Mitochondrial Dysfunction in Heart Failure with Preserved Ejection Fraction

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Abstract

Heart failure with preserved ejection fraction (HFpEF) is a complex syndrome with an increasingly recognized heterogeneity in pathophysiology. Exercise intolerance is the hallmark of HFpEF, and appears to be caused by both cardiac, as well as peripheral abnormalities in the arterial tree and skeletal muscle. Mitochondrial abnormalities can significantly contribute to impaired oxygen utilization, and the resulting exercise intolerance in HFpEF. We review key aspects of the complex biology of this organelle, the clinical relevance of mitochondrial function, the methods that are currently available to assess mitochondrial function in humans, and the evidence supporting a role for mitochondrial dysfunction in the pathophysiology of HFpEF. We also discuss the role of mitochondrial function as a therapeutic target, some key considerations for the design of early-phase clinical trials using agents that specifically target mitochondrial function to improve symptoms in patients with HFpEF, and ongoing trials with mitochondrial agents in HFpEF.

Keywords

Introduction

Heart failure is a complex and heterogeneous clinical syndrome that results in significant morbidity and mortality for patients and a heavy societal and economic burden. According to NHANES data, between 2011 and 2014, there were 6.5 million Americans over the age of 20 living with heart failure. The prevalence is projected to increase by a further 46% to over 8 million adults by 2030.[1] Approximately half of patients with heart failure have heart failure with preserved ejection fraction (HFpEF).[2] Epidemiologic data indicate that the prevalence of HFpEF is increasing over time as our population ages and epidemics of obesity, diabetes and metabolic syndrome worsen.[2] Despite numerous trials over the last few

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decades, at present there are no evidence-based available pharmacologic therapies that significantly impact outcomes in HFpEF.

An incomplete understanding of the pathophysiologic underpinnings of HFpEF represents a significant barrier to therapeutic advances in the field. Difficulties stem from heterogeneity within the HFpEF population, with research suggesting multiple unique phenotypes rather than a unified disease state.[3] The presence of distinct phenotypes complicates the design of clinical trials and may reduce the likelihood of successful outcomes, as relatively indiscriminate enrollment may result in blunted average responses to interventions that target specific pathologic processes found in certain phenotypic subgroups.

Despite this phenotypic heterogeneity, exercise intolerance is common to all patients with HFpEF and is the primary driver of morbidity and reduced quality of life in this population.[4] The normal physiologic adaptation to exercise involves a delicate systemic coordination between the cardiac pump, the respiratory system, and the arterial system, with the goal of delivering inhaled oxygen to mitochondria in skeletal and cardiac muscle. This oxygen can then be used by the mitochondria to generate adenosine triphosphate (ATP) and in turn, fuel locomotion, ventilation and cardiac contraction and relaxation. Peripheral abnormalities can therefore impact the threshold at which a diseased heart exhibits frank failure, and mitochondrial abnormalities (whether due to disease states directly impacting the mitochondria, deconditioning, or both) may impact both peripheral muscle (skeletal, respiratory) and myocardial function.

Peak exercise oxygen consumption (VO_2), a widely validated measure of exercise capacity, is consistently reduced in studies of HFpEF patients[5], indicating dysfunction in the oxygen delivery and utilization system. According to the Fick equation, peak VO_2 is the product of cardiac output (CO) and arteriovenous oxygen (A-VO_2Diff) difference, a marker of peripheral oxygen extraction. As such, a reduction in peak VO_2 can be a consequence of reduced delivery of oxygen to the systemic circulation by the heart or reduced peripheral utilization of oxygen.[6]

It had classically been thought that exercise intolerance and reduced VO_2 in HFpEF is primarily due to inadequate augmentation of cardiac output in response to exercise. Early evidence supported this theory and showed that patients with HFpEF had an inability to increase end-diastolic volume or stroke volume in response to exercise[7] or exhibited impaired chronotropic reserve.[8] However, more recent studies have demonstrated that peripheral dysfunction is also an important cause of exercise intolerance. Haykowsky *et al.* found that reduced cardiac output during exercise accounted for only 50% of the reduction in peak VO_2 in HFpEF, with reduced arterio-venous oxygen difference (A-VO_2Diff) accounting for the other half.[5] In fact, the strongest predictor of peak VO_2 was change in A-VO_2Diff between resting and peak exercise. Similarly, Bhella *et al.* showed that that indices of cardiac reserve were not impaired in well-compensated HFpEF patients compared to healthy controls during exercise.[9] A meta-analysis of six randomized controlled studies further demonstrated that, while exercise training significantly improved cardiorespiratory fitness in patients with HFpEF, resting LV function was largely unchanged, suggesting that the improved fitness may have been derived from changes in the periphery.[10] Furthermore,

in one study that simultaneously assessed cardiac output and VO_2 during exercise, it was found that exercise training improved peak VO_2 predominantly via increased A-VO_2Diff during exercise, rather than increased cardiac output,[11] indicating a peripheral rather than a central hemodynamic basis for the benefit of this intervention.

In sum, these studies indicate that peripheral abnormalities are important mediators of exercise intolerance in HFpEF. Among these abnormalities, mitochondrial dysfunction appears to be an important pathophysiologic contributor. Current heart failure therapies, including beta blockers, angiotensin-converting enzyme inhibitors, angiotensin receptor blockers, and mineralocorticoid receptor antagonists, appear to lead to a nonspecific decrease in mitochondrial reactive oxygen species production.[12] However, direct targeting of mitochondrial function as a therapeutic intervention has to date been insufficient explored. This review provides a discussion of mitochondrial abnormalities in HFpEF, currently available methods to assess mitochondrial function in humans, the role of mitochondrial function as a therapeutic target, key considerations for the design of early-phase clinical trials using mitochondrial-targeting agents, and completed or ongoing clinical trials that using novel therapies that target mitochondria.

Skeletal Muscle Mitochondrial Abnormalities in HFpEF

There is an increasing body of literature suggesting significant structural and functional abnormalities of skeletal muscle in patients with HFpEF. Kitzman *et al.* reported a shift towards fewer slow-twitch type I fibers (which have greater mitochondrial density and oxidative capacity), as well as a reduced capillary to fiber ratio in biopsy tissue from the vastus lateralis muscle among older patients with HFpEF. Both of these abnormalities were significantly related to peak VO_2 on multivariate analysis.[13] It was also shown that, compared with healthy controls, patients with HFpEF have a reduced percent of total and lean leg mass on DEXA scanning. Importantly, peak VO_2 indexed to lean body mass was significantly reduced in HFpEF, indicating a reduced oxygen utilization even after accounting for the reduced skeletal muscle mass.[14] While the above findings were important in illustrating significant skeletal muscle abnormalities in HFpEF, they did not directly assess mitochondrial content or oxidative capacity.

