e. human immortalized cardiomyocytes (AC16 cells) and primary cell line of neonatal rat ventricular cardiomyocytes (NRVMs) with increasing concentration of BOH for 24 hours resulted in a concomitant increase in whole-protein Kbhb (***Figure 17 and 18***). Protein Kbhb was also visible when protein lysates, instead of living cells, were incubated in silico for 6 hours with increasing concentrations of BOH. However, if thermal denaturation was applied before the incubation, the increase in Kbhb was no more visible (***Figure 19***). The denaturation/in silico protocol we adopted was originally designed to evaluate the enzymatic vs non-enzymatic nature of acetylation[205]. In silico incubation of denatured protein lysates with increasing concentration of acetyl-CoA resulted in a significant increase of acetylation, suggesting a non-enzymatic component in this

post translational modification[205]. In our work, incubation of protein lysates with BOH provided opposite results, proposing a pivotal role for one or more enzymes in Kbhb.

To elucidate which enzymes could be involved in Kbhb, we started from the evaluation of proteins involved in acetylation and de-acetylation. De-acetylating enzyme had been already proposed as de-Kbhb for histones[147] but little is known about installing enzymes and, in particular, the effect of these enzymes on proteins other than histones.

Among acetylating enzymes, and mitochondrial ones in particular, of interest is GCN5L1. This enzyme is implicated in acetylation of proteins involved in metabolic pathways[228]. Moreover, a role for GCN5L1 in the inhibition of cardiac glucose oxidation in aged mice with diet-induced obesity has been proposed[229]. In AC16 cells, GCN5L1 silencing reduced Kbhb in cells treated with BOH, compared with controls. This finding supports a role for GCN5L1 in Kbhb, and not only in acetylation, stimulating a wider range of effects to be considered when evaluating the consequences of GCN5L1 modulation, at least in cardiac metabolism.

We then moved to the evaluation of potential Kbhb removing enzymes. We evaluated SIRT3 and SIRT5 and in both cases, overexpression of the enzymes reduced Kbhb while silencing resulted in a higher signal. Sirtuins, and SIRT3 in particular, have been previously described as critical components of the complex enzymatic machinery involved in acetylation regulation in several cardiovascular diseases[230-232], including HFpEF[67, 194]. However, SIRT3 has been evaluated only as de-acetylating enzyme. In our work, we uncovered a new role for SIRT3, which may be implicated in regulation of cardiac metabolism via Kbhb modulation.

Once achieved a solid platform for Kbhb modulation in cell lines, we moved back to the evaluation of the effect of Kbhb on GOT2. At first, we evaluated if BOH treatment was specifically affecting GOT2 also in vitro. Data from mass spectrometry in mice pointed out that the site with higher Kbhb increase between HFpEF mice and chow was lysine 396 (K396). We therefore generated a vector containing a custom flag-tagged GOT2 protein where K396 was muted into arginine (K396R). We then transfected HEK293T cells with both the flag-tagged wild type isoform of the enzyme (WT) and the mutant isoform (K396R). After transfection and BOH treatment, we immunoprecipitated the protein lysates for flag and we blotted for Kbhb. As expected, Kbhb signal significantly increased in WT transfected and BOH treated cells (***Figure 24***). Kbhb in the K396R mutant was also

increased in BOH treated cells, but the difference with PBS-treated cells was not significant (***Figure 24***).

Moving to the evaluation of the enzyme activity, we measured GOT2 activity in normal cell lines, after treatment with BOH for 24 hours. In basal conditions, BOH treatment didn't result in a significant change in enzyme activity. To test the reliability of the assay, we treated AC16 with AOA, an established amino acid transaminase inhibitor[233] and we observed a significant reduction in GOT2 activity. A reduction was observed also after a 24 hours incubation in 1% hypoxia. This is not surprising, since it was already described a role for HIF1α in GOT1 and GOT2 activity inhibition[215]. Testing the effect of BOH on GOT2 activity after hypoxia, we found that in this condition BOH increased the enzyme activity. To test if this effect was dependent on GOT2 Kbhb, we compared the enzyme activity under hypoxia and BOH treatment in cells transfected with the WT GOT2 and the K396R mutant. The experiment, confirmed an increase of GOT2 activity only in WT transfected cells while in K396R a trend toward reduced activity was observed.

This finding shades a new light on the complex interplay between MAS and glycolysis in hypoxic conditions. During hypoxia, glycolytic rate increases to optimize glucose utilization. This shift from glucose oxidation to glycolysis is mediated by HIFs[213, 214]. However, the inhibitory effect of HIF1α on GOT2 activity[215] potentially hamper the MAS dependent shuttling of glycolysis derived NADH into mitochondria (***Figure 29***).

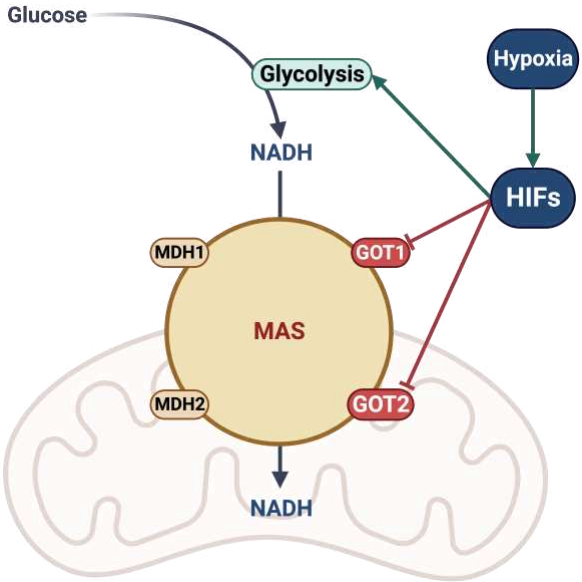

Figure 29 MAS - hypoxia interplay mediated by HIFs

Kbhb may play a role in this context: enhancing GOT2 activity, contributes revert the inhibitory effect of HIF, allowing more NADH to be used for ATP production.

In conclusion, HFpEF is a deadly syndrome where a limited understanding of underlying pathophysiological mechanisms contributes to the insufficient therapeutic arsenal currently available. Increasing evidence underpin the importance of obesity and metabolic syndrome to HFpEF pathogenesis, defining the so-called cardiometabolic HFpEF phenotype. Recently, a reliable mouse model of cardiometabolic HFpEF was established as a reliable platform to find the missing pieces in HFpEF jigsaw. Of particular interest in HFpEF pathogenesis is the role for cardiac metabolism derangements. Whether the same lost in metabolic flexibility described in HFrEF, also develops in HFpEF is still unclear. Moreover, if energy metabolites act as signaling molecules in the landscape of post translational modification is still under investigation. Unclear, in the end, is the role of ketone bodies: a rescue fuel proposed as a therapeutic option in HFrEF but with a largely unknown biology in HFpEF.

Using the "2-hit" cardiometabolic HFpEF model, we confirmed that glucose oxidation is hampered in cardiometabolic HFpEF. We analyzed the BOH catabolic pathway, revealing a reduction in the oxidation of this substrate in the heart. Moving to the analysis of the signaling role of this metabolite, for the first time we described an extensive increase in Kbhb among several mitochondrial protein in HFpEF hearts. Among these targets, we found MDH2 and GOT2, key proteins involved in the MAS. Surprisingly, an increase in MAS activity was observed in HFpEF. Modeling Kbhb in vitro, we uncovered a role for this post translational modification in restoring GOT2 under hypoxia, arguably contributing to the MAS activity modulation in HFpEF.

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Mass spectrometry data were collected by Pauline Fahjen (Max-Delbrück-Center for Molecular Medicine, Mass spectroscopy and Proteomics, Berlin, Germany.)

REFERENCES

1. Braunwald E, Ross J, Jr., Sonnenblick EH. Mechanisms of contraction of the normal and failing heart. *N Engl J Med* 1967;**277**:1012-1022 contd.
2. Braunwald E. Heart failure. *JACC Heart Fail* 2013;**1**:1-20.
3. McDonagh TA, Metra M, Adamo M, Gardner RS, Baumbach A, Bohm M, Burri H, Butler J, Celutkiene J, Chioncel O, Cleland JGF, Coats AJS, Crespo-Leiro MG, Farmakis D, Gilard M, Heymans S, Hoes AW, Jaarsma T, Jankowska EA, Lainscak M, Lam CSP, Lyon AR, McMurray JJV, Mebazaa A, Mindham R, Muneretto C, Francesco Piepoli M, Price S, Rosano GMC, Ruschitzka F, Kathrine Skibelund A, Group ESCSD. 2021 ESC Guidelines for the diagnosis and treatment of acute and chronic heart failure. *Eur Heart J* 2021;**42**:3599-3726.
4. Bozkurt B, Coats AJS, Tsutsui H, Abdelhamid CM, Adamopoulos S, Albert N, Anker SD, Atherton J, Bohm M, Butler J, Drazner MH, Michael Felker G, Filippatos G, Fiuzat M, Fonarow GC, Gomez-Mesa JE, Heidenreich P, Imamura T, Jankowska EA, Januzzi J, Khazanie P, Kinugawa K, Lam CSP, Matsue Y, Metra M, Ohtani T, Francesco Piepoli M, Ponikowski P, Rosano GMC, Sakata Y, Seferovic P, Starling RC, Teerlink JR, Vardeny O, Yamamoto K, Yancy C, Zhang J, Zieroth S. Universal definition and classification of heart failure: a report of the Heart Failure Society of America, Heart Failure Association of the European Society of Cardiology, Japanese Heart Failure Society and Writing Committee of the Universal Definition of Heart Failure: Endorsed by the Canadian Heart Failure Society, Heart Failure Association of India, Cardiac Society of Australia and New Zealand, and Chinese Heart Failure Association. *Eur J Heart Fail* 2021;**23**:352-380.
5. Meyer S, Brouwers FP, Voors AA, Hillege HL, de Boer RA, Gansevoort RT, van der Harst P, Rienstra M, van Gelder IC, van Veldhuisen DJ, van Gilst WH, van der Meer P. Sex differences in new-onset heart failure. *Clin Res Cardiol* 2015;**104**:342-350.
6. Brouwers FP, de Boer RA, van der Harst P, Voors AA, Gansevoort RT, Bakker SJ, Hillege HL, van Veldhuisen DJ, van Gilst WH. Incidence and epidemiology of new onset heart failure with preserved vs. reduced ejection fraction in a community-based cohort: 11-year follow-up of PREVEND. *Eur Heart J* 2013;**34**:1424-1431.
7. Conrad N, Judge A, Tran J, Mohseni H, Hedgecott D, Crespillo AP, Allison M, Hemingway H, Cleland JG, McMurray JJV, Rahimi K. Temporal trends and patterns in heart failure incidence: a population-based study of 4 million individuals. *Lancet* 2018;**391**:572-580.
8. van Riet EE, Hoes AW, Wagenaar KP, Limburg A, Landman MA, Rutten FH. Epidemiology of heart failure: the prevalence of heart failure and ventricular dysfunction in older adults over time. A systematic review. *Eur J Heart Fail* 2016;**18**:242-252.
9. Bibbins-Domingo K, Pletcher MJ, Lin F, Vittinghoff E, Gardin JM, Arynchyn A, Lewis CE, Williams OD, Hulley SB. Racial differences in incident heart failure among young adults. *N Engl J Med* 2009;**360**:1179-1190.
10. Writing Group M, Mozaffarian D, Benjamin EJ, Go AS, Arnett DK, Blaha MJ, Cushman M, Das SR, de Ferranti S, Despres JP, Fullerton HJ, Howard VJ, Huffman MD, Isasi CR, Jimenez MC, Judd SE, Kissela BM, Lichtman JH, Lisabeth LD, Liu S, Mackey RH, Magid DJ, McGuire DK, Mohler ER, 3rd, Moy CS, Muntner P, Mussolino ME, Nasir K, Neumar RW, Nichol G, Palaniappan L, Pandey DK, Reeves MJ, Rodriguez CJ, Rosamond W, Sorlie PD, Stein J, Towfighi A, Turan TN, Virani SS, Woo D, Yeh RW, Turner MB, American Heart Association Statistics C, Stroke Statistics S. Heart Disease and

Stroke Statistics-2016 Update: A Report From the American Heart Association. *Circulation* 2016;**133**:e38-360.

