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LEADING EDGE TRANSLATIONAL RESEARCH

# Functional Impact of Alternative Metabolic Substrates in Failing Human Cardiomyocytes

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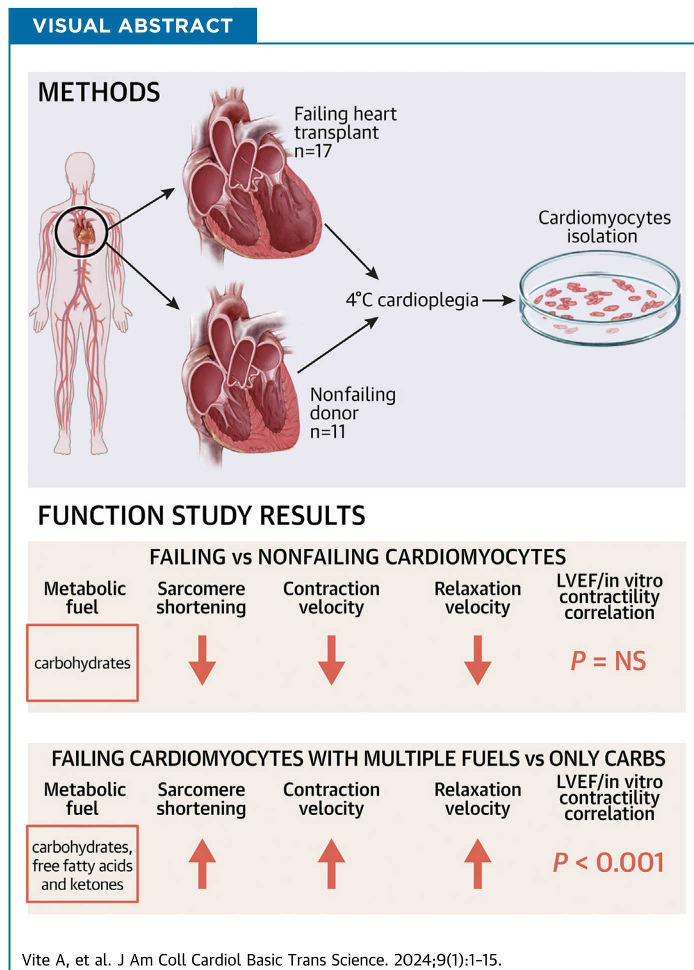
VISUAL ABSTRACT

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## HIGHLIGHTS

- **Alterations in the heart's ability to utilize metabolic fuel substrates may contribute to heart failure.**
- **Failing human cardiomyocytes exhibit metabolic inflexibility, with reduced contractility when glucose is the sole substrate.**
- **Contractility of failing human cardiomyocytes improves significantly when given a physiologic mix of substrates.**
- **Future studies of contractility in diseased hearts should incorporate a physiologic mix of fuel substrates.**

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**ABBREVIATIONS AND ACRONYMS**

**3-OHB** = beta-hydroxybutyrate

**BCAA** = branch chain amino acids

**BDM** = 2,3-butanedione monoxime

**CV** = contraction velocity

**FA** = fatty acid

**FFA** = free fatty acid

**G/L/P** = glucose/lactate/pyruvate

**HF** = failing heart

**KHB** = Krebs-Henseleit buffer solution

**LV** = left ventricular

**LVEF** = left ventricular ejection fraction

**NF** = nonfailing heart

**RV** = relaxation velocity

**SL** = sarcomere length

## SUMMARY

Recent studies suggest that metabolic dysregulation in patients with heart failure might contribute to myocardial contractile dysfunction. To understand the correlation between function and energy metabolism, we studied the impact of different fuel substrates on human nonfailing or failing cardiomyocytes. Consistent with the concept of metabolic flexibility, nonfailing myocytes exhibited excellent contractility in all fuels provided. However, impaired contractility was observed in failing myocytes when carbohydrates alone were used but was improved when additional substrates were added. This study demonstrates the functional significance of fuel utilization shifts in failing human cardiomyocytes. (J Am Coll Cardiol Basic Trans Science 2024;9:1–15) © 2024 The Authors. Published by Elsevier on behalf of the American College of Cardiology Foundation. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