Mitochondrial function was directly assessed by Bowen *et al.* in a rat model of HFpEF.[15] In this study, female salt-sensitive rats were separated into three groups: low-salt diet, high-salt diet or high-salt with treadmill exercise training. Diaphragm muscle biopsy from high-salt diet rats, who developed a HFpEF-like phenotype, demonstrated a fiber type shift from fast (type II) to slow (type I) twitch, with impaired mitochondrial respiration, indicating electron transport chain dysfunction. Interestingly, soleus muscle biopsy did not demonstrate a change in fiber-type ratio, but did demonstrate reduced activity of citrate synthase, a key Krebs cycle enzyme that has been well validated as a proxy for mitochondrial content and oxidative capacity.[16] Exercise training attenuated the reduction of both soleus citrate synthase activity and diaphragmatic mitochondrial respiration. This rat model may not recapitulate the key features of skeletal muscle dysfunction in HFpEF, since human HFpEF has been shown to exhibit a shift towards fewer slow-twitch type I fibers in vastus lateralis muscle, as discussed above. Additionally, a biphasic pattern of response to pressure overload

(an early increase in mitochondrial oxidative phosphorylation, with a subsequent decline that coincides with transition from cardiac hypertrophy to HF) has been reported in models of HFrEF; however, whether this pattern occurs in the transition to human HFpEF is unknown. [17]

Molina *et al.* subsequently studied the expression of important mitochondrial proteins (as markers of mitochondrial content and oxidative capacity) among elderly HFpEF patients. Mitochondrial content in vastus lateralis muscle tissue (as indicated by expression of porin, a mitochondrial membrane protein) was significantly reduced (46% lower) in the HFpEF group compared to sedentary healthy controls.[18] HFpEF subjects also had a significantly lower expression of both citrate synthase and mitofusin 2, a key regulator of mitochondrial fusion. Both porin and mitofusin 2 expression exhibited a moderate significant direct correlation with peak VO_2. These studies made a convincing case for the presence of skeletal muscle mitochondrial dysregulation in HFpEF, but further research was needed to assess the impact of such abnormalities on energetics during exercise.

Novel advanced imaging techniques provided additional insights into dysfunctional skeletal muscle energetics and further demonstrated the link between mitochondrial dysregulation and exercise intolerance. Recently, Weiss *et al.* compared 12 patients with HFpEF with 11 healthy controls, using ^{31}P magnetic resonance spectroscopy (MRS) during a plantar flexion exercise stress test to assess skeletal muscle energetics. They found that, while resting MRS measurements were not significantly different from healthy controls, patients with HFpEF exhibited very early and rapid depletion of high-energy phosphates during exercise, and significantly decreased maximal mitochondrial oxidative capacity.[19] Whereas it is tempting to interpret this as evidence of intrinsic mitochondrial dysfunction, it should be noted that *in vivo* measurements of oxidative capacity interrogate the integrated function of the oxygen transport chain, including local oxygen diffusion capacity and intrinsic mitochondrial function. Therefore, impaired microvascular vasodilation and/or microvascular rarefaction (which can directly impact oxygen diffusion capacity), in addition to intrinsic mitochondrial dysfunction, could cause or contribute to these observations.

Whereas patients with HF can exhibit skeletal muscle deconditioning as a result of chronically reduced physical activity, it is important to note that the skeletal myopathy seen in HFpEF, and more broadly in heart failure, cannot be fully explained by deconditioning. While deconditioning likely contributes to HF myopathy, disuse atrophy causes an opposite fiber type shift than that reported in human HFpEF; deconditioning typically results in preferential decrease in type II fibers.[20]

In summary, available data demonstrates abnormalities in skeletal muscle mitochondrial content and structure, and a significant energetic impairment during exertion in HFpEF. This suggests that mitochondrial dysfunction may contribute to exercise intolerance in HFpEF patients and introduces a potential novel therapeutic target in this condition. The etiology and time-course leading to mitochondrial dysfunction in HFpEF is unclear; possible etiologies include chronic elevation of sympathetic tone, oxidative stress, overexpression of pro-inflammatory cytokines, or factors associated with the metabolic syndrome known to be associated with mitochondrial abnormalities, such as insulin resistance.[21, 22] Given the

potential role of this organelle in the pathophysiology of HFpEF, there is significant interest in novel therapeutics that directly target mitochondria; however, there are important aspects of mitochondrial biology, function and phenotyping that need to be considered when designing and interpreting early-phase mechanistic clinical trials with agents that impact mitochondrial function in HFpEF.

Cardiac Muscle Mitochondrial Abnormalities in HFpEF

There is also evidence that cardiomyocyte mitochondria in HFpEF have structural and energetic abnormalities. The proposed pathophysiology involves an increase in reactive oxygen species and mitochondrial damage, which results in a mismatch between ATP production and demand while also activating downstream signaling pathways that can result in cardiac remodeling, inflammation and diastolic dysfunction.[23] A mouse-model of cardiac hypertrophy and diastolic dysfunction induced by abdominal aortic banding showed an overall decrease in cardiac mitochondrial metabolism and ATP production.[24] A recent study in rats utilized a high fat, high sucrose diet to induce metabolic heart disease, characterized by left ventricular hypertrophy and diastolic dysfunction. Hearts isolated from rats with metabolic heart disease showed decreased rate of ATP synthesis measured by[31]P- magnetic resonance spectroscopy.

Abnormal myocardial mitochondrial energetics have also been demonstrated in human subjects using non-invasive imaging techniques. A study by Phan, et al., used[31]P-magnetic resonance spectroscopy at rest to evaluate *in vivo* myocardial energetics in 37 subjects with HFpEF and 20 control subjects. They found that subjects with HFpEF had significantly reduced energy reserves, as indicated by creatine phosphate/adenosine triphosphate (PCr/ATP) ratio.[25] Of note, like in peripheral muscle, in vivo measurements of oxidative capacity may be impacted by factors that govern the oxygen transport upstream of the mitochondrial respiratory chain, including myocardial microvascular rarefaction, which is a feature of HFpEF.[26] The relative causal impact of cardiac vs. skeletal mitochondrial dysfunction on exercise intolerance is unclear, but it is likely that therapeutic agents that improve mitochondrial function will likely enhance both cardiac function and peripheral oxygen utilization.

Comparison with HFrEF

Patients with HFrEF have been shown to have a number of skeletal muscle abnormalities that have also been shown in HFpEF, including reductions in type I oxidative fibers, citrate synthase activity, mitochondrial volume and content, and capillary to fiber ratio.[27, 28]

However, despite these similarities, there are differences in mitochondrial abnormalities between patients with HFpEF and HFrEF. Hunter *et al.* utilized mass spectrometry to quantify levels of 63 circulating metabolites among patients with HFpEF, HFrEF, and no HF. They found that long chain acyl carnitine levels, a marker of impaired fatty acid oxidation, were elevated in all subjects with heart failure; however, they were significantly higher in HFrEF than in HFpEF.[29] In addition to this metabolic difference, prior studies indicate a greater degree of impairment in peripheral mitochondria in patients with HFpEF. As

mentioned above, Weiss *et al.* used[31]P MRS imaging to show that subjects with HFpEF deplete PCr more rapidly than subjects with HFrEF during plantar flexion exercise.[19] Additionally, a study utilizing invasive hemodynamics during cardiopulmonary exercise testing demonstrated that patients with HFpEF have more impaired peripheral oxygen extraction compared to patients with HFrEF.[30]

Methods for the Assessment of Mitochondrial Function

A variety of techniques can be applied to interrogate several aspects of mitochondrial biology, including mitochondrial biogenesis (content and turnover dynamics), ATP synthesis and mitochondrial oxygen consumption/utilization, as schematized in Figure 1 and Table 1.