11. Chen J, Dharmarajan K, Wang Y, Krumholz HM. National trends in heart failure hospital stay rates, 2001 to 2009. *J Am Coll Cardiol* 2013;**61**:1078-1088.
12. Roth GA, Forouzanfar MH, Moran AE, Barber R, Nguyen G, Feigin VL, Naghavi M, Mensah GA, Murray CJ. Demographic and epidemiologic drivers of global cardiovascular mortality. *N Engl J Med* 2015;**372**:1333-1341.
13. Vos T, Flaxman AD, Naghavi M, Lozano R, Michaud C, Ezzati M, Shibuya K, Salomon JA, Abdalla S, Aboyans V, Abraham J, Ackerman I, Aggarwal R, Ahn SY, Ali MK, Alvarado M, Anderson HR, Anderson LM, Andrews KG, Atkinson C, Baddour LM, Bahalim AN, Barker-Collo S, Barrero LH, Bartels DH, Basanez MG, Baxter A, Bell ML, Benjamin EJ, Bennett D, Bernabe E, Bhalla K, Bhandari B, Bikbov B, Bin Abdulhak A, Birbeck G, Black JA, Blencowe H, Blore JD, Blyth F, Bolliger I, Bonaventure A, Boufous S, Bourne R, Boussinesq M, Braithwaite T, Brayne C, Bridgett L, Brooker S, Brooks P, Brugha TS, Bryan-Hancock C, Bucello C, Buchbinder R, Buckle G, Budke CM, Burch M, Burney P, Burstein R, Calabria B, Campbell B, Canter CE, Carabin H, Carapetis J, Carmona L, Cella C, Charlson F, Chen H, Cheng AT, Chou D, Chugh SS, Coffeng LE, Colan SD, Colquhoun S, Colson KE, Condon J, Connor MD, Cooper LT, Corriere M, Cortinovis M, de Vaccaro KC, Couser W, Cowie BC, Criqui MH, Cross M, Dabhadkar KC, Dahiya M, Dahodwala N, Damsere-Derry J, Danaei G, Davis A, De Leo D, Degenhardt L, Dellavalle R, Delossantos A, Denenberg J, Derrett S, Des Jarlais DC, Dharmaratne SD, Dherani M, Diaz-Torne C, Dolk H, Dorsey ER, Driscoll T, Duber H, Ebel B, Edmond K, Elbaz A, Ali SE, Erskine H, Erwin PJ, Espindola P, Ewoigbokhan SE, Farzadfar F, Feigin V, Felson DT, Ferrari A, Ferri CP, Fevre EM, Finucane MM, Flaxman S, Flood L, Foreman K, Forouzanfar MH, Fowkes FG, Franklin R, Fransen M, Freeman MK, Gabbe BJ, Gabriel SE, Gakidou E, Ganatra HA, Garcia B, Gaspari F, Gillum RF, Gmel G, Gosselin R, Grainger R, Groeger J, Guillemin F, Gunnell D, Gupta R, Haagsma J, Hagan H, Halasa YA, Hall W, Haring D, Haro JM, Harrison JE, Havmoeller R, Hay RJ, Higashi H, Hill C, Hoen B, Hoffman H, Hotez PJ, Hoy D, Huang JJ, Ibeanusi SE, Jacobsen KH, James SL, Jarvis D, Jasrasaria R, Jayaraman S, Johns N, Jonas JB, Karthikeyan G, Kassebaum N, Kawakami N, Keren A, Khoo JP, King CH, Knowlton LM, Kobusingye O, Koranteng A, Krishnamurthi R, Lalloo R, Laslett LL, Lathlean T, Leasher JL, Lee YY, Leigh J, Lim SS, Limb E, Lin JK, Lipnick M, Lipshultz SE, Liu W, Loane M, Ohno SL, Lyons R, Ma J, Mabweijano J, MacIntyre MF, Malekzadeh R, Mallinger L, Manivannan S, Marcenes W, March L, Margolis DJ, Marks GB, Marks R, Matsumori A, Matzopoulos R, Mayosi BM, McAnulty JH, McDermott MM, McGill N, McGrath J, Medina-Mora ME, Meltzer M, Mensah GA, Merriman TR, Meyer AC, Miglioli V, Miller M, Miller TR, Mitchell PB, Mocumbi AO, Moffitt TE, Mokdad AA, Monasta L, Montico M, Moradi-Lakeh M, Moran A, Morawska L, Mori R, Murdoch ME, Mwaniki MK, Naidoo K, Nair MN, Naldi L, Narayan KM, Nelson PK, Nelson RG, Nevitt MC, Newton CR, Nolte S, Norman P, Norman R, O'Donnell M, O'Hanlon S, Olives C, Omer SB, Ortblad K, Osborne R, Ozgediz D, Page A, Pahari B, Pandian JD, Rivero AP, Patten SB, Pearce N, Padilla RP, Perez-Ruiz F, Perico N, Pesudovs K, Phillips D, Phillips MR, Pierce K, Pion S, Polanczyk GV, Polinder S, Pope CA, 3rd, Popova S, Porrini E, Pourmalek F, Prince M, Pullan RL, Ramaiah KD, Ranganathan D, Razavi H, Regan M, Rehm JT, Rein DB, Remuzzi G, Richardson K, Rivara FP, Roberts T, Robinson C, De Leon FR, Ronfani L, Room R, Rosenfeld LC, Rushton L, Sacco RL, Saha S, Sampson U, Sanchez-Riera L, Sanman E, Schwebel DC, Scott JG, Segui-Gomez M, Shahraz S, Shepard DS, Shin H, Shivakoti R, Singh D, Singh GM,

Singh JA, Singleton J, Sleet DA, Sliwa K, Smith E, Smith JL, Stapelberg NJ, Steer A, Steiner T, Stolk WA, Stovner LJ, Sudfeld C, Syed S, Tamburlini G, Tavakkoli M, Taylor HR, Taylor JA, Taylor WJ, Thomas B, Thomson WM, Thurston GD, Tleyjeh IM, Tonelli M, Towbin JA, Truelsen T, Tsilimbaris MK, Ubeda C, Undurraga EA, van der Werf MJ, van Os J, Vavilala MS, Venketasubramanian N, Wang M, Wang W, Watt K, Weatherall DJ, Weinstock MA, Weintraub R, Weisskopf MG, Weissman MM, White RA, Whiteford H, Wiersma ST, Wilkinson JD, Williams HC, Williams SR, Witt E, Wolfe F, Woolf AD, Wulf S, Yeh PH, Zaidi AK, Zheng ZJ, Zonies D, Lopez AD, Murray CJ, AlMazroa MA, Memish ZA. Years lived with disability (YLDs) for 1160 sequelae of 289 diseases and injuries 1990-2010: a systematic analysis for the Global Burden of Disease Study 2010. *Lancet* 2012;**380**:2163-2196.

14. Heidenreich PA, Bozkurt B, Aguilar D, Allen LA, Byun JJ, Colvin MM, Deswal A, Drazner MH, Dunlay SM, Evers LR, Fang JC, Fedson SE, Fonarow GC, Hayek SS, Hernandez AF, Khazanie P, Kittleson MM, Lee CS, Link MS, Milano CA, Nnacheta LC, Sandhu AT, Stevenson LW, Vardeny O, Vest AR, Yancy CW. 2022 AHA/ACC/HFSA Guideline for the Management of Heart Failure: A Report of the American College of Cardiology/American Heart Association Joint Committee on Clinical Practice Guidelines. *Circulation* 2022;**145**:e895-e1032.
15. AHA medical/scientific statement. 1994 revisions to classification of functional capacity and objective assessment of patients with diseases of the heart. *Circulation* 1994;**90**:644-645.
16. Beer M, Seyfarth T, Sandstede J, Landschutz W, Lipke C, Kostler H, von Kienlin M, Harre K, Hahn D, Neubauer S. Absolute concentrations of high-energy phosphate metabolites in normal, hypertrophied, and failing human myocardium measured noninvasively with (31)P-SLOOP magnetic resonance spectroscopy. *J Am Coll Cardiol* 2002;**40**:1267-1274.
17. Conway MA, Allis J, Ouwerkerk R, Niioka T, Rajagopalan B, Radda GK. Detection of low phosphocreatine to ATP ratio in failing hypertrophied human myocardium by 31P magnetic resonance spectroscopy. *Lancet* 1991;**338**:973-976.
18. Kato T, Niizuma S, Inuzuka Y, Kawashima T, Okuda J, Tamaki Y, Iwanaga Y, Narazaki M, Matsuda T, Soga T, Kita T, Kimura T, Shioi T. Analysis of metabolic remodeling in compensated left ventricular hypertrophy and heart failure. *Circ Heart Fail* 2010;**3**:420-430.
19. Pieske B, Tschope C, de Boer RA, Fraser AG, Anker SD, Donal E, Edelmann F, Fu M, Guazzi M, Lam CSP, Lancellotti P, Melenovsky V, Morris DA, Nagel E, Pieske-Kraigher E, Ponikowski P, Solomon SD, Vasan RS, Rutten FH, Voors AA, Ruschitzka F, Paulus WJ, Seferovic P, Filippatos G. How to diagnose heart failure with preserved ejection fraction: the HFA-PEFF diagnostic algorithm: a consensus recommendation from the Heart Failure Association (HFA) of the European Society of Cardiology (ESC). *Eur J Heart Fail* 2020;**22**:391-412.
20. Lupon J, Gavidia-Bovadilla G, Ferrer E, de Antonio M, Perera-Lluna A, Lopez-Ayerbe J, Domingo M, Nunez J, Zamora E, Moliner P, Santiago-Vacas E, Santesmases J, Bayes-Genis A. Heart Failure With Preserved Ejection Fraction Infrequently Evolves Toward a Reduced Phenotype in Long-Term Survivors. *Circ Heart Fail* 2019;**12**:e005652.
21. Yusuf S, Pfeffer MA, Swedberg K, Granger CB, Held P, McMurray JJ, Michelson EL, Olofsson B, Ostergren J, Investigators C, Committees. Effects of candesartan in patients with chronic heart failure and preserved left-ventricular ejection fraction: the CHARM-Preserved Trial. *Lancet* 2003;**362**:777-781.