The beating heart has a high rate of energy consumption with a very limited capacity to store adenosine triphosphate (ATP). As a result, the myocardium is in constant need of metabolic substrates to generate the ATP required for normal function.[1-3] One key adaptation to these challenges is the ability of the heart to utilize a wide variety of metabolic fuel substrates for production of ATP, a feature termed metabolic flexibility. The normal myocardium is omnivorous and readily metabolizes fatty acids (FAs), glucose, lactate, ketone bodies, pyruvate, and amino acids.[3,4] Under typical conditions, the majority of ATP generation is derived from FAs and metabolic flexibility permits normal function when there are alterations or restrictions in metabolic fuel availability.[4]

In the failing heart (HF), a variety of derangements in metabolic fuel utilization have been described and recently reviewed.[3] The overwhelming majority of studies examining alterations in metabolic substrate utilization in HFs have employed nonhuman animal models. These models permit characterizations of temporal dynamics and analyses of metabolic flux with the use of labeled substrates, isolated-perfused hearts, and manipulations of specific molecular targets to derive insights into causal mechanisms and the functional significance of disease-associated metabolic adaptations.[5-7] There are far fewer studies directly examining fuel utilization in human HFs, and most of these involve restricted characterizations using in vivo imaging techniques or static molecular and metabolomic characterizations of explanted tissues.[8-12] Despite the essential linkage between energy metabolism and contractility, only rare studies have used acute changes in cardiomyocytes contractility as a readout for evaluating the functional impact of changes in metabolic fuel utilization.[13] To address these gaps, we evaluated the effects of physiologic metabolic fuels on the contractility of cardiomyocytes isolated from human HFs and nonfailing hearts (NFs).

Recognizing that nearly all prior studies examining isolated cardiomyocyte physiology have used carbohydrates as sole metabolic substrates, we compared a mix of glucose, lactate, and pyruvate (G/L/P) with fuel alternatives including free fatty acids (FFAs), beta-hydroxybutyrate (3-OHB), and a physiologic mix of G/L/P, FFAs, and 3-OHB (MIX). Contractility in cardiomyocytes from human HFs was markedly dependent on availability of the alternate substrates in addition to G/L/P alone. Strong correlations between in vivo cardiac performance and ex vivo cardiomyocyte contractility under conditions with the most physiologic combination of fuels affirm the relevance of using cardiomyocyte contractility as a functional readout. Metabolomic and gene expression profiling provided additional insights into the molecular shifts that contribute to impaired metabolic flexibility in human HFs.

## METHODS

**HUMAN HEART PROCUREMENT AND TISSUE SAMPLES.** Procurement of human myocardial tissue was performed under protocols and ethical regulations approved by Institutional Review Boards at the

The authors attest they are in compliance with human studies committees and animal welfare regulations of the authors' institutions and Food and Drug Administration guidelines, including patient consent where appropriate. For more information, visit the Author Center.

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University of Pennsylvania and the Gift-of-Life Donor Program (Pennsylvania) as previously described.[14,15] HFs were obtained at the time of cardiac transplantation. NFs were obtained at the time of organ donation from cadaveric donors. In vivo function, based on left ventricular ejection fraction (LVEF), was assessed via echocardiography in all hearts. At the time of tissue procurement, all hearts were arrested in situ with the use of ice-cold blood-containing high-potassium cardioplegic solution. Explanted hearts were transported to the laboratory on wet ice in cold Krebs-Henseleit buffer solution (KHB). On arrival in the laboratory, the hearts were perfused a second time with 1 L of the cardioplegic solution. Myocytes were isolated from the myocardial sample as described below. For further details on classification and descriptive statistics of each donor heart, see Supplemental Tables 1 and 2.