Assessment of Mitochondrial biogenesis

Mitochondrial biogenesis is the process by which mitochondrial size and number are increased to escalate capacity for ATP production. Stimulating mitochondrial biogenesis in skeletal muscle to improve the bio-energetic capacity during exercise, is thus an important goal for novel therapeutics. During mitochondrial biogenesis, an increase in mitochondrial content as well as the dynamic remodeling of the mitochondrial proteome occurs. Turnover and removal of aged or damaged mitochondria occurs through mitophagy; this process involves induction of general autophagic mechanisms with selective priming of mitochondria for removal.[31]

Mitochondrial content: Mitochondrial volume and content have been assessed using transmission electron microscopy of muscle biopsy samples for decades. Indeed, there is considerable evidence that mitochondrial content assessed in this manner is reduced in heart failure and correlates with exercise capacity. However, this technique is time-consuming, requires expensive equipment, and does not provide information regarding mitochondrial function.

Biomarkers isolated from muscle tissue samples are often used as surrogate measures of mitochondrial content. Activity of citrate synthase, which catalyzes the first reaction of the citric acid cycle, is the most commonly used biomarker for mitochondrial function and is thought to be the most reliable indicator of mitochondrial content.[18] Activity of this enzyme is measurable via a commercially available spectrophotometric assay kit (Sigma-Aldrich, St. Louis MO, USA). Similar assays for activities of electron transport chain complexes I-IV, including cytochrome c oxidase, have also been developed.[32]

In addition to enzyme activity, expression of various mitochondrial structural proteins and transcription factors can provide an estimate of mitochondrial content; commonly used proteins include mitofusin I/II[18], silent information regulator 1 (SIRT1)[33], peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α)[34], nuclear respiratory factor 1/2 (NRF1/2), estrogen-related receptor alpha (ERRα)[35], and succinate dehydrogenase[36]. Protein expression can be measured directly with Western blotting or through measurement of mRNA transcripts with real-time polymerase chain reaction (PCR).[33]

The content of cardiolipin, a phospholipid found only in the mitochondrial inner membrane, appears to be an excellent marker of mitochondrial content.[16] Mitochondrial DNA (mtDNA) content can be quantified as well via polymerase chain reaction (PCR). Levels of mtDNA have been shown to increase in response to exercise training proportionally to citrate synthase and mitochondrial volume density[37]; however, recent evidence suggests that it may not be a reliable indicator of mitochondrial content.[16]

Mitochondrial turnover dynamics: In addition to measuring fixed concentrations of mitochondrial proteins, assessment of dynamic changes in the mitochondrial transcriptome and proteome allow for a more specific and temporally-resolved assessment of mitochondrial biogenesis in response to stimuli. Mitochondrial proteins are continually remodeled with frequent turnover, allowing for quality control of proteins and optimal mitochondrial function.[38] The synthesis and turnover rates of mitochondrial proteins thus reflect the overall quality of the mitochondria as a whole. However, mitochondrial proteins are particularly susceptible to damage by reactive oxygen species (ROS), given that most ROS are generated in the mitochondria during oxidative phosphorylation.[39] As such, it becomes even more important to ensure that turnover continues to occur during pathologic states like heart failure. Indeed, it is thought that accumulation of protein damage from ROS and decreased mitochondrial protein turnover are key aspects of the pathophysiology of heart failure.[40]

It is possible to assess dynamic mitochondrial proteome-wide changes through measuring changes in the rates of protein synthesis or turnover. Novel stable isotope labeling techniques with high-resolution mass spectrometry have allowed for global assessments of mitochondrial proteome dynamics. Heavy water (^2H_2O) labeling has been used to assess turnover rates of mitochondrial proteins in a rat model, finding dramatic variations in half-lives; half-lives ranging from hours to months were calculated for the 458 mitochondrial proteins that were characterized.[41] This same technique applied to a rat model of heart failure found bidirectional changes in half-lives of cardiac mitochondrial proteins, with several proteins involved in fatty acid oxidation, electron transport chain (ETC) function, and ATP synthesis showing increased turnover and other oxidative proteins showing decreased turnover.[42] This approach has been extended to humans *in vivo*, with a study by Wang et al. showing the safety of a ^2H_2O administration protocol for large scale tracking of serum proteome dynamics.[43] The potential of this technique for characterizing patients with HFpEF and their response to novel therapeutics requires further study.

Assessment of ATP synthesis

In addition to evaluating changes in mitochondrial biogenesis, changes in the functional ability of mitochondria to generate ATP can be measured *ex vivo* and *in vivo*.

Ex vivo measurements of mitochondrial ATP synthesis can be performed via bioluminescence in mitochondria isolated from tissue samples.[44] In brief, fresh tissue is sampled through percutaneous needle muscle biopsy and mitochondria are separated rapidly by centrifugation and suspended in a reaction mixture, which contains firefly luciferin-luciferase ATP-monitoring reagent, substrates for oxidation and ADP. ATP molecules

produced by mitochondria react with firefly luciferase and create light signal proportional to the concentration of ATP in solution. The quantity of photons produced is then quantified using a luminometer. Whereas this technique is intuitive, *ex vivo* measurements of mitochondrial ATP synthesis obtained via skeletal muscle biopsy may not reflect their function *in vivo*, which depends on integrated oxygen transport and diffusion mechanisms. In addition, the need for single or serial skeletal muscle biopsies represents a barrier to subject participation in clinical trials.

A number of novel non-invasive imaging methods for measuring oxidative capacity and ATP production have been well described as markers of *in vivo* mitochondrial function. Unlike studies of mitochondria explanted from tissue biopsy, these imaging methods allow for *in vivo*, serial and real-time measurements of oxidative phosphorylation capacity during or immediately after exercise.