22. Cleland JG, Tendera M, Adamus J, Freemantle N, Polonski L, Taylor J, Investigators P-C. The perindopril in elderly people with chronic heart failure (PEP-CHF) study. *Eur Heart J* 2006;**27**:2338-2345.
23. Pitt B, Pfeffer MA, Assmann SF, Boineau R, Anand IS, Claggett B, Clausell N, Desai AS, Diaz R, Fleg JL, Gordeev I, Harty B, Heitner JF, Kenwood CT, Lewis EF, O'Meara E, Probstfield JL, Shaburishvili T, Shah SJ, Solomon SD, Sweitzer NK, Yang S, McKinlay SM, Investigators T. Spironolactone for heart failure with preserved ejection fraction. *N Engl J Med* 2014;**370**:1383-1392.
24. Solomon SD, McMurray JJV, Anand IS, Ge J, Lam CSP, Maggioni AP, Martinez F, Packer M, Pfeffer MA, Pieske B, Redfield MM, Rouleau JL, van Veldhuisen DJ, Zannad F, Zile MR, Desai AS, Claggett B, Jhund PS, Boytsov SA, Comin-Colet J, Cleland J, Dungen HD, Goncalvesova E, Katova T, Kerr Saraiva JF, Lelonek M, Merkely B, Senni M, Shah SJ, Zhou J, Rizkala AR, Gong J, Shi VC, Lefkowitz MP, Investigators P-H, Committees. Angiotensin-Neprilysin Inhibition in Heart Failure with Preserved Ejection Fraction. *N Engl J Med* 2019;**381**:1609-1620.
25. Kuno T, Ueyama H, Fujisaki T, Briasouli A, Takagi H, Briasoulis A. Meta-Analysis Evaluating the Effects of Renin-Angiotensin-Aldosterone System Blockade on Outcomes of Heart Failure With Preserved Ejection Fraction. *Am J Cardiol* 2020;**125**:1187-1193.
26. Salah HM, Fudim M, Al'Aref SJ, Khan MS, Almarzooq ZI, Devabhaktuni SR, Mentz RJ, Butler J, Greene SJ. Meta-Analysis of Efficacy of Sacubitril/Valsartan in Heart Failure With Preserved Ejection Fraction. *Am J Cardiol* 2021;**145**:165-168.
27. Massie BM, Carson PE, McMurray JJ, Komajda M, McKelvie R, Zile MR, Anderson S, Donovan M, Iverson E, Staiger C, Ptaszynska A, Investigators IP. Irbesartan in patients with heart failure and preserved ejection fraction. *N Engl J Med* 2008;**359**:2456-2467.
28. Anker SD, Butler J, Filippatos G, Ferreira JP, Bocchi E, Bohm M, Brunner-La Rocca HP, Choi DJ, Chopra V, Chuquiure-Valenzuela E, Giannetti N, Gomez-Mesa JE, Janssens S, Januzzi JL, Gonzalez-Juanatey JR, Merkely B, Nicholls SJ, Perrone SV, Pina IL, Ponikowski P, Senni M, Sim D, Spinar J, Squire I, Taddei S, Tsutsui H, Verma S, Vinereanu D, Zhang J, Carson P, Lam CSP, Marx N, Zeller C, Sattar N, Jamal W, Schnaidt S, Schnee JM, Brueckmann M, Pocock SJ, Zannad F, Packer M, Investigators EM-PT. Empagliflozin in Heart Failure with a Preserved Ejection Fraction. *N Engl J Med* 2021;**385**:1451-1461.
29. Solomon SD, McMurray JJV, Claggett B, de Boer RA, DeMets D, Hernandez AF, Inzucchi SE, Kosiborod MN, Lam CSP, Martinez F, Shah SJ, Desai AS, Jhund PS, Belohlavek J, Chiang CE, Borleffs CJW, Comin-Colet J, Dobreanu D, Drozdz J, Fang JC, Alcocer-Gamba MA, Al Habeeb W, Han Y, Cabrera Honorio JW, Janssens SP, Katova T, Kitakaze M, Merkely B, O'Meara E, Saraiva JFK, Tereshchenko SN, Thierer J, Vaduganathan M, Vardeny O, Verma S, Pham VN, Wilderang U, Zaozerska N, Bachus E, Lindholm D, Petersson M, Langkilde AM, Committees DT, Investigators. Dapagliflozin in Heart Failure with Mildly Reduced or Preserved Ejection Fraction. *N Engl J Med* 2022;**387**:1089-1098.
30. Kosiborod MN, Abildstrom SZ, Borlaug BA, Butler J, Rasmussen S, Davies M, Hovingh GK, Kitzman DW, Lindegaard ML, Moller DV, Shah SJ, Treppendahl MB, Verma S, Abhayaratna W, Ahmed FZ, Chopra V, Ezekowitz J, Fu M, Ito H, Lelonek M, Melenovsky V, Merkely B, Nunez J, Perna E, Schou M, Senni M, Sharma K, Van der Meer P, von Lewinski D, Wolf D, Petrie MC, Committees ST-HT, Investigators. Semaglutide in Patients with Heart Failure with Preserved Ejection Fraction and Obesity. *N Engl J Med* 2023;**389**:1069-1084.

31. Dunlay SM, Roger VL, Redfield MM. Epidemiology of heart failure with preserved ejection fraction. *Nat Rev Cardiol* 2017;**14**:591-602.
32. Sidney S, Go AS, Jaffe MG, Solomon MD, Ambrosy AP, Rana JS. Association Between Aging of the US Population and Heart Disease Mortality From 2011 to 2017. *JAMA Cardiol* 2019;**4**:1280-1286.
33. Flegal KM, Kruszon-Moran D, Carroll MD, Fryar CD, Ogden CL. Trends in Obesity Among Adults in the United States, 2005 to 2014. *JAMA* 2016;**315**:2284-2291.
34. Mills KT, Stefanescu A, He J. The global epidemiology of hypertension. *Nat Rev Nephrol* 2020;**16**:223-237.
35. Sax DR, Rana JS, Mark DG, Huang J, Collins SP, Liu D, Storrow AB, Reed ME, Network KC. Outcomes among acute heart failure emergency department patients by preserved vs. reduced ejection fraction. *ESC Heart Fail* 2021;**8**:2889-2898.
36. Lam CSP, Gamble GD, Ling LH, Sim D, Leong KTG, Yeo PSD, Ong HY, Jaufeerally F, Ng TP, Cameron VA, Poppe K, Lund M, Devlin G, Troughton R, Richards AM, Doughty RN. Mortality associated with heart failure with preserved vs. reduced ejection fraction in a prospective international multi-ethnic cohort study. *Eur Heart J* 2018;**39**:1770-1780.
37. Shah SJ, Katz DH, Selvaraj S, Burke MA, Yancy CW, Gheorghiade M, Bonow RO, Huang CC, Deo RC. Phenomapping for novel classification of heart failure with preserved ejection fraction. *Circulation* 2015;**131**:269-279.
38. Cohen JB, Schrauben SJ, Zhao L, Basso MD, Cvijic ME, Li Z, Yarde M, Wang Z, Bhattacharya PT, Chirinos DA, Prenner S, Zamani P, Seiffert DA, Car BD, Gordon DA, Margulies K, Cappola T, Chirinos JA. Clinical Phenogroups in Heart Failure With Preserved Ejection Fraction: Detailed Phenotypes, Prognosis, and Response to Spironolactone. *JACC Heart Fail* 2020;**8**:172-184.
39. Segar MW, Patel KV, Ayers C, Basit M, Tang WHW, Willett D, Berry J, Grodin JL, Pandey A. Phenomapping of patients with heart failure with preserved ejection fraction using machine learning-based unsupervised cluster analysis. *Eur J Heart Fail* 2020;**22**:148-158.
40. Messerli FH, Rimoldi SF, Bangalore S. The Transition From Hypertension to Heart Failure: Contemporary Update. *JACC Heart Fail* 2017;**5**:543-551.
41. Schiattarella GG, Alcaide P, Condorelli G, Gillette TG, Heymans S, Jones EAV, Kallikourdis M, Lichtman A, Marelli-Berg F, Shah S, Thorp EB, Hill JA. Immunometabolic Mechanisms of Heart Failure with Preserved Ejection Fraction. *Nat Cardiovasc Res* 2022;**1**:211-222.
42. Kitzman DW, Lam CSP. Obese Heart Failure With Preserved Ejection Fraction Phenotype: From Pariah to Central Player. *Circulation* 2017;**136**:20-23.
43. Tromp J, Claggett BL, Liu J, Jackson AM, Jhund PS, Kober L, Widimsky J, Boytsov SA, Chopra VK, Anand IS, Ge J, Chen CH, Maggioni AP, Martinez F, Packer M, Pfeffer MA, Pieske B, Redfield MM, Rouleau JL, Van Veldhuisen DJ, Zannad F, Zile MR, Rizkala AR, Inubushi-Molessa A, Lefkowitz MP, Shi VC, McMurray JJV, Solomon SD, Lam CSP, Investigators P-H. Global Differences in Heart Failure With Preserved Ejection Fraction: The PARAGON-HF Trial. *Circ Heart Fail* 2021;**14**:e007901.
44. Haykowsky MJ, Nicklas BJ, Brubaker PH, Hundley WG, Brinkley TE, Upadhya B, Becton JT, Nelson MD, Chen H, Kitzman DW. Regional Adipose Distribution and its Relationship to Exercise Intolerance in Older Obese Patients Who Have Heart Failure With Preserved Ejection Fraction. *JACC Heart Fail* 2018;**6**:640-649.
45. Sorimachi H, Obokata M, Takahashi N, Reddy YNV, Jain CC, Verbrugge FH, Koepp KE, Khosla S, Jensen MD, Borlaug BA. Pathophysiologic importance of visceral adipose tissue in women with heart failure and preserved ejection fraction. *Eur Heart J* 2021;**42**:1595-1605.