**LEFT VENTRICULAR CARDIOMYOCYTE ISOLATION.** Myocytes were isolated as described previously.[14,15] Briefly, after the anterior portion of the left ventricular (LV) apex was dissected, a small catheter (18-22 g) was placed into the lumen of the distal left anterior descending coronary artery. Cut vessels on the dissected tissue were suture ligated. The tissue was placed into a Langendorff incubation chamber to maintain tissue temperature at 37 °C. The cannulated LV tissue was then perfused with $Ca^{2+}$-free solution (KHB containing 20 mmol/L 2,3-butanedione monoxime (BDM) and 10 mmol/L taurine) for 5 minutes. Then, 200 mL of KHB containing 294 U/mL collagenase, 20 mmol/L BDM, and 10 mmol/L taurine was perfused for 25 minutes. $Ca^{2+}$ was reintroduced stepwise over the course of 10 minutes into the collagenase solution by adding $CaCl_2$ solution up to 1 mmol/L concentration. Finally, the tissue was perfused for 5 minutes with a rinse solution (KHB containing 10 mmol/L taurine, 20 mmol/L BDM, 1 mmol/L $CaCl_2$, and 1% bovine serum albumin). The tissue was then removed from the cannula, and myocardial tissue was minced in the rinsing solution and triturated with glass pipets. The resulting cell suspension was filtered through 280-μm nylon mesh (Component Supply U-CMN-280), centrifuged (100*g* for 3 minutes), and resuspended in the rinsing solution.

**CARDIOMYOCYTE CONTRACTILITY.** For ex vivo measurements of contractility, viable cells were enriched by gravity sedimentation for 10 minutes, and the resulting loose pellet was transferred to a fresh tube and resuspended in normal Tyrode solution (140 mmol/L NaCl, 0.5 mmol/L $MgCl_2$, 0.33 mmol/L $NaH_2PO_4$, 5 mmol/L HEPES, 1 mmol/L $CaCl_2$, and 5 mmol/L KCl). The cells in Tyrode solution had the following combinations of metabolic substrates added:

1. G/L/P: 5 mmol/L glucose + 100 μmol/L pyruvate + 1 mmol/L lactate.
2. FFA: G/L/P + 200 μmol/L oleic acid (water soluble; Sigma O1257) + 100 μmol/L palmitic acid (BSA-conjugated; Sigma P0500) + 50 μmol/L L-carnitine + 100 μmol/L linoleic acid (water soluble; Sigma L5900).[13]
3. 3-OHB: G/L/P + 3 mmol/L (R)-(−)-3-hydroxybutyric acid sodium salt (Sigma 298360).[16]
4. MIX: G/L/P + FFA + 3-OHB

After addition of the metabolites, the pH was adjusted to 7.4. When specified, isoproterenol (1 μmol/L; Sigma I-2760) was added to the Tyrode solutions. The cells were left in the different Tyrode solutions for at least 15 minutes before contractility measurements.

Cardiomyocytes were subjected to field stimulation at 1 Hz with a myopacer (IonOptix MYP100) and custom-fabricated carbon electrodes lowered into the media bath. Sarcomere length changes were measured after 10-15 seconds of 1 Hz pacing to achieve steady state by a high-speed video image acquisition with a Nikon PU-2000 inverted confocal microscope with a ×40 objective and subsequent Fourier transform analysis (IonWizard, IonOptix). To obtain a representative contractility average, a minimum of 5 steady state contractions for each myocyte were recorded and analyzed for each condition. All experiments were performed in a closed chamber at 37°C. Contractility is measured as the absolute change in sarcomere length (SL) from rest to peak contraction (ΔSL).

**WESTERN BLOTTING.** Frozen LV tissues were pulverized with the use of a Freezer Mill, then proteins were extracted into RIPA buffer supplemented with protease and phosphatase inhibitors cocktail (Roche Diagnostics). Aliquots of supernates were mixed with 4× Laemmli sample buffer (Bio-Rad #1610747), boiled for 5 minutes at 95°C, and resolved on sodium dodecyl sulfate-polyacrylamide gel electrophoresis Tris-glycine gels (Bio-Rad). Proteins were transferred to a membrane on Mini Trans-Blot Cell (Bio-Rad), blocked for 1 hour in 5% milk in phosphate-buffered saline solution containing 0.05% Tween-20 (PBST), and probed with the corresponding primary antibodies (Supplemental Table 3) overnight at 4°C. Membranes were then rinsed with PBST 3 times for 5 minutes, and incubated with secondary antibodies (below) in PBST for 1 hour at room temperature.

Membranes were rinsed again with PBST and then imaged on an Odyssey Imager. Image analysis was performed with the use of FIJI. RPL5 and Ponceau were used as loading control samples.