Magnetic resonance spectroscopy (MRS) can detect ^{31}P, which is commonly found in high-energy intracellular substrates. This requires MRI phosphorus coils (i.e., coils "tuned" to detect ^{31}P signal), which are different from standard clinical hydrogen coils. Exercise protocols can be performed while subjects are inside the magnet, causing demand for ATP that is met by breakdown of phosphocreatine (PCr). After depletion of PCr and cessation of exercise, oxidative phosphorylation within the muscle leads to ATP production. The recovery rate of PCr after exercise depletion reflects generation of ATP and can be used to calculate the maximum *in vivo* mitochondrial oxidative capacity (ATP_{max}).[45]

Phosphorous spectroscopy has been extensively validated through comparison with other markers of mitochondrial function. ATP_{max} measured with ^{31}P MRS has been correlated with gold-standard *in vitro* measures of mitochondrial respiration from muscle biopsy. Lanza *et al.* first provided this evidence in a study that used high-resolution respirometry of mitochondria isolated from muscle biopsy to show agreement between *in vitro* organelle oxygen consumption and *in vivo* oxidative capacity.[46] Coen *et al.* expanded on these observations by comparing *in vivo* ATP_{max} with respiration from permeabilized muscle fibers.[47] ^{31}P MRS can also be performed during exercise and recovery for dynamic assessment of mitochondrial capacity with high temporal resolution in stressed skeletal muscle (Figure 2A). As detailed above, Weiss *et al.* utilized this technique to show that subjects with HFpEF deplete PCr more rapidly than subjects with HFrEF and healthy controls during plantar flexion exercise; they also showed poor oxidative capacity in these subjects through assessment of PCr recovery rates.[19] The main limitations of ^{31}P MRS are its poor spatial resolution, its low signal-to-noise ratio, and the lack of wide availability of phosphorus coils.

Creatine chemical exchange saturation transfer (CrCEST) is a highly innovative novel MRI technique that addresses various limitations of ^{31}P MRS and allows for assessments of muscle oxidative capacity with high spatial (anatomic) resolution, high signal-to-noise ratio, and the use of hydrogen coils. Figure 2B shows an example of a CrCEST acquisition. CrCEST utilizes the magnetic saturation of creatine hydrogens, which are continuously exchanged (and transferred) to water molecules. This technique can perform fast, spatially-resolved measurements of creatine concentrations in tissue, and thus determine the kinetics

of rate of free creatine recovery after exercise, which is a mirror image of PCr recovery (Figure 2C).[48] Debrosse *et al.* utilized CrCEST in a study of subjects with genetic mitochondrial diseases and found a significantly prolonged rate of free creatine decline, consistent with poor mitochondrial oxidative function.[49]

Assessment of Mitochondrial Oxygen Consumption

Mitochondrial respiration involves a series of cellular processes that convert energy stored in macronutrients into ATP, utilizing oxygen as the final electron acceptor of the electron transport chain. Rates of oxygen consumption thus reflect mitochondrial capacity for ATP synthesis. High-resolution respirometry allows for the assessment of oxygen consumption in intact or permeabilized cells or isolated mitochondria. Unlike static measurements (such as concentrations of enzymes, DNA, RNA and various signaling molecules), respirometry allows for dynamic measurements of mitochondrial function at the level of the organelle. Respiration is measured by a temperature-controlled device, known as an oxygraph, that measures changes in oxygen tension inside a sealed incubation chamber; oxygen consumption is then derived from the change in oxygen concentration.[50] Oxygen consumption can also be measured after the addition of various oxidative substrates. Notably, to ensure mitochondrial structural integrity, respiratory function must be measured on fresh tissue and not on frozen samples. Coen *et al.* reported that *ex vivo* oxygen consumption of permeabilized fibers obtained from vastus lateralis muscle biopsy, as measured by respirometry, significantly correlated with peak VO_2 and maximal ATP production capacity (as measured by ^{31}P MRS) in older adults.[47]

An interesting low-cost non-invasive method for the assessment of skeletal muscle mitochondrial function was developed by Ryan *et al* using near-infrared spectroscopy (NIRS).[51] NIRS is a non-invasive minimal-risk method, which measures changes in optical absorption among the oxy and deoxy fractions of hemoglobin and myoglobin in tissue. These can be used to assess local oxygen consumption. When NIRS is used in tandem with repeated intermittent arterial occlusions via a cuff inflation, oxygen delivery is interrupted and the decline in tissue oxygenation measured by NIRS is considered a function of oxygen consumption. The rate of oxygen consumption can be assessed at various time points after a local exercise transient (such as repeated handgrip contractions with NIRS interrogation of the forearm), and the rate at which the consumption rate recovers to baseline can be quantified (Figure 3). This technique has been reported to be reproducible and was cross-validated with ^{31}P-MRS assessments in a study of healthy individuals after short-duration plantar flexion exercise.[52] However, NIRS is significantly limited by depth and fat attenuation. Figure 3 demonstrates an example of a forearm NIRS intermittent occlusion study in a patient with HFpEF.

Assessment of cardiac mitochondrial function

Many of the above described techniques have been utilized for the assessment of mitochondrial function in the heart. However, these measurements present unique challenges when compared to the equivalent studies in skeletal tissue. Foremost among these is the difficulty of obtaining routine endomyocardial biopsy samples for research purposes. Given

the invasiveness of obtaining heart tissue, most studies have utilized tissue that was obtained for clinical purposes or explanted hearts after transplantation.

Due to these barriers to obtaining cardiac tissue, significant attention has been paid to utilizing non-invasive imaging for assessments of cardiac metabolism. [31]P magnetic resonance imaging has been used in prior studies for assessment of myocardial mitochondrial function; unfortunately, data collected using older 3T MRI coils showed a relative low sensitivity, leading to poor spatial resolution and long acquisition times.[53] More recent studies using 7T coils have shown a higher signal-to-noise ratio and more precise quantification of ^{31}P spectra.[54] CrCEST has been applied for the assessment of myocardial mitochondrial function in an animal model of myocardial infarction, but significant technical development is required before it can be utilized in humans.[55]

Considerations for Early Phase Clinical Trial Design with Mitochondrial Agents in HFpEF

Treatment duration

Mitochondrial biology has important implications for clinical trial design. In particular, the kinetics of maximum effect and return to baseline after a pharmacologic intervention should be carefully considered.

Time Course of the Onset of Drug Effects

The goal of achieving a near-maximal effect prior to the end-point read outs ("on" effects) is paramount in deciding the duration of the active treatment phase in both cross-over and parallel arm early-phase trials. The goal of treatment with mitochondrial agents in HFpEF is to improve exercise capacity by increasing mitochondrial biogenesis and function. Mitochondrial biogenesis involves both an increase in mitochondrial content and remodeling of the mitochondrial proteome. It is well established in the literature that sustained exercise protocols increase mitochondrial content.[56] Recent data indicate that exercise-induced adaptations in the mitochondrial transcriptome and proteome occur within days of stimuli, indicating that mitochondrial biogenesis can occur much earlier than previously believed.

A recent study of the mitochondrial proteome in human skeletal muscle indicated extensive remodeling in response to exercise in as little as 7–14 days, suggesting increased mitochondrial biogenesis and increased oxidative capacity; as an example, citrate synthase activity increased by 35% at 7 days and subsequently plateaued.[57] Citrate synthase activity has been shown to be highly correlated and concordant with mitochondrial content and cristae density.[16] A subsequent analysis of mitochondrial transcriptional regulation in response to short-term exercise demonstrated elevations of both mRNA and protein content of established regulators of mitochondrial biogenesis, including PGC-1α after 1 day and ERRα after three days.[58] Increased levels of citrate synthase, cytochrome c, and cytochrome c oxidase subunit IV expression were found within 3–7 days following start of exercise training. Elevation of mitofusin-2 and transcription factor A mitochondrial factor mRNA expression was seen throughout training, though no change in protein content was measured.