46. Owan TE, Hodge DO, Herges RM, Jacobsen SJ, Roger VL, Redfield MM. Trends in prevalence and outcome of heart failure with preserved ejection fraction. *N Engl J Med* 2006;**355**:251-259.
47. Meta-analysis Global Group in Chronic Heart F. The survival of patients with heart failure with preserved or reduced left ventricular ejection fraction: an individual patient data meta-analysis. *Eur Heart J* 2012;**33**:1750-1757.
48. Quiroz R, Doros G, Shaw P, Liang CS, Gauthier DF, Sam F. Comparison of characteristics and outcomes of patients with heart failure preserved ejection fraction versus reduced left ventricular ejection fraction in an urban cohort. *Am J Cardiol* 2014;**113**:691-696.
49. Tromp J, Shen L, Jhund PS, Anand IS, Carson PE, Desai AS, Granger CB, Komajda M, McKelvie RS, Pfeffer MA, Solomon SD, Kober L, Swedberg K, Zile MR, Pitt B, Lam CSP, McMurray JJV. Age-Related Characteristics and Outcomes of Patients With Heart Failure With Preserved Ejection Fraction. *J Am Coll Cardiol* 2019;**74**:601-612.
50. Capone F, Vettor R, Schiattarella GG. Cardiometabolic HFpEF: NASH of the Heart. *Circulation* 2023;**147**:451-453.
51. Salah HM, Pandey A, Soloveva A, Abdelmalek MF, Diehl AM, Moylan CA, Wegermann K, Rao VN, Hernandez AF, Tedford RJ, Parikh KS, Mentz RJ, McGarrah RW, Fudim M. Relationship of Nonalcoholic Fatty Liver Disease and Heart Failure With Preserved Ejection Fraction. *JACC Basic Transl Sci* 2021;**6**:918-932.
52. Lopaschuk GD, Ussher JR, Folmes CD, Jaswal JS, Stanley WC. Myocardial fatty acid metabolism in health and disease. *Physiol Rev* 2010;**90**:207-258.
53. Schiattarella GG, Altamirano F, Kim SY, Tong D, Ferdous A, Piristine H, Dasgupta S, Wang X, French KM, Villalobos E, Spurgin SB, Waldman M, Jiang N, May HI, Hill TM, Luo Y, Yoo H, Zaha VG, Lavandero S, Gillette TG, Hill JA. Xbp1s-FoxO1 axis governs lipid accumulation and contractile performance in heart failure with preserved ejection fraction. *Nat Commun* 2021;**12**:1684.
54. Wu CK, Lee JK, Hsu JC, Su MM, Wu YF, Lin TT, Lan CW, Hwang JJ, Lin LY. Myocardial adipose deposition and the development of heart failure with preserved ejection fraction. *Eur J Heart Fail* 2020;**22**:445-454.
55. Wei J, Nelson MD, Szczepaniak EW, Smith L, Mehta PK, Thomson LE, Berman DS, Li D, Bairey Merz CN, Szczepaniak LS. Myocardial steatosis as a possible mechanistic link between diastolic dysfunction and coronary microvascular dysfunction in women. *Am J Physiol Heart Circ Physiol* 2016;**310**:H14-19.
56. Mahmod M, Pal N, Rayner J, Holloway C, Raman B, Dass S, Levelt E, Ariga R, Ferreira V, Banerjee R, Schneider JE, Rodgers C, Francis JM, Karamitsos TD, Frenneaux M, Ashrafian H, Neubauer S, Rider O. The interplay between metabolic alterations, diastolic strain rate and exercise capacity in mild heart failure with preserved ejection fraction: a cardiovascular magnetic resonance study. *J Cardiovasc Magn Reson* 2018;**20**:88.
57. Martin LA, Kennedy BE, Karten B. Mitochondrial cholesterol: mechanisms of import and effects on mitochondrial function. *J Bioenerg Biomembr* 2016;**48**:137-151.
58. Taegtmeyer H. Energy metabolism of the heart: from basic concepts to clinical applications. *Curr Probl Cardiol* 1994;**19**:59-113.
59. Stanley WC, Recchia FA, Lopaschuk GD. Myocardial substrate metabolism in the normal and failing heart. *Physiol Rev* 2005;**85**:1093-1129.
60. Korvald C, Elvenes OP, Myrmel T. Myocardial substrate metabolism influences left ventricular energetics in vivo. *Am J Physiol Heart Circ Physiol* 2000;**278**:H1345-1351.

61. Randle PJ, Garland PB, Hales CN, Newsholme EA. The glucose fatty-acid cycle. Its role in insulin sensitivity and the metabolic disturbances of diabetes mellitus. *Lancet* 1963;**1**:785-789.
62. Fukushima A, Lopaschuk GD. Cardiac fatty acid oxidation in heart failure associated with obesity and diabetes. *Biochim Biophys Acta* 2016;**1861**:1525-1534.
63. Lopaschuk GD, Tsang H. Metabolism of palmitate in isolated working hearts from spontaneously diabetic "BB" Wistar rats. *Circ Res* 1987;**61**:853-858.
64. Herrero P, Peterson LR, McGill JB, Matthew S, Lesniak D, Dence C, Gropler RJ. Increased myocardial fatty acid metabolism in patients with type 1 diabetes mellitus. *J Am Coll Cardiol* 2006;**47**:598-604.
65. Alrob OA, Sankaralingam S, Ma C, Wagg CS, Fillmore N, Jaswal JS, Sack MN, Lehner R, Gupta MP, Michelakis ED, Padwal RS, Johnstone DE, Sharma AM, Lopaschuk GD. Obesity-induced lysine acetylation increases cardiac fatty acid oxidation and impairs insulin signalling. *Cardiovasc Res* 2014;**103**:485-497.
66. Meyer JG, Softic S, Basisty N, Rardin MJ, Verdin E, Gibson BW, Ilkayeva O, Newgard CB, Kahn CR, Schilling B. Temporal dynamics of liver mitochondrial protein acetylation and succinylation and metabolites due to high fat diet and/or excess glucose or fructose. *PLoS One* 2018;**13**:e0208973.
67. Deng Y, Xie M, Li Q, Xu X, Ou W, Zhang Y, Xiao H, Yu H, Zheng Y, Liang Y, Jiang C, Chen G, Du D, Zheng W, Wang S, Gong M, Chen Y, Tian R, Li T. Targeting Mitochondria-Inflammation Circuit by beta-Hydroxybutyrate Mitigates HFpEF. *Circ Res* 2021;**128**:232-245.
68. Fukushima A, Alrob OA, Zhang L, Wagg CS, Altamimi T, Rawat S, Rebeyka IM, Kantor PF, Lopaschuk GD. Acetylation and succinylation contribute to maturational alterations in energy metabolism in the newborn heart. *Am J Physiol Heart Circ Physiol* 2016;**311**:H347-363.
69. Karwi QG, Uddin GM, Ho KL, Lopaschuk GD. Loss of Metabolic Flexibility in the Failing Heart. *Front Cardiovasc Med* 2018;**5**:68.
70. Davila-Roman VG, Vedala G, Herrero P, de las Fuentes L, Rogers JG, Kelly DP, Gropler RJ. Altered myocardial fatty acid and glucose metabolism in idiopathic dilated cardiomyopathy. *J Am Coll Cardiol* 2002;**40**:271-277.
71. Tuunanen H, Engblom E, Naum A, Nagren K, Hesse B, Airaksinen KE, Nuutila P, Iozzo P, Ukkonen H, Opie LH, Knuuti J. Free fatty acid depletion acutely decreases cardiac work and efficiency in cardiomyopathic heart failure. *Circulation* 2006;**114**:2130-2137.
72. Sung MM, Das SK, Levasseur J, Byrne NJ, Fung D, Kim TT, Masson G, Boisvenue J, Soltys CL, Oudit GY, Dyck JR. Resveratrol treatment of mice with pressure-overload-induced heart failure improves diastolic function and cardiac energy metabolism. *Circ Heart Fail* 2015;**8**:128-137.
73. Byrne NJ, Levasseur J, Sung MM, Masson G, Boisvenue J, Young ME, Dyck JR. Normalization of cardiac substrate utilization and left ventricular hypertrophy precede functional recovery in heart failure regression. *Cardiovasc Res* 2016;**110**:249-257.
74. Doenst T, Pytel G, Schrepper A, Amorim P, Farber G, Shingu Y, Mohr FW, Schwarzer M. Decreased rates of substrate oxidation ex vivo predict the onset of heart failure and contractile dysfunction in rats with pressure overload. *Cardiovasc Res* 2010;**86**:461-470.
75. Neglia D, De Caterina A, Marraccini P, Natali A, Ciardetti M, Vecoli C, Gastaldelli A, Ciociaro D, Pellegrini P, Testa R, Menichetti L, L'Abbate A, Stanley WC, Recchia FA. Impaired myocardial metabolic reserve and substrate selection flexibility during stress in patients with idiopathic dilated cardiomyopathy. *Am J Physiol Heart Circ Physiol* 2007;**293**:H3270-3278.

76. Finck BN, Kelly DP. Peroxisome proliferator-activated receptor gamma coactivator-1 (PGC-1) regulatory cascade in cardiac physiology and disease. *Circulation* 2007;**115**:2540-2548.
77. Madrazo JA, Kelly DP. The PPAR trio: regulators of myocardial energy metabolism in health and disease. *J Mol Cell Cardiol* 2008;**44**:968-975.
78. Sack MN, Rader TA, Park S, Bastin J, McCune SA, Kelly DP. Fatty acid oxidation enzyme gene expression is downregulated in the failing heart. *Circulation* 1996;**94**:2837-2842.
79. Kanda H, Nohara R, Hasegawa K, Kishimoto C, Sasayama S. A nuclear complex containing PPARalpha/RXRalpha is markedly downregulated in the hypertrophied rat left ventricular myocardium with normal systolic function. *Heart Vessels* 2000;**15**:191-196.
80. Huss JM, Kelly DP. Nuclear receptor signaling and cardiac energetics. *Circ Res* 2004;**95**:568-578.
81. Finck BN, Kelly DP. PGC-1 coactivators: inducible regulators of energy metabolism in health and disease. *J Clin Invest* 2006;**116**:615-622.
82. Hopkins TA, Sugden MC, Holness MJ, Kozak R, Dyck JR, Lopaschuk GD. Control of cardiac pyruvate dehydrogenase activity in peroxisome proliferator-activated receptor-alpha transgenic mice. *Am J Physiol Heart Circ Physiol* 2003;**285**:H270-276.
83. Schummer CM, Werner U, Tennagels N, Schmoll D, Haschke G, Juretschke HP, Patel MS, Gerl M, Kramer W, Herling AW. Dysregulated pyruvate dehydrogenase complex in Zucker diabetic fatty rats. *Am J Physiol Endocrinol Metab* 2008;**294**:E88-96.
84. Karbowska J, Kochan Z, Smolenski RT. Peroxisome proliferator-activated receptor alpha is downregulated in the failing human heart. *Cell Mol Biol Lett* 2003;**8**:49-53.
85. Sihag S, Cresci S, Li AY, Sucharov CC, Lehman JJ. PGC-1alpha and ERRalpha target gene downregulation is a signature of the failing human heart. *J Mol Cell Cardiol* 2009;**46**:201-212.
86. Peterson LR, Herrero P, Schechtman KB, Racette SB, Waggoner AD, Kisrieva-Ware Z, Dence C, Klein S, Marsala J, Meyer T, Gropler RJ. Effect of obesity and insulin resistance on myocardial substrate metabolism and efficiency in young women. *Circulation* 2004;**109**:2191-2196.
87. Mazumder PK, O'Neill BT, Roberts MW, Buchanan J, Yun UJ, Cooksey RC, Boudina S, Abel ED. Impaired cardiac efficiency and increased fatty acid oxidation in insulin-resistant ob/ob mouse hearts. *Diabetes* 2004;**53**:2366-2374.
88. Buchanan J, Mazumder PK, Hu P, Chakrabarti G, Roberts MW, Yun UJ, Cooksey RC, Litwin SE, Abel ED. Reduced cardiac efficiency and altered substrate metabolism precedes the onset of hyperglycemia and contractile dysfunction in two mouse models of insulin resistance and obesity. *Endocrinology* 2005;**146**:5341-5349.
89. Zhang L, Jaswal JS, Ussher JR, Sankaralingam S, Wagg C, Zaugg M, Lopaschuk GD. Cardiac insulin-resistance and decreased mitochondrial energy production precede the development of systolic heart failure after pressure-overload hypertrophy. *Circ Heart Fail* 2013;**6**:1039-1048.
90. Mori J, Alrob OA, Wagg CS, Harris RA, Lopaschuk GD, Oudit GY. ANG II causes insulin resistance and induces cardiac metabolic switch and inefficiency: a critical role of PDK4. *Am J Physiol Heart Circ Physiol* 2013;**304**:H1103-1113.
91. Riehle C, Wende AR, Zaha VG, Pires KM, Wayment B, Olsen C, Bugger H, Buchanan J, Wang X, Moreira AB, Doenst T, Medina-Gomez G, Litwin SE, Lelliott CJ, Vidal-Puig A, Abel ED. PGC-1beta deficiency accelerates the