**CITRATE SYNTHASE ACTIVITY.** Frozen LV tissue from NF and HF patients were homogenized for 10 minutes at 4 °C into the Lysis buffer (20 mmol/L Tris-HCl, 137 mmol/L NaCl, 1% Triton X-100, 2 mmol/L EDTA, and protease inhibitor) in a 1:5 ratio of tissue mass to lysis buffer. The extract was then centrifuged at 10,000*g* for 10 minutes at 4 °C. The supernate was transferred to a clean tube, and protein concentration was measured by means of the bicinchoninic acid method. Protein was diluted to 4 μg/μL with molecular grade water. Citrate synthase quantification was performed according to the manufacturer's protocol (Sigma-Aldrich #CS0720). The absorbance at 412 nm was obtained with the use of a SpectraMax M5 plate reader.

**LV TISSUE METABOLITE MEASUREMENT.** Approximately 100 mg of each frozen heart sample was lyophilized overnight, powdered, and weighed to make homogenates for each targeted liquid chromatography-mass spectrometry metabolomics assay (acylcarnitines, amino acids, organic acids, nucleotides, and malonyl and acetyl CoA). Metabolites were extracted in cold aqueous and organic solvent mixtures according to validated optimized protocols and quantified with the use of an Agilent 1290 Infinity HPLC/6495B triple-quadrupole mass spectrometer as previously described.[10]

**QUANTITATIVE POLYMERASE CHAIN REACTION.** Frozen LV tissues were pulverized with the use of the SPEX cryohomogenizer; 700 μL QIAzol Lysis Reagent (Qiagen) was added to each sample before the homogenization protocol of 2 cycles of 2 minutes at 20 Hz agitation. The RNA was then extracted with the use of the Qiagen mRNeasy kit, following the manufacturer's instructions. Isolated RNA was diluted to 0.1 μg/μL for use in complementary DNA synthesis. Quantitative polymerase chain reaction was performed as described previously[15] with the use of the appropriate primers (Supplemental Table 3).

**STATISTICAL ANALYSIS.** All hearts with successful cardiomyocyte isolation have been included without selection. For Figures 1 and 2, N represents the number of hearts and n the number of individual cells used to calculate the summary statistics that are provided in Supplemental Tables 5 to 7. For Figure 3, a Pearson correlation analysis was used to compare our continuous variables. For Figures 4 to 7, all experiments were replicated in multiple NF and HF hearts in each condition, indicated by N in Supplemental Tables 8 to 10. Statistical analysis and graphing were performed with the use of OriginPro 8.5 (version 2021, OriginLab). Data are presented as the mean ± SD in box-and-whisker plots or mean ± SEM in line graphs, as specified in the figure legends. Unpaired 2-tailed Student's *t*-tests were used to compare 2 groups, and 1-way analysis of variance followed by Tukey's post hoc test was used to correct for multiple comparisons sharing a single control condition (Supplemental Tables 4 to 9). A $P$ value of $<0.05$ was considered to be statistically significant.