The above data suggests that treatment for as little as one to two weeks could be feasible to see initial effects on mitochondrial function; it is unclear, however, when changes in mitochondrial content/function and particularly, their effects on whole-system aerobic capacity and other relevant endpoints such as quality of life, would reach a sustained steady state. Additionally, the HFpEF patient population may have a more limited capacity to stimulate mitochondrial biogenesis requiring treatment over longer time periods than non-HF patients. Given such uncertainties, it seems prudent to consider treatment durations of at least several (>4–6) weeks if feasible.

Washout of mitochondrial effects

In addition to the half-life of specific drugs, the longevity of newly formed mitochondria (which in turn determine the time-period required for mitochondrial density to return to baseline after discontinuation of therapy), is a key factor to consider, particularly when cross-over designs are being contemplated. Unfortunately, data regarding the mitochondrial half-life in various organisms and tissue types are scarce, and to our knowledge, no data are available in HFpEF patients. It is however clear than even within the same organism, mitochondrial half-lives differ greatly by organ/tissue type, as shown in Table 2. Further research is needed regarding the dynamics of mitochondrial turnover in human HFpEF. Given this uncertainty, results from crossover studies evaluating mitochondrial-targeting agents should be interpreted in the context of the limitations of this study design.

Strategies for modulation of mitochondrial function

Stimulation of mitochondrial biogenesis

Human and animal model data suggest that mitochondrial biogenesis is reduced in heart failure. The exact mechanisms behind this are unclear; however, evidence points to both downregulation of the PGC1-α pathway or defective mitochondrial DNA replication. Accumulating evidence suggests that stimulation of mitochondrial biogenesis is possible through activation of adenosine monophosphate-activated kinase (AMPK) and the nitric oxide/soluble guanylyl cyclase/cyclic guanosine monophosphate pathways.

AMPK has been shown to induce mitochondrial biogenesis through direct phosphorylation of PGC1-α production[63]. It also appears that AMPK coordinates mitochondrial biogenesis through epigenetic regulation of nuclear genes involved in nucleosome remodeling.[64] A number of current therapies indirectly target AMPK and have cardioprotective effects, including metformin, telmisartan, thiazolidinediones and statins.[65–67] Metformin, in particular, appears to reduce progression of HF in animal models and is currently being tested in an upcoming study of functional capacity and mean pulmonary artery pressures in subjects with HFpEF (NCT03629340; Table 3).[68] Direct AMPK activators are in various stages of development, including 5-aminoimidazole-4-carboxamide riboside (AICAR), A-769662, and PT-1.[69] However, development of AMPK activators is complicated by the heterogeneous expression and effects of its various subunits and isoforms. For example, gain-of-function mutations in the γ2 subunit of AMPK appears to induce familial hypertrophic cardiomyopathy.[66]

Nitric oxide, through a cyclic guanosine monophosphate dependent (cGMP) pathway, has also been shown to activate mitochondrial biogenesis.[84] Nitric oxide signaling can be increased through two pathways: direct modulation of soluble guanylyl cyclase (sGC), which synthesizes cGMP, and through targeting of the endogenous nitrate-nitrite-NO pathway. Although pre-clinical and early phase studies were promising, increasing NO through the soluble guanylyl cyclase system with phosphodiesterase-5 inhibitors was not effective in improving clinical or exercise status in patients with HFpEF.[85] Vericiguat, a direct sGC stimulator, appeared to exert positive effects on quality of life in the SOCRATES PRESERVED trial, an effect that is now being tested in a larger trial (VITALITY-HFpEF; NCT03547583).[86] The CAPACITY-HFpEF trial is testing another sGC stimulator, IW-1973 (NCT03254485) (Table 3). However, these trials are not assessing the mechanism of any potential effect of sGC stimulation on clinical outcomes. Of note, these agents could impact symptoms through several non-cardiac effects, including improvements in conduit artery function, which is highly NO-dependent.[87]

Targeting the nitrate-nitrite-NO pathway also appears to be an efficacious means for increasing nitric oxide levels. Despite encouraging results with single-dose administration trials of inhaled nitrite,[88] the recent INDIE-HFpEF phase IIb trial did not demonstrate a benefit of this agent on quality of life or aerobic capacity. The very short half-life of this agent (~35 minutes) may have substantially limited any potential efficacy in this agent, which was administered three times daily. In contrast to inorganic nitrite, orally-administered inorganic nitrate demonstrates a longer half-life and favorable pharmacokinetics for sustained administration.[89] The use of oral inorganic nitrate has shown promise in single and repeated-dose administration in early phase studies, and a larger phase IIb trial (KNO$_3$CK OUT HFpEF) is ongoing.[89, 90] (Table 3). Of note, it is likely that the effect of inorganic nitrate on exercise capacity is mediated by arterial, rather than mitochondrial effects since reductions in arterial wave reflections were the main correlate of increases in peak VO2 with this agent.[75]

Resveratrol, a polyphenol found in red wine, has interestingly been shown to stimulate mitochondrial biogenesis through both AMPK and nitric oxide-dependent mechanisms.[71, 72] Animal model data suggests that resveratrol can reduce cardiac dysfunction and improve mitochondrial biogenesis in hypertension-mediated heart failure.[91] A study of 40 human subjects post-myocardial infarction demonstrated efficacy in improvement of diastolic function.[92] Resveratrol has been studied in HFpEF with a completed study assessing the effect of grape seed extract on endothelial function, however, the results have not yet been published (Table 3).

Reduction of oxidative stress

Elevated and pathologic reactive oxygen species production has been implicated in a number of cardiometabolic disorders, including heart failure.[93] However, trials of antioxidant therapies in HF have resulted in varying levels of success.

MitoQ, a lipophilic quinol that accumulates in the mitochondrial matrix and scavenges ROS, has been extensively studied as a mitochondrial antioxidant. A recently published study showed that in rats with pressure-overload induced heart failure, MitoQ reduced hydrogen

peroxide levels and improved mitochondrial respiration.[94] A human study of patients with chronic hepatitis C showed a significant improvement in hepatic function without severe side effects.[95] However, the efficacy of MitoQ may be limited as its uptake into the mitochondria relies on an intermembrane potential difference, which is reduced in heart failure.[66]

Szeto-Schiller (SS) peptides are small amino acid sequences that are rapidly taken up by mitochondria due to their high affinity for cardiolipin, a phospholipid found in the inner mitochondrial membrane. The SS-31 variant of SS peptides (also called elamipretide or Bendavia), in particular, has shown benefit as a cardioprotective antioxidant with reduced mitochondrial ROS production and reduces maladaptive remodeling.[70] There is also evidence that elamipretide improves skeletal muscle function and exercise capacity.[96] Results from long-term phase II efficacy studies of Elamipretide in humans are pending.