transition to heart failure in pressure overload hypertrophy. *Circ Res* 2011;**109**:783-793.
92. Degens H, de Brouwer KF, Gilde AJ, Lindhout M, Willemsen PH, Janssen BJ, van der Vusse GJ, van Bilsen M. Cardiac fatty acid metabolism is preserved in the compensated hypertrophic rat heart. *Basic Res Cardiol* 2006;**101**:17-26.
93. Akki A, Smith K, Seymour AM. Compensated cardiac hypertrophy is characterised by a decline in palmitate oxidation. *Mol Cell Biochem* 2008;**311**:215-224.
94. Zhabyeyev P, Gandhi M, Mori J, Basu R, Kassiri Z, Clanachan A, Lopaschuk GD, Oudit GY. Pressure-overload-induced heart failure induces a selective reduction in glucose oxidation at physiological afterload. *Cardiovasc Res* 2013;**97**:676-685.
95. Mori J, Basu R, McLean BA, Das SK, Zhang L, Patel VB, Wagg CS, Kassiri Z, Lopaschuk GD, Oudit GY. Agonist-induced hypertrophy and diastolic dysfunction are associated with selective reduction in glucose oxidation: a metabolic contribution to heart failure with normal ejection fraction. *Circ Heart Fail* 2012;**5**:493-503.
96. Funada J, Betts TR, Hodson L, Humphreys SM, Timperley J, Frayn KN, Karpe F. Substrate utilization by the failing human heart by direct quantification using arterio-venous blood sampling. *PLoS One* 2009;**4**:e7533.
97. Lopaschuk GD, Wambolt RB, Barr RL. An imbalance between glycolysis and glucose oxidation is a possible explanation for the detrimental effects of high levels of fatty acids during aerobic reperfusion of ischemic hearts. *J Pharmacol Exp Ther* 1993;**264**:135-144.
98. Diakos NA, Navankasattusas S, Abel ED, Rutter J, McCreath L, Ferrin P, McKellar SH, Miller DV, Park SY, Richardson RS, Deberardinis R, Cox JE, Kfoury AG, Selzman CH, Stehlik J, Fang JC, Li DY, Drakos SG. Evidence of Glycolysis Up-Regulation and Pyruvate Mitochondrial Oxidation Mismatch During Mechanical Unloading of the Failing Human Heart: Implications for Cardiac Reloading and Conditioning. *JACC Basic Transl Sci* 2016;**1**:432-444.
99. Gupte AA, Hamilton DJ, Cordero-Reyes AM, Youker KA, Yin Z, Estep JD, Stevens RD, Wenner B, Ilkayeva O, Loebe M, Peterson LE, Lyon CJ, Wong ST, Newgard CB, Torre-Amione G, Taegtmeyer H, Hsueh WA. Mechanical unloading promotes myocardial energy recovery in human heart failure. *Circ Cardiovasc Genet* 2014;**7**:266-276.
100. Dodd MS, Atherton HJ, Carr CA, Stuckey DJ, West JA, Griffin JL, Radda GK, Clarke K, Heather LC, Tyler DJ. Impaired in vivo mitochondrial Krebs cycle activity after myocardial infarction assessed using hyperpolarized magnetic resonance spectroscopy. *Circ Cardiovasc Imaging* 2014;**7**:895-904.
101. Ussher JR, Koves TR, Jaswal JS, Zhang L, Ilkayeva O, Dyck JR, Muoio DM, Lopaschuk GD. Insulin-stimulated cardiac glucose oxidation is increased in high-fat diet-induced obese mice lacking malonyl CoA decarboxylase. *Diabetes* 2009;**58**:1766-1775.
102. Sankaralingam S, Abo Alrob O, Zhang L, Jaswal JS, Wagg CS, Fukushima A, Padwal RS, Johnstone DE, Sharma AM, Lopaschuk GD. Lowering body weight in obese mice with diastolic heart failure improves cardiac insulin sensitivity and function: implications for the obesity paradox. *Diabetes* 2015;**64**:1643-1657.
103. Abel ED, Kaulbach HC, Tian R, Hopkins JC, Duffy J, Doetschman T, Minnemann T, Boers ME, Hadro E, Oberste-Berghaus C, Quist W, Lowell BB, Ingwall JS, Kahn BB. Cardiac hypertrophy with preserved contractile function after selective deletion of GLUT4 from the heart. *J Clin Invest* 1999;**104**:1703-1714.
104. Cahill GF, Jr. Fuel metabolism in starvation. *Annu Rev Nutr* 2006;**26**:1-22.

105. Cotter DG, Schugar RC, Crawford PA. Ketone body metabolism and cardiovascular disease. *Am J Physiol Heart Circ Physiol* 2013;**304**:H1060-1076.
106. Yurista SR, Chong CR, Badimon JJ, Kelly DP, de Boer RA, Westenbrink BD. Therapeutic Potential of Ketone Bodies for Patients With Cardiovascular Disease: JACC State-of-the-Art Review. *J Am Coll Cardiol* 2021;**77**:1660-1669.
107. Capozzi ME, Coch RW, Koech J, Astapova, II, Wait JB, Encisco SE, Douros JD, El K, Finan B, Sloop KW, Herman MA, D'Alessio DA, Campbell JE. The Limited Role of Glucagon for Ketogenesis During Fasting or in Response to SGLT2 Inhibition. *Diabetes* 2020;**69**:882-892.
108. Opie LH, Knuuti J. The adrenergic-fatty acid load in heart failure. *J Am Coll Cardiol* 2009;**54**:1637-1646.
109. Paolisso G, De Riu S, Marrazzo G, Verza M, Varricchio M, D'Onofrio F. Insulin resistance and hyperinsulinemia in patients with chronic congestive heart failure. *Metabolism* 1991;**40**:972-977.
110. Lommi J, Kupari M, Koskinen P, Naveri H, Leinonen H, Pulkki K, Harkonen M. Blood ketone bodies in congestive heart failure. *J Am Coll Cardiol* 1996;**28**:665-672.
111. Sengenes C, Berlan M, De Glisezinski I, Lafontan M, Galitzky J. Natriuretic peptides: a new lipolytic pathway in human adipocytes. *FASEB J* 2000;**14**:1345-1351.
112. Fukao T, Song XQ, Mitchell GA, Yamaguchi S, Sukegawa K, Orii T, Kondo N. Enzymes of ketone body utilization in human tissues: protein and messenger RNA levels of succinyl-coenzyme A (CoA):3-ketoacid CoA transferase and mitochondrial and cytosolic acetoacetyl-CoA thiolases. *Pediatr Res* 1997;**42**:498-502.
113. Williamson DH, Bates MW, Page MA, Krebs HA. Activities of enzymes involved in acetoacetate utilization in adult mammalian tissues. *Biochem J* 1971;**121**:41-47.
114. Kassovska-Bratinova S, Fukao T, Song XQ, Duncan AM, Chen HS, Robert MF, Perez-Cerda C, Ugarte M, Chartrand C, Vobecky S, Kondo N, Mitchell GA. Succinyl CoA: 3-oxoacid CoA transferase (SCOT): human cDNA cloning, human chromosomal mapping to 5p13, and mutation detection in a SCOT-deficient patient. *Am J Hum Genet* 1996;**59**:519-528.
115. Chen W, Sharma G, Jiang W, Maptue NR, Malloy CR, Sherry AD, Khemtong C. Metabolism of hyperpolarized (13) C-acetoacetate to beta-hydroxybutyrate detects real-time mitochondrial redox state and dysfunction in heart tissue. *NMR Biomed* 2019;**32**:e4091.
116. Sikder K, Shukla SK, Patel N, Singh H, Rafiq K. High Fat Diet Upregulates Fatty Acid Oxidation and Ketogenesis via Intervention of PPAR-gamma. *Cell Physiol Biochem* 2018;**48**:1317-1331.
117. Rardin MJ, Newman JC, Held JM, Cusack MP, Sorensen DJ, Li B, Schilling B, Mooney SD, Kahn CR, Verdin E, Gibson BW. Label-free quantitative proteomics of the lysine acetylome in mitochondria identifies substrates of SIRT3 in metabolic pathways. *Proc Natl Acad Sci U S A* 2013;**110**:6601-6606.
118. Fukao T, Lopaschuk GD, Mitchell GA. Pathways and control of ketone body metabolism: on the fringe of lipid biochemistry. *Prostaglandins Leukot Essent Fatty Acids* 2004;**70**:243-251.
119. Kashiwaya Y, Sato K, Tsuchiya N, Thomas S, Fell DA, Veech RL, Passonneau JV. Control of glucose utilization in working perfused rat heart. *J Biol Chem* 1994;**269**:25502-25514.
120. Sato K, Kashiwaya Y, Keon CA, Tsuchiya N, King MT, Radda GK, Chance B, Clarke K, Veech RL. Insulin, ketone bodies, and mitochondrial energy transduction. *FASEB J* 1995;**9**:651-658.

121. Kashiwaya Y, Pawlosky R, Markis W, King MT, Bergman C, Srivastava S, Murray A, Clarke K, Veech RL. A ketone ester diet increases brain malonyl-CoA and Uncoupling proteins 4 and 5 while decreasing food intake in the normal Wistar Rat. *J Biol Chem* 2010;**285**:25950-25956.
122. Gibb AA, Hill BG. Metabolic Coordination of Physiological and Pathological Cardiac Remodeling. *Circ Res* 2018;**123**:107-128.
123. Du Z, Shen A, Huang Y, Su L, Lai W, Wang P, Xie Z, Xie Z, Zeng Q, Ren H, Xu D. 1H-NMR-based metabolic analysis of human serum reveals novel markers of myocardial energy expenditure in heart failure patients. *PLoS One* 2014;**9**:e88102.
124. Horton JL, Davidson MT, Kurishima C, Vega RB, Powers JC, Matsuura TR, Petucci C, Lewandowski ED, Crawford PA, Muoio DM, Recchia FA, Kelly DP. The failing heart utilizes 3-hydroxybutyrate as a metabolic stress defense. *JCI Insight* 2019;**4**.
125. Bedi KC, Jr., Snyder NW, Brandimarto J, Aziz M, Mesaros C, Worth AJ, Wang LL, Javaheri A, Blair IA, Margulies KB, Rame JE. Evidence for Intramyocardial Disruption of Lipid Metabolism and Increased Myocardial Ketone Utilization in Advanced Human Heart Failure. *Circulation* 2016;**133**:706-716.
126. Schugar RC, Moll AR, Andre d'Avignon D, Weinheimer CJ, Kovacs A, Crawford PA. Cardiomyocyte-specific deficiency of ketone body metabolism promotes accelerated pathological remodeling. *Mol Metab* 2014;**3**:754-769.
127. Uchihashi M, Hoshino A, Okawa Y, Ariyoshi M, Kaimoto S, Tateishi S, Ono K, Yamanaka R, Hato D, Fushimura Y, Honda S, Fukai K, Higuchi Y, Ogata T, Iwai-Kanai E, Matoba S. Cardiac-Specific Bdh1 Overexpression Ameliorates Oxidative Stress and Cardiac Remodeling in Pressure Overload-Induced Heart Failure. *Circ Heart Fail* 2017;**10**.
128. Rudolph W, Maas D, Richter J, Hasinger F, Hofmann H, Dohrn P. [on the Significance of Acetoacetate and Beta-Hydroxybutyrate in Human Myocardial Metabolism]. *Klin Wochenschr* 1965;**43**:445-451.
129. Williamson JR, Krebs HA. Acetoacetate as fuel of respiration in the perfused rat heart. *Biochem J* 1961;**80**:540-547.
130. Wieland O, Funcke H, Loffler G. Interconversion of pyruvate dehydrogenase in rat heart muscle upon perfusion with fatty acids or ketone bodies. *FEBS Lett* 1971;**15**:295-298.
131. Hiltunen JK, Hassinen IE. Energy-linked regulation of glucose and pyruvate oxidation in isolated perfused rat heart. Role of pyruvate dehydrogenase. *Biochim Biophys Acta* 1976;**440**:377-390.
132. Kerbey AL, Randle PJ, Cooper RH, Whitehouse S, Pask HT, Denton RM. Regulation of pyruvate dehydrogenase in rat heart. Mechanism of regulation of proportions of dephosphorylated and phosphorylated enzyme by oxidation of fatty acids and ketone bodies and of effects of diabetes: role of coenzyme A, acetyl-coenzyme A and reduced and oxidized nicotinamide-adenine dinucleotide. *Biochem J* 1976;**154**:327-348.
133. Gormsen LC, Svart M, Thomsen HH, Sondergaard E, Vendelbo MH, Christensen N, Tolbod LP, Harms HJ, Nielsen R, Wiggers H, Jessen N, Hansen J, Botker HE, Moller N. Ketone Body Infusion With 3-Hydroxybutyrate Reduces Myocardial Glucose Uptake and Increases Blood Flow in Humans: A Positron Emission Tomography Study. *J Am Heart Assoc* 2017;**6**.
134. Rijzewijk LJ, van der Meer RW, Lamb HJ, de Jong HW, Lubberink M, Romijn JA, Bax JJ, de Roos A, Twisk JW, Heine RJ, Lammertsma AA, Smit JW, Diamant M. Altered myocardial substrate metabolism and decreased diastolic function in nonischemic human diabetic cardiomyopathy: studies with cardiac positron emission tomography and magnetic resonance imaging. *J Am Coll Cardiol* 2009;**54**:1524-1532.