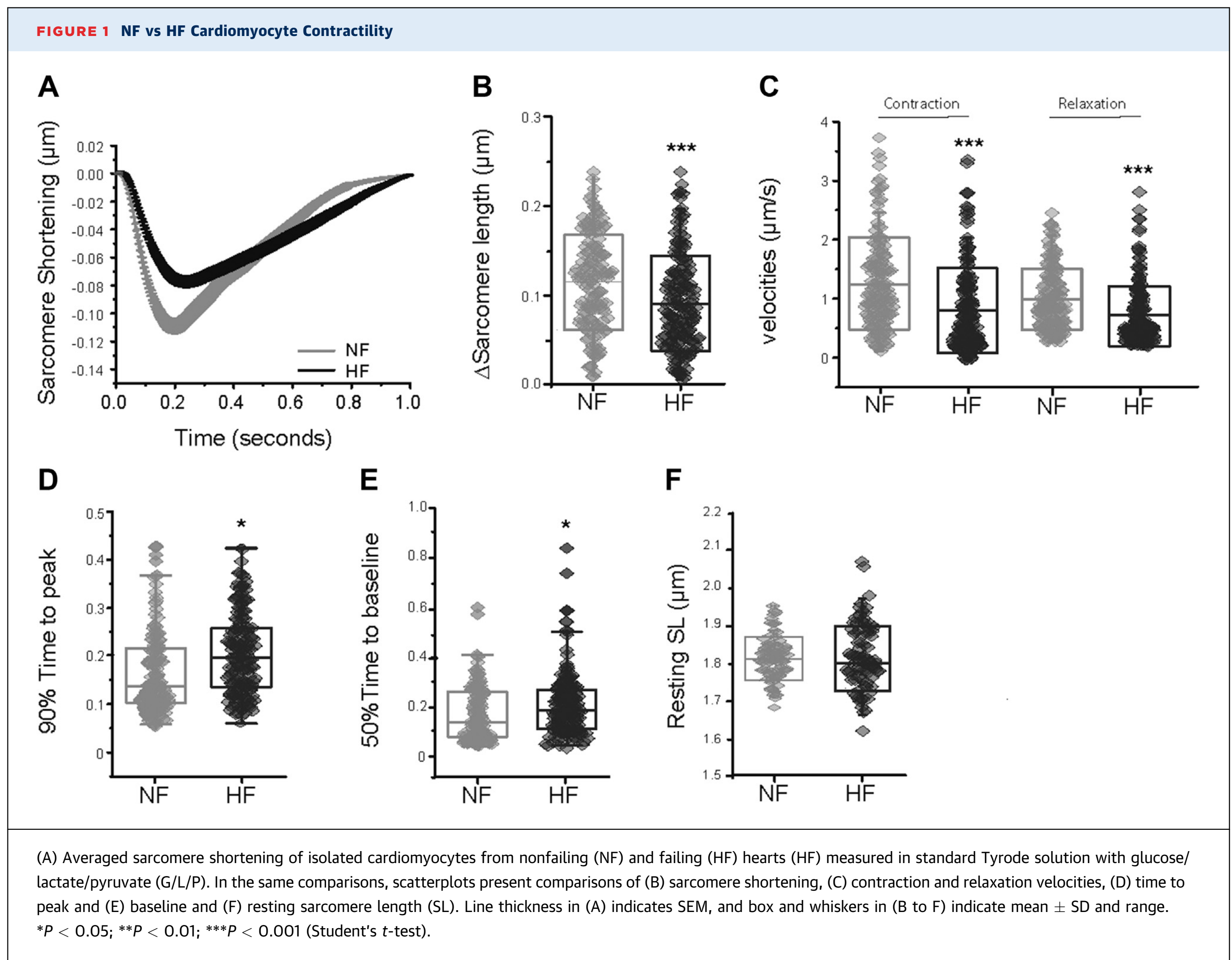
## RESULTS

As shown in Figures 1A and 1B, with G/L/P, sarcomere shortening is substantially greater in adult human NF compared with HF cardiomyocytes ($\Delta SL_{NF}$ 0.119 ± 0.05 μm vs $\Delta SL_{HF}$ 0.096 ± 0.05 μm; $P < 0.001$). In addition, contraction velocity (CV) and relaxing velocity (RV) are significantly higher in NF vs HF cardiomyocytes ($CV_{NF}$ 1.278 ± 0.79 μm/s vs $CV_{HF}$ 0.852 ± 0.67 μm/s; $RV_{NF}$ 1.088 ± 0.061 vs $RV_{HF}$ 0.738 ± 0.053; $P < 0.001$) (Figures 1C to 1F).

In NF cardiomyocytes, a relatively small increase in average sarcomere shortening was observed with the addition of FFAs to G/L/P ($\Delta SL_{G/L/P}$ 0.119 ± 0.004 μm vs $\Delta SL_{FFA}$ 0.144 ± 0.005 μm; $P < 0.001$) (Figures 2A and 2C). There were also modest but significant increases in CV ($CV_{G/L/P}$ 1.278 ± 0.79 μm/s vs $CV_{FFA}$ 1.569 ± 0.80 μm/s; $P < 0.001$) and RV ($RV_{G/L/P}$ 1.088 ± 0.74 μm/s vs $RV_{FFA}$ 1.584 ± 0.97 μm/s; $P < 0.001$) with addition of FFAs alone or as part of the MIX of substrates (Figures 2D and 2E). In addition, resting sarcomere length was greater in the FFA and MIX groups, suggesting more complete cellular relaxation in NF hearts (Figure 2F). However, there was no effect of the addition of 3-OHB on either contractility or velocities in the NF cardiomyocytes.