Other antioxidant therapies include free radical scavengers and superoxide dismutase mimetics like mitoTEMPO and EUK8/EUK132.[97, 98] Maintenance of mitochondrial iron homeostasis and reduction of iron content is also thought to result in inhibition of free radical formation and reduced oxidative stress; targeted therapies of targeted mitochondrial iron chelators like deferiprone and idebenone have undergone human testing and led to partial reversal of cardiomyopathy in patients with Freidrich's ataxia.[99]

CONCLUSION

Current evidence supports mitochondrial function as an important factor contributing to the pathophysiology of HFpEF. As a result, there are a number of ongoing or completed early phase clinical trials of agents that seek to improve mitochondrial function in this population (Table 3) by acting on a number of different aspects of mitochondrial function (Figure 4). However, these trials largely utilize endpoints that indirectly assess the improvement of mitochondrial function through downstream effects on exercise capacity. While exercise capacity is a clinically important outcome, it may be vital to better assess how these novel therapeutics impact mitochondrial oxidative function, and whether dysfunction in other organ systems become limiting in the presence of substantially improved mitochondrial function. Direct measures of mitochondrial function have the potential to determine whether the pharmacologic effects of new drugs mimic those seen in animal models, and to enhance our understanding of how (and in whom) to target mitochondrial dysfunction in HFpEF. As described above, there are numerous measures of mitochondrial function that can be utilized in human studies, allowing for assessments of mitochondrial function at a level of detail never before possible.

However, more studies are needed to clarify both the role of abnormal mitochondrial function in HFpEF and the impact of mitochondrial therapies on morbidity and mortality in this patient population. Additionally, future studies will be needed to clarify the extent of mitochondrial pathology among the various phenotypic subgroups of patients with HFpEF. As these studies are being planned and conducted, it will be vital to consider the unique biology of the mitochondria and how we can best stimulate this powerhouse of the cell to improve the lives of patients with HFpEF.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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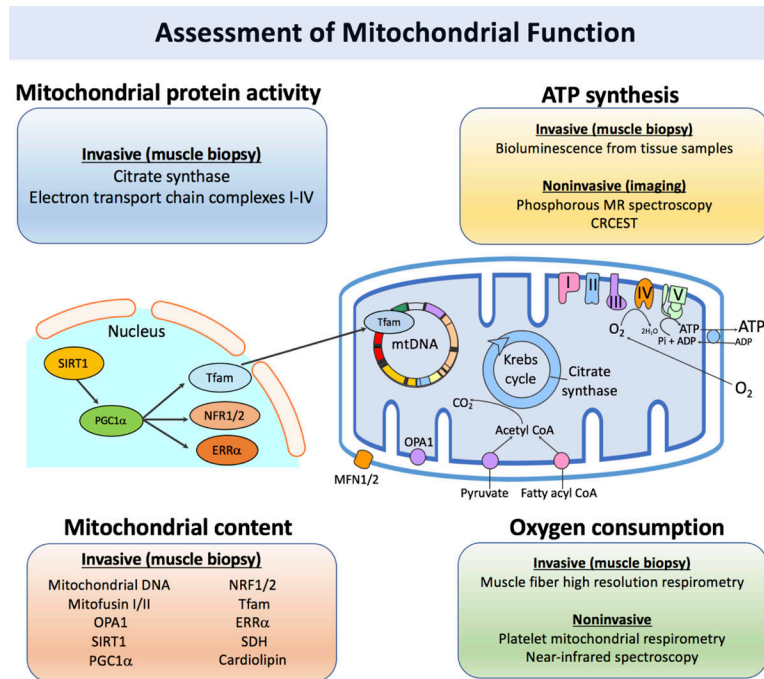
Figure 1. Utilizing multiple aspects of mitochondrial biology for the assessment of functional changes
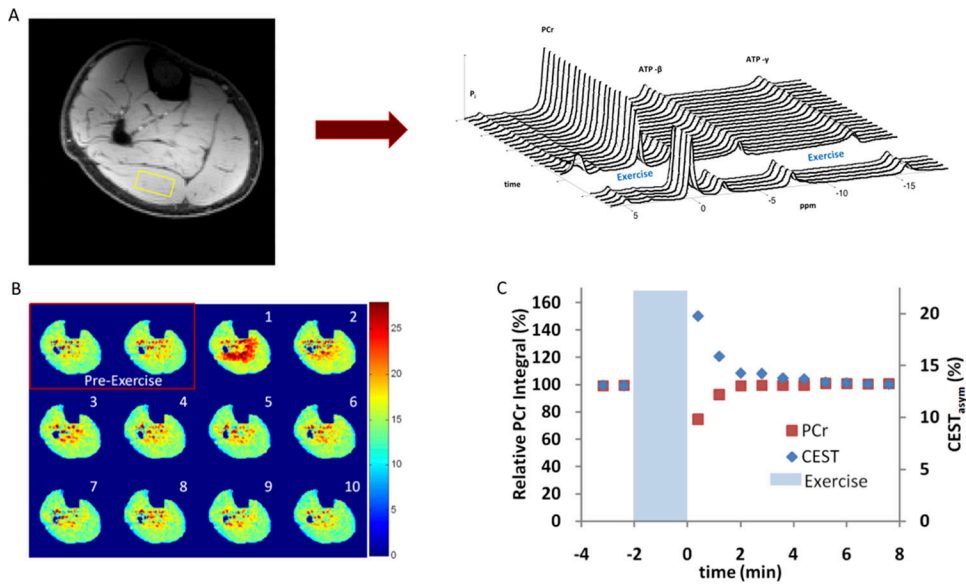
Figure 2. Utilizing phosphorous MRS and CrCEST for the quantification of oxidative capacity in lower limbs during exercise.