135. Karwi QG, Zhang L, Altamimi TR, Wagg CS, Patel V, Uddin GM, Joerg AR, Padwal RS, Johnstone DE, Sharma A, Oudit GY, Lopaschuk GD. Weight loss enhances cardiac energy metabolism and function in heart failure associated with obesity. *Diabetes Obes Metab* 2019;**21**:1944-1955.
136. 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.
137. Kolwicz SC, Jr., Purohit S, Tian R. Cardiac metabolism and its interactions with contraction, growth, and survival of cardiomyocytes. *Circ Res* 2013;**113**:603-616.
138. Le Page LM, Rider OJ, Lewis AJ, Ball V, Clarke K, Johansson E, Carr CA, Heather LC, Tyler DJ. Increasing Pyruvate Dehydrogenase Flux as a Treatment for Diabetic Cardiomyopathy: A Combined 13C Hyperpolarized Magnetic Resonance and Echocardiography Study. *Diabetes* 2015;**64**:2735-2743.
139. Gopal K, Almutairi M, Al Batran R, Eaton F, Gandhi M, Ussher JR. Cardiac-Specific Deletion of Pyruvate Dehydrogenase Impairs Glucose Oxidation Rates and Induces Diastolic Dysfunction. *Front Cardiovasc Med* 2018;**5**:17.
140. Zordoky BN, Sung MM, Ezekowitz J, Mandal R, Han B, Bjorndahl TC, Bouatra S, Anderson T, Oudit GY, Wishart DS, Dyck JR, Alberta H. Metabolomic fingerprint of heart failure with preserved ejection fraction. *PLoS One* 2015;**10**:e0124844.
141. Hunter WG, Kelly JP, McGarrah RW, 3rd, Khouri MG, Craig D, Haynes C, Ilkayeva O, Stevens RD, Bain JR, Muehlbauer MJ, Newgard CB, Felker GM, Hernandez AF, Velazquez EJ, Kraus WE, Shah SH. Metabolomic Profiling Identifies Novel Circulating Biomarkers of Mitochondrial Dysfunction Differentially Elevated in Heart Failure With Preserved Versus Reduced Ejection Fraction: Evidence for Shared Metabolic Impairments in Clinical Heart Failure. *J Am Heart Assoc* 2016;**5**.
142. Youm YH, Nguyen KY, Grant RW, Goldberg EL, Bodogai M, Kim D, D'Agostino D, Planavsky N, Lupfer C, Kanneganti TD, Kang S, Horvath TL, Fahmy TM, Crawford PA, Biragyn A, Alnemri E, Dixit VD. The ketone metabolite beta-hydroxybutyrate blocks NLRP3 inflammasome-mediated inflammatory disease. *Nat Med* 2015;**21**:263-269.
143. Forsythe CE, Phinney SD, Fernandez ML, Quann EE, Wood RJ, Bibus DM, Kraemer WJ, Feinman RD, Volek JS. Comparison of low fat and low carbohydrate diets on circulating fatty acid composition and markers of inflammation. *Lipids* 2008;**43**:65-77.
144. Xie Z, Zhang D, Chung D, Tang Z, Huang H, Dai L, Qi S, Li J, Colak G, Chen Y, Xia C, Peng C, Ruan H, Kirkey M, Wang D, Jensen LM, Kwon OK, Lee S, Pletcher SD, Tan M, Lombard DB, White KP, Zhao H, Li J, Roeder RG, Yang X, Zhao Y. Metabolic Regulation of Gene Expression by Histone Lysine beta-Hydroxybutyrylation. *Mol Cell* 2016;**62**:194-206.
145. Dmitrieva-Posocco O, Wong AC, Lundgren P, Golos AM, Descamps HC, Dohnalova L, Cramer Z, Tian Y, Yueh B, Eskiocak O, Egervari G, Lan Y, Liu J, Fan J, Kim J, Madhu B, Schneider KM, Khoziainova S, Andreeva N, Wang Q, Li N, Furth EE, Bailis W, Kelsen JR, Hamilton KE, Kaestner KH, Berger SL, Epstein JA, Jain R, Li M, Beyaz S, Lengner CJ, Katona BW, Grivennikov SI, Thaiss CA, Levy M. beta-Hydroxybutyrate suppresses colorectal cancer. *Nature* 2022;**605**:160-165.
146. Liu K, Li F, Sun Q, Lin N, Han H, You K, Tian F, Mao Z, Li T, Tong T, Geng M, Zhao Y, Gu W, Zhao W. p53 beta-hydroxybutyrylation attenuates p53 activity. *Cell Death Dis* 2019;**10**:243.

147. Zhang X, Cao R, Niu J, Yang S, Ma H, Zhao S, Li H. Molecular basis for hierarchical histone de-beta-hydroxybutyrylation by SIRT3. *Cell Discov* 2019;**5**:35.
148. Shi L, Tu BP. Acetyl-CoA and the regulation of metabolism: mechanisms and consequences. *Curr Opin Cell Biol* 2015;**33**:125-131.
149. Hirschey MD, Zhao Y. Metabolic Regulation by Lysine Malonylation, Succinylation, and Glutarylation. *Mol Cell Proteomics* 2015;**14**:2308-2315.
150. Wang Y, Sano S, Yura Y, Ke Z, Sano M, Oshima K, Ogawa H, Horitani K, Min KD, Miura-Yura E, Kour A, Evans MA, Zuriaga MA, Hirschi KK, Fuster JJ, Pietras EM, Walsh K. Tet2-mediated clonal hematopoiesis in nonconditioned mice accelerates age-associated cardiac dysfunction. *JCI Insight* 2020;**5**.
151. Borlaug BA. The pathophysiology of heart failure with preserved ejection fraction. *Nat Rev Cardiol* 2014;**11**:507-515.
152. Borlaug BA, Olson TP, Lam CS, Flood KS, Lerman A, Johnson BD, Redfield MM. Global cardiovascular reserve dysfunction in heart failure with preserved ejection fraction. *J Am Coll Cardiol* 2010;**56**:845-854.
153. Fleg JL, Morrell CH, Bos AG, Brant LJ, Talbot LA, Wright JG, Lakatta EG. Accelerated longitudinal decline of aerobic capacity in healthy older adults. *Circulation* 2005;**112**:674-682.
154. Lakatta EG, Levy D. Arterial and cardiac aging: major shareholders in cardiovascular disease enterprises: Part II: the aging heart in health: links to heart disease. *Circulation* 2003;**107**:346-354.
155. Strait JB, Lakatta EG. Aging-associated cardiovascular changes and their relationship to heart failure. *Heart Fail Clin* 2012;**8**:143-164.
156. Beere PA, Russell SD, Morey MC, Kitzman DW, Higginbotham MB. Aerobic exercise training can reverse age-related peripheral circulatory changes in healthy older men. *Circulation* 1999;**100**:1085-1094.
157. Roh JD, Houstis N, Yu A, Chang B, Yeri A, Li H, Hobson R, Lerchenmuller C, Vujic A, Chaudhari V, Damilano F, Platt C, Zlotoff D, Lee RT, Shah R, Jerosch-Herold M, Rosenzweig A. Exercise training reverses cardiac aging phenotypes associated with heart failure with preserved ejection fraction in male mice. *Aging Cell* 2020;**19**:e13159.
158. Dai DF, Santana LF, Vermulst M, Tomazela DM, Emond MJ, MacCoss MJ, Gollahon K, Martin GM, Loeb LA, Ladiges WC, Rabinovitch PS. Overexpression of catalase targeted to mitochondria attenuates murine cardiac aging. *Circulation* 2009;**119**:2789-2797.
159. Sindler AL, Fleenor BS, Calvert JW, Marshall KD, Zigler ML, Lefer DJ, Seals DR. Nitrite supplementation reverses vascular endothelial dysfunction and large elastic artery stiffness with aging. *Aging Cell* 2011;**10**:429-437.
160. Brenner DA, Apstein CS, Saupe KW. Exercise training attenuates age-associated diastolic dysfunction in rats. *Circulation* 2001;**104**:221-226.
161. Walker EM, Jr., Nillas MS, Mangiarua EI, Cansino S, Morrison RG, Perdue RR, Triest WE, Wright GL, Studeny M, Wehner P, Rice KM, Blough ER. Age-associated changes in hearts of male Fischer 344/Brown Norway F1 rats. *Ann Clin Lab Sci* 2006;**36**:427-438.
162. Boluyt MO, Converso K, Hwang HS, Mikkor A, Russell MW. Echocardiographic assessment of age-associated changes in systolic and diastolic function of the female F344 rat heart. *J Appl Physiol (1985)* 2004;**96**:822-828.
163. Pacher P, Mabley JG, Liaudet L, Evgenov OV, Marton A, Hasko G, Kollai M, Szabo C. Left ventricular pressure-volume relationship in a rat model of advanced aging-associated heart failure. *Am J Physiol Heart Circ Physiol* 2004;**287**:H2132-2137.