We next looked at the effect of substrate supplementation in cardiomyocytes isolated from 17 HFs. As noted above and detailed in Supplemental Table 2, no selection of patient pathology, age, or cardiac performance (eg, LVEF) has been made, and every successful isolation was included in this experiment. The addition of 3-OHB or FFAs both substantially enhanced average sarcomere shortening and rates of contraction and relaxation compared with the G/L/P condition (Figures 2B and 2C). As shown in Figure 2 and Supplemental Figure 1, the physiologic combination of substrates, MIX, resulted in similar sarcomere shortening between NF and HF cardiomyocytes ($\Delta SL_{NF}$ 0.134 ± 0.05 μm vs $\Delta SL_{HF}$ 0.129 ± 0.07 μm) while also rescuing CV ($CV_{NF}$ 1.476 ± 0.79 μm/s vs

**FIGURE 1** NF vs HF Cardiomyocyte Contractility

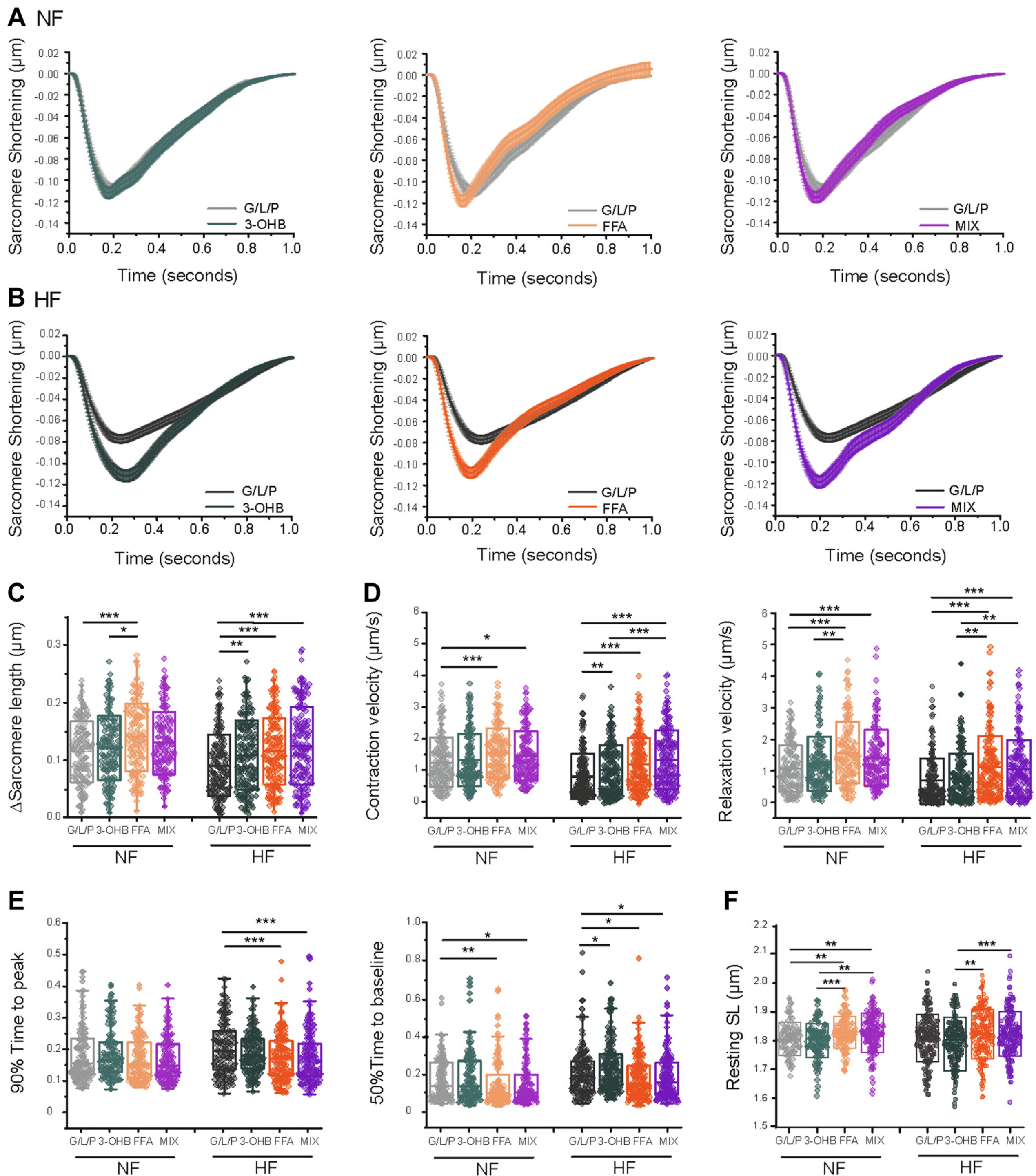



(A) Averaged sarcomere shortening of isolated cardiomyocytes from nonfailing (NF) and failing (HF) hearts (HF) measured in standard Tyrode solution with glucose/lactate/pyruvate (G/L/P). In the same comparisons, scatterplots present comparisons of (B) sarcomere shortening, (C) contraction and relaxation velocities, (D) time to peak and (E) baseline and (F) resting sarcomere length (SL). Line thickness in (A) indicates SEM, and box and whiskers in (B to F) indicate mean ± SD and range. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ (Student's *t*-test).

$CV_{HF}$ 1.359 ± 0.88 μm/s) and significantly improving RV ($RV_{NF}$ 1.419 ± 0.88 μm/s vs $RV_{HF}$ 1.130 ± 0.89 μm/s) (**Figures 2D and 2E**). In a subset of hearts, we assessed the effects of the alternative metabolic fuel substrates in responses to the beta-adrenergic agonist isoproterenol at a saturating dose of 1 μmol/L. In NF cardiomyocytes (Supplemental Figure 2), isoproterenol induced a significant increment in sarcomere shortening in the presence of G/L/P alone but not with the other fuel combinations. In HF cardiomyocytes (Supplemental Figure 3), isoproterenol also induced a significant increment in sarcomere shortening in the presence of G/L/P alone, but no further increments in the extent or rates of shortening beyond the increases observed with the other substrate combinations before isoproterenol.