Panel A shows a representative image from a phosphorous MR spectroscopy study of the lower limb. The phosphorous spectra are analyzed for each voxel before and after exercise, allowing quantification of phosphocreatine recovery as a marker of oxidative capacity. Panel B shows an example image from a CrCEST study with pre-exercise baseline and post-exercise muscle-group specific increase in free creatine signal and subsequent decay during recovery. Panel C provides a comparison of signal recovery to baseline during the post-exercise period in both phosphorous MRS and CrCEST. Reproduced from Kogan, et al.[100]
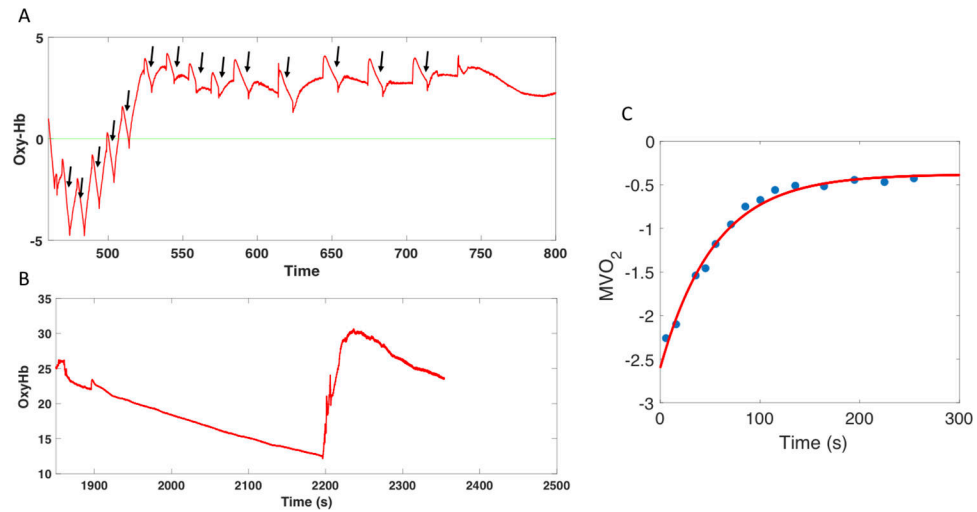
Figure 3. Use of Near Infrared Spectroscopy for the assessment of skeletal muscle oxygen consumption.

Panel A shows measurements of oxygenated hemoglobin during intermittent occlusions post-exercise (to calculate individual slopes, indicated by arrows). This signal is calibrated according to an ischemic occlusion (panel B). A subsequent exponential fit of the slopes (panel C) allows for the measurement of oxygen consumption (MVO_2).

Figure 4. Activity of mitochondria-targeted therapies

Table 1.

Methods for the assessment of mitochondrial function

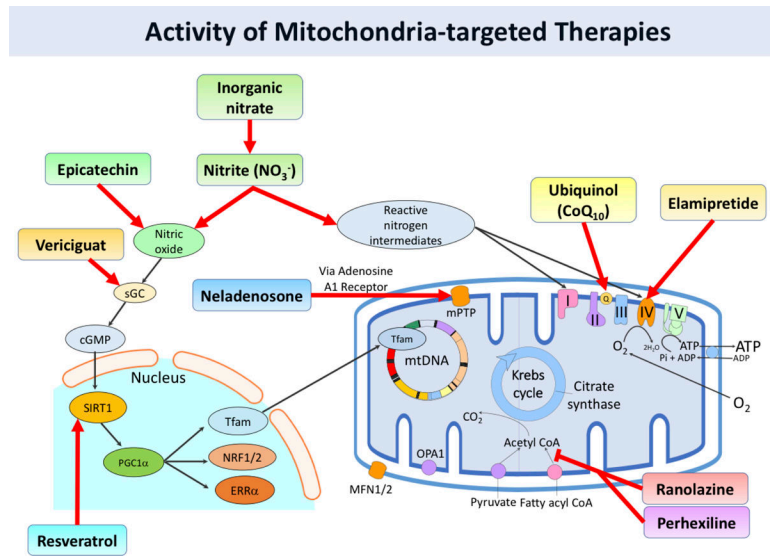| Technique	Mitochondrial site	Invasiveness
Mitochondrial biogenesis		
Transmission electron microscopy	Peripheral, cardiac	Biopsy
Enzyme activity	Peripheral, cardiac	Biopsy
Mitochondrial protein content	Peripheral, cardiac	Biopsy
Mitochondrial DNA content	Peripheral, cardiac	Biopsy
Proteome turnover dynamics	Peripheral, cardiac	Biopsy
ATP synthesis		
Bioluminescence	Peripheral, cardiac	Biopsy
Phosphorous magnetic resonance spectroscopy	Peripheral, cardiac	Non-invasive (magnetic resonance)
Creatine chemical exchange saturation transfer (CrCEST)	Peripheral	Non-invasive (magnetic resonance imaging)
Oxygen consumption		
High resolution respirometry	Peripheral	Biopsy (muscle), or non-invasive (serum leukocytes or platelets)
Near-infrared spectroscopy (NIRS)	Peripheral	Non-invasive

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Table 2.

Overview of estimated mitochondrial protein half-lives

Organism	Cell/tissue type	Mitochondrial protein $t_{1/2}$
Mouse	Cardiomyocyte	15–18 days[41, 59]
Mouse	Hepatocyte	2–4 days[59]
Mouse	Brain	6–10 days[60]
Rat	Cardiomyocyte	~ 30 days[61]
Rat	Skeletal muscle	~ 7 days[62]

Table 3.

Ongoing and completed clinical trials testing the efficacy of agents that may impact mitochondrial function in HFpEF

Drug (Study Acronym)	Status at time of publication	Study Design	Patient population	Study Phase	Mitochondrial mechanism of Action	Relevant Study Endpoints	Results
Elamipretide	Completed (NCT02814097)	Randomized, double-blinded, placebo controlled, parallel-arm	Target enrollment 46	2	Szeto-Schiller peptide that binds to cardiolipin on inner mitochondrial membrane and prevents conversion of cytochrome c into peroxidase, stabilizes electron transport chain, lowers reactive oxygen species levels.[70]	Primary: change in E/e' at rest and exercise measured with echocardiography after 4 weeks of daily subcutaneous study drug/placebo injection	Pending
Resveratrol (contained in grape-seed extract) (GRAPEVINE-HF)	Completed (NCT01185067)	Randomized, double-blinded, placebo controlled, crossover	Target enrollment 15	1	AMPK and SIRT1 mediated activation of nitric oxide synthase and PGC1-α-induced mitochondrial biogenesis.[71,72]	Primary: brachial artery flow-mediated dilation after 6 weeks of oral study drug/placebo Secondary: maximal exercise capacity and oxygen consumption	Pending
Neladenoson bialanate (PANACHE)	Ongoing (NCT03098979)	Randomized, double-blinded, placebo controlled, parallel-arm	Target enrollment 288	2	Adenosine A1 receptor agonist that leads to improved mitochondrial function via reduced opening of mitochondrial permeability transition pore (mPTP).[73]	Primary: absolute change in 6-minute walk distance after 20 weeks of oral study drug/placebo	Pending
Inorganic nitrate-rich beetroot juice	Completed (NCT01919177)	Randomized, double-blinded, placebo controlled, crossover design	17	2	Key non-mitochondrial mechanisms of action (such as vascular effects) have been demonstrated, but mitochondrial effects are also possible (reduced mitochondrial proton leak, stimulation of biogenesis via cGMP; improved efficiency of oxidative phosphorylation).[74]	Primary: change in peak exercise VO$_2$ after single dose of inorganic nitrate Secondary: change in skeletal muscle mitochondrial oxidative capacity measured with near-infrared spectroscopy	Single dose (18 mmol) of nitrate-rich beetroot juice increased peak VO$_2$, increased exercise cardiac output and reduced arterial wave reflections, with a non-significant trend towards improved mitochondrial oxidation.[75] In this trial, the reduction in arterial wave reflections was the main predictor of improvement in peak VO$_2$.
Potassium nitrate (KNO$_3$)	Completed (NCT02256345)	Randomized, double-blinded, placebo-controlled	KNO$_3$: 9, placebo: 3	2	Key non-mitochondrial mechanisms of action (such as vascular effects) have been demonstrated, but mitochondrial effects are also possible (reduced mitochondrial proton leak, stimulation of biogenesis via cGMP; improved efficiency of oxidative phosphorylation).[74]	The main objective of this study was to assess the safety, population-specific pharmacokinetics and dose-response effects with sustained administration of KNO$_3$. Efficacy endpoints included the change in peak VO$_2$ (primary), exercise capacity and quality of life. Mitochondrial function was assessed with NIRS.	KNO$_3$ was safe and demonstrated favorable pharmacokinetics for sustained administration in this population. Non-significant trend towards improvement in peak VO$_2$, with significant improvements in exercise duration, and Kansas City Cardiomyopathy