164. Fannin J, Rice KM, Thulluri S, Dornon L, Arvapalli RK, Wehner P, Blough ER. Age-associated alterations of cardiac structure and function in the female F344xBN rat heart. *Age (Dordr)* 2014;**36**:9684.
165. Shah SJ, Borlaug BA, Kitzman DW, McCulloch AD, Blaxall BC, Agarwal R, Chirinos JA, Collins S, Deo RC, Gladwin MT, Granzier H, Hummel SL, Kass DA, Redfield MM, Sam F, Wang TJ, Desvigne-Nickens P, Adhikari BB. Research Priorities for Heart Failure With Preserved Ejection Fraction: National Heart, Lung, and Blood Institute Working Group Summary. *Circulation* 2020;**141**:1001-1026.
166. Kasiakogias A, Rosei EA, Camafort M, Ehret G, Faconti L, Ferreira JP, Brguljan J, Januszewicz A, Kahan T, Manolis A, Tsioufis K, Weber T, von Lueder TG, Smiseth OA, Wachtell K, Kjeldsen SE, Zannad F, Mancia G, Kreutz R. Hypertension and heart failure with preserved ejection fraction: position paper by the European Society of Hypertension. *J Hypertens* 2021;**39**:1522-1545.
167. Shah SJ, Kitzman DW, Borlaug BA, van Heerebeek L, Zile MR, Kass DA, Paulus WJ. Phenotype-Specific Treatment of Heart Failure With Preserved Ejection Fraction: A Multiorgan Roadmap. *Circulation* 2016;**134**:73-90.
168. Shah SJ. Precision Medicine for Heart Failure with Preserved Ejection Fraction: An Overview. *J Cardiovasc Transl Res* 2017;**10**:233-244.
169. Becher PM, Lindner D, Miteva K, Savvatis K, Zietsch C, Schmack B, Van Linthout S, Westermann D, Schultheiss HP, Tschope C. Role of heart rate reduction in the prevention of experimental heart failure: comparison between If-channel blockade and beta-receptor blockade. *Hypertension* 2012;**59**:949-957.
170. Murdoch CE, Chaubey S, Zeng L, Yu B, Ivetic A, Walker SJ, Vanhoutte D, Heymans S, Grieve DJ, Cave AC, Brewer AC, Zhang M, Shah AM. Endothelial NADPH oxidase-2 promotes interstitial cardiac fibrosis and diastolic dysfunction through proinflammatory effects and endothelial-mesenchymal transition. *J Am Coll Cardiol* 2014;**63**:2734-2741.
171. Ichihara S, Senbonmatsu T, Price E, Jr., Ichiki T, Gaffney FA, Inagami T. Angiotensin II type 2 receptor is essential for left ventricular hypertrophy and cardiac fibrosis in chronic angiotensin II-induced hypertension. *Circulation* 2001;**104**:346-351.
172. Matsumoto E, Sasaki S, Kinoshita H, Kito T, Ohta H, Konishi M, Kuwahara K, Nakao K, Itoh N. Angiotensin II-induced cardiac hypertrophy and fibrosis are promoted in mice lacking Fgf16. *Genes Cells* 2013;**18**:544-553.
173. Regan JA, Mauro AG, Carbone S, Marchetti C, Gill R, Mezzaroma E, Valle Raleigh J, Salloum FN, Van Tassell BW, Abbate A, Toldo S. A mouse model of heart failure with preserved ejection fraction due to chronic infusion of a low subpressor dose of angiotensin II. *Am J Physiol Heart Circ Physiol* 2015;**309**:H771-778.
174. Westermann D, Becher PM, Lindner D, Savvatis K, Xia Y, Frohlich M, Hoffmann S, Schultheiss HP, Tschope C. Selective PDE5A inhibition with sildenafil rescues left ventricular dysfunction, inflammatory immune response and cardiac remodeling in angiotensin II-induced heart failure in vivo. *Basic Res Cardiol* 2012;**107**:308.
175. Ogata T, Miyauchi T, Sakai S, Takanashi M, Irukayama-Tomobe Y, Yamaguchi I. Myocardial fibrosis and diastolic dysfunction in deoxycorticosterone acetate-salt hypertensive rats is ameliorated by the peroxisome proliferator-activated receptor-alpha activator fenofibrate, partly by suppressing inflammatory responses associated with the nuclear factor-kappa-B pathway. *J Am Coll Cardiol* 2004;**43**:1481-1488.

176. Matsumura Y, Kuro T, Konishi F, Takaoka M, Gariepy CE, Yanagisawa M. Enhanced blood pressure sensitivity to DOCA-salt treatment in endothelin ET(B) receptor-deficient rats. *Br J Pharmacol* 2000;**129**:1060-1062.
177. Mohammed SF, Ohtani T, Korinek J, Lam CS, Larsen K, Simari RD, Valencik ML, Burnett JC, Jr., Redfield MM. Mineralocorticoid accelerates transition to heart failure with preserved ejection fraction via "nongenomic effects". *Circulation* 2010;**122**:370-378.
178. Bowen TS, Eisenkolb S, Drobner J, Fischer T, Werner S, Linke A, Mangner N, Schuler G, Adams V. High-intensity interval training prevents oxidant-mediated diaphragm muscle weakness in hypertensive mice. *FASEB J* 2017;**31**:60-71.
179. Hulsmans M, Sager HB, Roh JD, Valero-Munoz M, Houstis NE, Iwamoto Y, Sun Y, Wilson RM, Wojtkiewicz G, Tricot B, Osborne MT, Hung J, Vinegoni C, Naxerova K, Sosnovik DE, Zile MR, Bradshaw AD, Liao R, Tawakol A, Weissleder R, Rosenzweig A, Swirski FK, Sam F, Nahrendorf M. Cardiac macrophages promote diastolic dysfunction. *J Exp Med* 2018;**215**:423-440.
180. Yoon S, Kim M, Lee H, Kang G, Bedi K, Margulies KB, Jain R, Nam KI, Kook H, Eom GH. S-Nitrosylation of Histone Deacetylase 2 by Neuronal Nitric Oxide Synthase as a Mechanism of Diastolic Dysfunction. *Circulation* 2021;**143**:1912-1925.
181. Valero-Munoz M, Backman W, Sam F. Murine Models of Heart Failure with Preserved Ejection Fraction: a "Fishing Expedition". *JACC Basic Transl Sci* 2017;**2**:770-789.
182. Wallner M, Eaton DM, Berretta RM, Liesinger L, Schittmayer M, Gindlhuber J, Wu J, Jeong MY, Lin YH, Borghetti G, Baker ST, Zhao H, Pfleger J, Blass S, Rainer PP, von Lewinski D, Bugger H, Mohsin S, Graier WF, Zirlik A, McKinsey TA, Birner-Gruenberger R, Wolfson MR, Houser SR. HDAC inhibition improves cardiopulmonary function in a feline model of diastolic dysfunction. *Sci Transl Med* 2020;**12**.
183. Gomes AC, Falcao-Pires I, Pires AL, Bras-Silva C, Leite-Moreira AF. Rodent models of heart failure: an updated review. *Heart Fail Rev* 2013;**18**:219-249.
184. Obokata M, Reddy YNV, Pislaru SV, Melenovsky V, Borlaug BA. Evidence Supporting the Existence of a Distinct Obese Phenotype of Heart Failure With Preserved Ejection Fraction. *Circulation* 2017;**136**:6-19.
185. Schauer A, Draskowski R, Jannasch A, Kirchhoff V, Goto K, Mannel A, Barthel P, Augstein A, Winzer E, Tugtekin M, Labeit S, Linke A, Adams V. ZSF1 rat as animal model for HFpEF: Development of reduced diastolic function and skeletal muscle dysfunction. *ESC Heart Fail* 2020;**7**:2123-2134.
186. Leite S, Oliveira-Pinto J, Tavares-Silva M, Abdellatif M, Fontoura D, Falcao-Pires I, Leite-Moreira AF, Lourenco AP. Echocardiography and invasive hemodynamics during stress testing for diagnosis of heart failure with preserved ejection fraction: an experimental study. *Am J Physiol Heart Circ Physiol* 2015;**308**:H1556-1563.
187. Abdellatif M, Trummer-Herbst V, Koser F, Durand S, Adao R, Vasques-Novoa F, Freundt JK, Voglhuber J, Pricolo MR, Kasa M, Turk C, Aprahamian F, Herrero-Galan E, Hofer SJ, Pendl T, Rech L, Kargl J, Anto-Michel N, Ljubojevic-Holzer S, Schipke J, Brandenberger C, Auer M, Schreiber R, Koyani CN, Heinemann A, Zirlik A, Schmidt A, von Lewinski D, Scherr D, Rainer PP, von Maltzahn J, Muhlfeld C, Kruger M, Frank S, Madeo F, Eisenberg T, Prokesch A, Leite-Moreira AF, Lourenco AP, Alegre-Cebollada J, Kiechl S, Linke WA, Kroemer G, Sedej S. Nicotinamide for the treatment of heart failure with preserved ejection fraction. *Sci Transl Med* 2021;**13**.
188. Leite S, Cerqueira RJ, Ibarrola J, Fontoura D, Fernandez-Celis A, Zannad F, Falcao-Pires I, Paulus WJ, Leite-Moreira AF, Rossignol P, Lopez-Andres N, Lourenco AP. Arterial Remodeling and Dysfunction in the ZSF1 Rat Model of

Heart Failure With Preserved Ejection Fraction. *Circ Heart Fail* 2019;**12**:e005596.
189. Hamdani N, Franssen C, Lourenco A, Falcao-Pires I, Fontoura D, Leite S, Plettig L, Lopez B, Ottenheijm CA, Becher PM, Gonzalez A, Tschope C, Diez J, Linke WA, Leite-Moreira AF, Paulus WJ. Myocardial titin hypophosphorylation importantly contributes to heart failure with preserved ejection fraction in a rat metabolic risk model. *Circ Heart Fail* 2013;**6**:1239-1249.
190. van Dijk CG, Oosterhuis NR, Xu YJ, Brandt M, Paulus WJ, van Heerebeek L, Duncker DJ, Verhaar MC, Fontoura D, Lourenco AP, Leite-Moreira AF, Falcao-Pires I, Joles JA, Cheng C. Distinct Endothelial Cell Responses in the Heart and Kidney Microvasculature Characterize the Progression of Heart Failure With Preserved Ejection Fraction in the Obese ZSF1 Rat With Cardiorenal Metabolic Syndrome. *Circ Heart Fail* 2016;**9**:e002760.
191. Espino-Gonzalez E, Tickle PG, Benson AP, Kissane RWP, Askew GN, Egginton S, Bowen TS. Abnormal skeletal muscle blood flow, contractile mechanics and fibre morphology in a rat model of obese-HFpEF. *J Physiol* 2021;**599**:981-1001.
192. Loos RJF, Yeo GSH. The genetics of obesity: from discovery to biology. *Nat Rev Genet* 2022;**23**:120-133.
193. Schiattarella GG, Altamirano F, Tong D, French KM, Villalobos E, Kim SY, Luo X, Jiang N, May HI, Wang ZV, Hill TM, Mammen PPA, Huang J, Lee DI, Hahn VS, Sharma K, Kass DA, Lavandero S, Gillette TG, Hill JA. Nitrosative stress drives heart failure with preserved ejection fraction. *Nature* 2019;**568**:351-356.
194. Tong D, Schiattarella GG, Jiang N, Altamirano F, Szweda PA, Elnwasany A, Lee DI, Yoo H, Kass DA, Szweda LI, Lavandero S, Verdin E, Gillette TG, Hill JA. NAD(+) Repletion Reverses Heart Failure With Preserved Ejection Fraction. *Circ Res* 2021;**128**:1629-1641.
195. Mesquita T, Zhang R, Cho JH, Zhang R, Lin YN, Sanchez L, Goldhaber JI, Yu JK, Liang JA, Liu W, Trayanova NA, Cingolani E. Mechanisms of Sinoatrial Node Dysfunction in Heart Failure With Preserved Ejection Fraction. *Circulation* 2022;**145**:45-60.
196. Kitakata H, Endo J, Hashimoto S, Mizuno E, Moriyama H, Shirakawa K, Goto S, Katsumata Y, Fukuda K, Sano M. Imeglimin prevents heart failure with preserved ejection fraction by recovering the impaired unfolded protein response in mice subjected to cardiometabolic stress. *Biochem Biophys Res Commun* 2021;**572**:185-190.
197. Zhang B, Xu Y, Cui X, Jiang H, Luo W, Weng X, Wang Y, Zhao Y, Sun A, Ge J. Alteration of m6A RNA Methylation in Heart Failure With Preserved Ejection Fraction. *Front Cardiovasc Med* 2021;**8**:647806.
198. Tong D, Schiattarella GG, Jiang N, May HI, Lavandero S, Gillette TG, Hill JA. Female Sex Is Protective in a Preclinical Model of Heart Failure With Preserved Ejection Fraction. *Circulation* 2019;**140**:1769-1771.
199. Crewe C, Kinter M, Szweda LI. Rapid inhibition of pyruvate dehydrogenase: an initiating event in high dietary fat-induced loss of metabolic flexibility in the heart. *PLoS One* 2013;**8**:e77280.
200. Parra V, Altamirano F, Hernandez-Fuentes CP, Tong D, Kyrychenko V, Rotter D, Pedrozo Z, Hill JA, Eisner V, Lavandero S, Schneider JW, Rothermel BA. Down Syndrome Critical Region 1 Gene, Rcan1, Helps Maintain a More Fused Mitochondrial Network. *Circ Res* 2018;**122**:e20-e33.
201. Caudal A, Tang X, Chavez JD, Keller A, Mohr JP, Bakhtina AA, Villet O, Chen H, Zhou B, Walker MA, Tian R, Bruce JE. Mitochondrial interactome quantitation reveals structural changes in metabolic machinery in the failing murine heart. *Nat Cardiovasc Res* 2022;**1**:855-866.