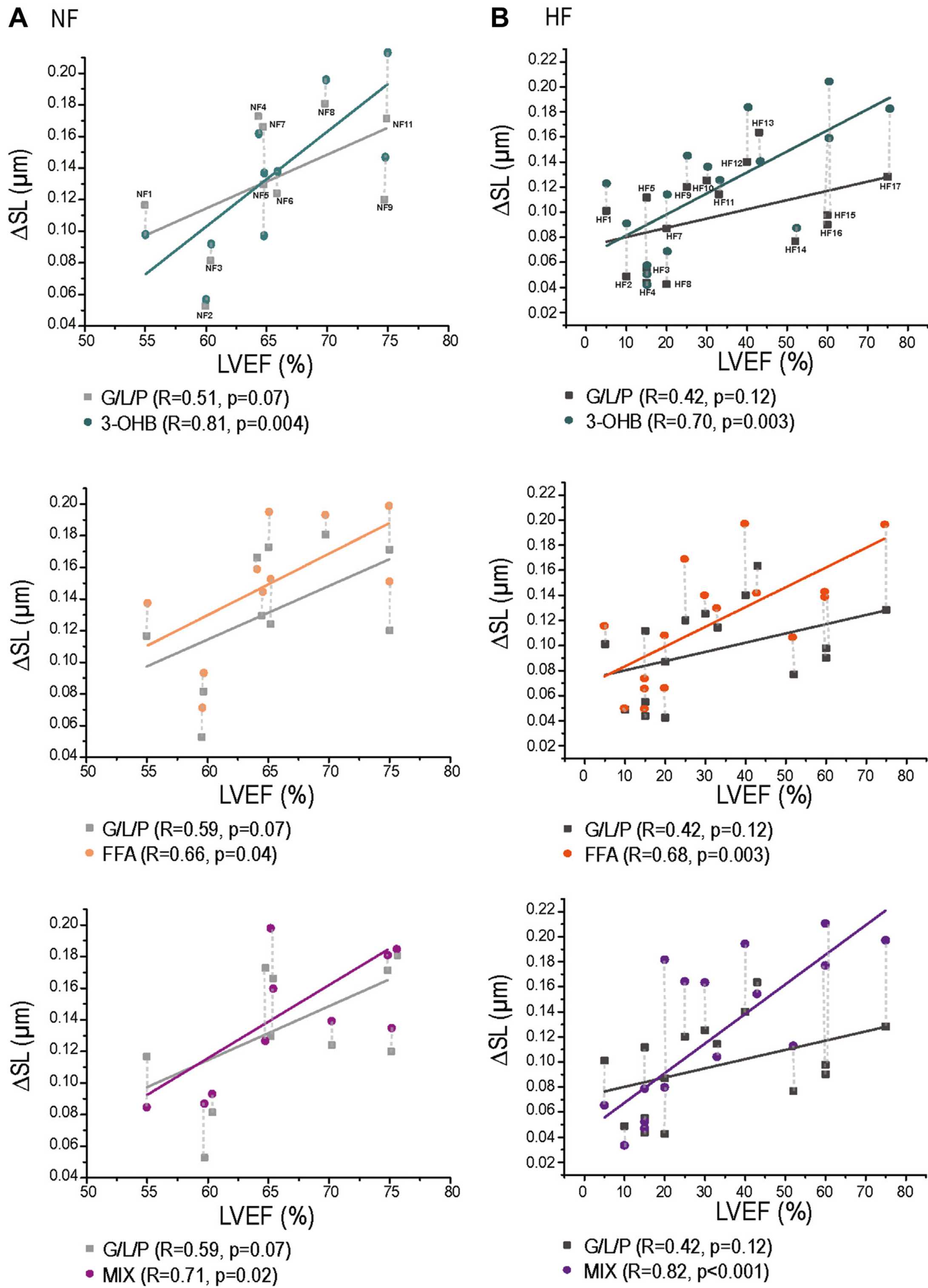
Exploiting the variability in our cohorts, we examined the relationships between the average ex vivo contractile performance for each subjects' cardiomyocytes and their respective in vivo contractility based on the echocardiography-derived LVEF. We found that the LVEF was positively correlated with the average cardiomyocyte sarcomere shortening during substrate supplementation (**Figure 3**). For NF subjects, ex vivo shortening was positively correlated with in vivo LVEF, with minimal impact of alternative fuels (**Figure 3A**). Among HF subjects, the correlation between cardiomyocyte shortening and LVEF was not significant, with a low slope when G/L/P was the only substrate, but there was a strong correlation with a steeper slope when G/L/P was supplemented with other fuels (**Figure 3B**), with a correlation coefficient up to 0.82 with the MIX substrate. Of note, cardiomyocyte contractility from subjects with LVEF <25% generally did not improve with the additional metabolic fuels. We observed no significant correlations between ex vivo shortening and LV mass (Supplemental Figure 4).