Drug (Study Acronym)	Status at time of publication	Study Design	Patient population	Study Phase	Mitochondrial mechanism of Action	Relevant Study Endpoints	Results
							Questionnaire total symptom and functional status scores. There was no evidence of improvement in mitochondrial oxidative function.
Potassium nitrate (KNO₃CK OUT HFPEF)	Ongoing, actively recruiting (NCT02840799)	Randomized, double-blinded, placebo controlled, crossover design	Target enrollment 76	2	Key non-mitochondrial mechanisms of action (such as vascular effects) have been demonstrated, but mitochondrial effects are also possible (reduced mitochondrial proton leak, stimulation of biogenesis via cGMP, improved efficiency of oxidative phosphorylation).[74]	Co-primary endpoints: change in total work performed and peak VO₂ during a maximal effort exercise test, after 6 weeks of oral treatment. Secondary: various mechanistic endpoints, including free creatine recovery kinetics measured with CrCEST.	Pending.
Intravenous Sodium Nitrite	Completed (NCT 01932606)	Randomized, double-blinded, placebo controlled, parallel-arm	NaNO₂: 14, placebo: 14	2	Key non-mitochondrial mechanisms of action (such as vascular effects) have been demonstrated, but mitochondrial effects are also possible (reduced mitochondrial proton leak, stimulation of biogenesis via cGMP, improved efficiency of oxidative phosphorylation).[74]	Primary: change in pulmonary capillary wedge pressure (PCWP) during exercise	After single intravenous dose of study drug, significant improvement of exercise PCWP by nitrite vs. placebo.[76]
Inhaled Sodium Nitrite	Completed (NCT02262078)	Randomized, double-blinded, placebo controlled, parallel-arm	NaNO₂: 13, placebo: 13	2	Key non-mitochondrial mechanisms of action (such as vascular effects) have been demonstrated, but mitochondrial effects are also possible (reduced mitochondrial proton leak, stimulation of biogenesis via cGMP, improved efficiency of oxidative phosphorylation).[74]	Primary: change in pulmonary capillary wedge pressure (PCWP) during exercise	After single inhaled dose of study drug, significant improvement of exercise PCWP by nitrite vs. placebo and reduction of resting PCWP.[77]
Inhaled (Nebulized) Sodium Nitrite (INDIE-HFpEF)	Completed (NCT02742129)	Randomized, double-blinded, placebo controlled, crossover design	105	2	Key non-mitochondrial mechanisms of action (such as vascular effects) have been demonstrated, but mitochondrial effects are also possible (reduced mitochondrial proton leak, stimulation of biogenesis via cGMP, improved efficiency	Primary: change in peak VO2 after 4 weeks of study drug/placebo	Three times daily dosing of inhaled sodium nitrate (NaNO₂) did not result in a change in exercise capacity vs. placebo (P=0.27). Final results unpublished; initial results recently presented.[78] The short half-life of nitrite (~35 minutes) from this

Drug (Study Acronym)	Status at time of publication	Study Design	Patient population	Study Phase	Mitochondrial mechanism of Action	Relevant Study Endpoints	Results
					of oxidative phosphorylation.[74]		delivery system may have contributed to neutral results with sustained administration. Oral potassium nitrate (being tested in KNO3CKOUT HFpEF) has a much longer half-life (~6–8 hours).
Ubiquinol (active coenzyme Q10)	Not yet recruiting (NCT03113793)	Randomized, double-blinded, placebo controlled, parallel-arm	Target enrollment 276	2	Mitochondrial inner membrane electron transport chain cofactor.[79]	Primary: change in health status defined by Kansas City Cardiomyopathy Questionnaire after 12 weeks of oral study drug/placebo Secondary: change in mitochondrial adenosine triphosphate (ATP) production measured using serum lactate/ATP ratio	Pending
Ranolazine (RALI-DHF)	Completed (NCT01163734)	Randomized, double-blinded, placebo controlled, parallel-arm	Ranolazine: n=12; placebo: n=8.	2	Partial inhibitor of fatty acid oxidation.[80]	Primary: change in hemodynamic parameters (LVEDP, PCWP, time-constant of relaxation tau) after 30-minute infusion of study drug/placebo	Improved LVEDP, pulmonary capillary wedge pressure. No change in relaxation parameters.[81]
Epicatechin	Completed (NCT02068040)	Non-randomized, open-label, single group	7	1	Nitric oxide-mediated stimulation of mitochondrial biogenesis.[82]	Primary: change in peak exercise VO₂, skeletal muscle metabolism (measured with magnetic resonance spectroscopy) after 3 months of oral study drug	Unpublished
Perhexiline	Completed(NCT00839228)	Randomized, double-blinded, placebo controlled, parallel-arm	70	2	Inhibition of mitochondrial fatty acid oxidation.[83]	Primary: change in peak exercise VO₂ after 3 months of oral study drug	Unpublished
Vericiguat (VITALITY-HFpEF)	Ongoing (NCT03547583)	Randomized, double-blinded, placebo-controlled, parallel-arm	Target enrollment: 735	2	Stimulation of soluble guanylate cyclase.	Primary: change in Kansas City Cardiomyopathy Questionnaire score after 24 weeks of oral study drug. Secondary: change in six-minute walk test.	Pending
IW-1973 (CAPACITY-HFpEF)	Ongoing (NCT03254485)	Randomized, double-blinded, placebo-controlled, parallel-arm	Target enrollment: 184	2	Stimulation of soluble guanylate cyclase.	Primary: incidence of treatment-emergent adverse events, change in peak VO₂ Secondary: change in six-minute walk test, ventilatory efficiency	Pending

SIRT 1 = sirtuin 1, PGC1-α = Peroxisome proliferator-activated receptor gamma coactivator 1-alpha, cGMP = cyclic guanosine monophosphate, VO₂ = oxygen consumption, NIRS = near infrared spectroscopy, CrCEST = creatine chemical exchange saturation transfer, LVEDP = left ventricular end-diastolic pressure

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