202. Wang T, Yao W, Li J, He Q, Shao Y, Huang F. Acetyl-CoA from inflammation-induced fatty acids oxidation promotes hepatic malate-aspartate shuttle activity and glycolysis. *Am J Physiol Endocrinol Metab* 2018;**315**:E496-E510.
203. Cao J, An D, Galduroz M, Zhuo J, Liang S, Eybye M, Frassetto A, Kuroda E, Funahashi A, Santana J, Mihai C, Benenato KE, Kumarasinghe ES, Sabnis S, Salerno T, Coughlan K, Miracco EJ, Levy B, Besin G, Schultz J, Lukacs C, Guey L, Finn P, Furukawa T, Giangrande PH, Saheki T, Martini PGV. mRNA Therapy Improves Metabolic and Behavioral Abnormalities in a Murine Model of Citrin Deficiency. *Mol Ther* 2019;**27**:1242-1251.
204. Davidson MM, Nesti C, Palenzuela L, Walker WF, Hernandez E, Protas L, Hirano M, Isaac ND. Novel cell lines derived from adult human ventricular cardiomyocytes. *J Mol Cell Cardiol* 2005;**39**:133-147.
205. Wagner GR, Payne RM. Widespread and enzyme-independent Nepsilon-acetylation and Nepsilon-succinylation of proteins in the chemical conditions of the mitochondrial matrix. *J Biol Chem* 2013;**288**:29036-29045.
206. Chambers KT, Leone TC, Sambandam N, Kovacs A, Wagg CS, Lopaschuk GD, Finck BN, Kelly DP. Chronic inhibition of pyruvate dehydrogenase in heart triggers an adaptive metabolic response. *J Biol Chem* 2011;**286**:11155-11162.
207. Selvaraj S, Kelly DP, Margulies KB. Implications of Altered Ketone Metabolism and Therapeutic Ketosis in Heart Failure. *Circulation* 2020;**141**:1800-1812.
208. Safer B. The Metabolic Significance of the Malate-Aspartate Cycle in Heart. *Circ Res* 1975;**37**:527-533.
209. LaNoue KF, Schoolwerth AC. Metabolite transport in mitochondria. *Annu Rev Biochem* 1979;**48**:871-922.
210. Park J, Chen Y, Tishkoff DX, Peng C, Tan M, Dai L, Xie Z, Zhang Y, Zwaans BM, Skinner ME, Lombard DB, Zhao Y. SIRT5-mediated lysine desuccinylation impacts diverse metabolic pathways. *Mol Cell* 2013;**50**:919-930.
211. D'Amario D, Migliaro S, Borovac JA, Restivo A, Vergallo R, Galli M, Leone AM, Montone RA, Niccoli G, Aspromonte N, Crea F. Microvascular Dysfunction in Heart Failure With Preserved Ejection Fraction. *Front Physiol* 2019;**10**:1347.
212. Warbrick I, Rabkin SW. Hypoxia-inducible factor 1-alpha (HIF-1alpha) as a factor mediating the relationship between obesity and heart failure with preserved ejection fraction. *Obes Rev* 2019;**20**:701-712.
213. Liu Y, Luo Q, Su Z, Xing J, Wu J, Xiang L, Huang Y, Pan H, Wu X, Zhang X, Li J, Yan F, Zhang H. Suppression of Myocardial Hypoxia-Inducible Factor-1alpha Compromises Metabolic Adaptation and Impairs Cardiac Function in Patients With Cyanotic Congenital Heart Disease During Puberty. *Circulation* 2021;**143**:2254-2272.
214. Semenza GL. Hypoxia-inducible factor 1 and cardiovascular disease. *Annu Rev Physiol* 2014;**76**:39-56.
215. Melendez-Rodriguez F, Urrutia AA, Lorendeau D, Rinaldi G, Roche O, Bogurcu-Seidel N, Ortega Muelas M, Mesa-Ciller C, Turiel G, Bouthelier A, Hernansanz-Agustin P, Elorza A, Escasany E, Li QOY, Torres-Capelli M, Tello D, Fuertes E, Fraga E, Martinez-Ruiz A, Perez B, Gimenez-Bachs JM, Salinas-Sanchez AS, Acker T, Sanchez Prieto R, Fendt SM, De Bock K, Aragones J. HIF1alpha Suppresses Tumor Cell Proliferation through Inhibition of Aspartate Biosynthesis. *Cell Rep* 2019;**26**:2257-2265 e2254.
216. Hartupee J, Mann DL. Neurohormonal activation in heart failure with reduced ejection fraction. *Nat Rev Cardiol* 2017;**14**:30-38.

217. Rashid AM, Khan MS, Fudim M, DeWald TA, DeVore A, Butler J. Management of Heart Failure With Reduced Ejection Fraction. *Curr Probl Cardiol* 2023;**48**:101596.
218. Aguilar-Gallardo JS, Correa A, Contreras JP. Cardio-renal benefits of sodium-glucose co-transporter 2 inhibitors in heart failure with reduced ejection fraction: mechanisms and clinical evidence. *Eur Heart J Cardiovasc Pharmacother* 2022;**8**:311-321.
219. Shah KS, Xu H, Matsouaka RA, Bhatt DL, Heidenreich PA, Hernandez AF, Devore AD, Yancy CW, Fonarow GC. Heart Failure With Preserved, Borderline, and Reduced Ejection Fraction: 5-Year Outcomes. *J Am Coll Cardiol* 2017;**70**:2476-2486.
220. Hoekstra T, Lesman-Leegte I, van Veldhuisen DJ, Sanderman R, Jaarsma T. Quality of life is impaired similarly in heart failure patients with preserved and reduced ejection fraction. *Eur J Heart Fail* 2011;**13**:1013-1018.
221. Shao D, Kolwicz SC, Jr., Wang P, Roe ND, Villet O, Nishi K, Hsu YA, Flint GV, Caudal A, Wang W, Regnier M, Tian R. Increasing Fatty Acid Oxidation Prevents High-Fat Diet-Induced Cardiomyopathy Through Regulating Parkin-Mediated Mitophagy. *Circulation* 2020;**142**:983-997.
222. Yurista SR, Matsuura TR, Sillje HHW, Nijholt KT, McDaid KS, Shewale SV, Leone TC, Newman JC, Verdin E, van Veldhuisen DJ, de Boer RA, Kelly DP, Westenbrink BD. Ketone Ester Treatment Improves Cardiac Function and Reduces Pathologic Remodeling in Preclinical Models of Heart Failure. *Circ Heart Fail* 2021;**14**:e007684.
223. Nielsen R, Moller N, Gormsen LC, Tolbod LP, Hansson NH, Sorensen J, Harms HJ, Frokiaer J, Eiskjaer H, Jespersen NR, Mellemkjaer S, Lassen TR, Pryds K, Botker HE, Wiggers H. Cardiovascular Effects of Treatment With the Ketone Body 3-Hydroxybutyrate in Chronic Heart Failure Patients. *Circulation* 2019;**139**:2129-2141.
224. Lee CF, Chavez JD, Garcia-Menendez L, Choi Y, Roe ND, Chiao YA, Edgar JS, Goo YA, Goodlett DR, Bruce JE, Tian R. Normalization of NAD+ Redox Balance as a Therapy for Heart Failure. *Circulation* 2016;**134**:883-894.
225. Karamanlidis G, Lee CF, Garcia-Menendez L, Kolwicz SC, Jr., Suthammarak W, Gong G, Sedensky MM, Morgan PG, Wang W, Tian R. Mitochondrial complex I deficiency increases protein acetylation and accelerates heart failure. *Cell Metab* 2013;**18**:239-250.
226. Pietrocola F, Galluzzi L, Bravo-San Pedro JM, Madeo F, Kroemer G. Acetyl coenzyme A: a central metabolite and second messenger. *Cell Metab* 2015;**21**:805-821.
227. Lu Z, Scott I, Webster BR, Sack MN. The emerging characterization of lysine residue deacetylation on the modulation of mitochondrial function and cardiovascular biology. *Circ Res* 2009;**105**:830-841.
228. Thapa D, Zhang M, Manning JR, Guimaraes DA, Stoner MW, O'Doherty RM, Shiva S, Scott I. Acetylation of mitochondrial proteins by GCN5L1 promotes enhanced fatty acid oxidation in the heart. *Am J Physiol Heart Circ Physiol* 2017;**313**:H265-H274.
229. Thapa D, Bugga P, Mushala BAS, Manning JR, Stoner MW, McMahon B, Zeng X, Cantrell PS, Yates N, Xie B, Edmunds LR, Jurczak MJ, Scott I. GCN5L1 impairs diastolic function in mice exposed to a high fat diet by restricting cardiac pyruvate oxidation. *Physiol Rep* 2022;**10**:e15415.
230. Dikalova AE, Pandey A, Xiao L, Arslanbaeva L, Sidorova T, Lopez MG, Billings FTt, Verdin E, Auwerx J, Harrison DG, Dikalov SI. Mitochondrial Deacetylase Sirt3 Reduces Vascular Dysfunction and Hypertension While Sirt3 Depletion in Essential Hypertension Is Linked to Vascular Inflammation and Oxidative Stress. *Circ Res* 2020;**126**:439-452.

231. Jin L, Geng L, Ying L, Shu L, Ye K, Yang R, Liu Y, Wang Y, Cai Y, Jiang X, Wang Q, Yan X, Liao B, Liu J, Duan F, Sweeney G, Woo CWH, Wang Y, Xia Z, Lian Q, Xu A. FGF21-Sirtuin 3 Axis Confers the Protective Effects of Exercise Against Diabetic Cardiomyopathy by Governing Mitochondrial Integrity. *Circulation* 2022;**146**:1537-1557.
232. Zeng H, Vaka VR, He X, Booz GW, Chen JX. High-fat diet induces cardiac remodelling and dysfunction: assessment of the role played by SIRT3 loss. *J Cell Mol Med* 2015;**19**:1847-1856.
233. Seglen PO, Solheim AE. Effects of aminooxyacetate, alanine and other amino acids on protein synthesis in isolated rat hepatocytes. *Biochim Biophys Acta* 1978;**520**:630-641.