**FIGURE 2 Cardiomyocyte Contractility in Different Metabolic Substrates**




Averaged sarcomere shortening of isolated cardiomyocytes from (A) NFs and (B) HFs. Colors represent alternative fuel substrates: glucose/lactate/pyruvate (G/L/P) (gray/black), G/L/P plus beta-hydroxybutyrate (3-OHB) (green), G/L/P plus free fatty acids (FFA) (orange), and all 3 (MIX) (purple). Scatterplots present within-group comparisons for different metabolic substrates for (C) SL, (D) contraction and relaxation velocities, time to (E) peak and baseline and (F) resting SL. Line thickness in (A and B) (indicates SEM, and box and whiskers in (B to F) plots indicate mean ± SD and range. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ (1-way analysis of variance with Tukey post hoc test). Abbreviations as in Figure 1.

**FIGURE 3** **Relationship Between In Vivo LVEF and Ex Vivo Cardiomyocyte Contractility in Different Metabolic Substrates**




Each labeled point represents a different heart in Supplemental Tables 1 and 2. For each heart, average sarcomere shortening in alternative fuel substrates is shown: G/L/P (gray), 3-OHB (green), FFA (orange) and MIX (purple) on isolated cardiomyocytes from (A) NFs and (B) HFs. Dotted lines denote comparisons between responses to 2 different substrates within the same heart. *R* value under each graph represents Pearson's correlation coefficient. LVEF = left ventricular ejection fraction; other abbreviations as in **Figures 1 and 2**.

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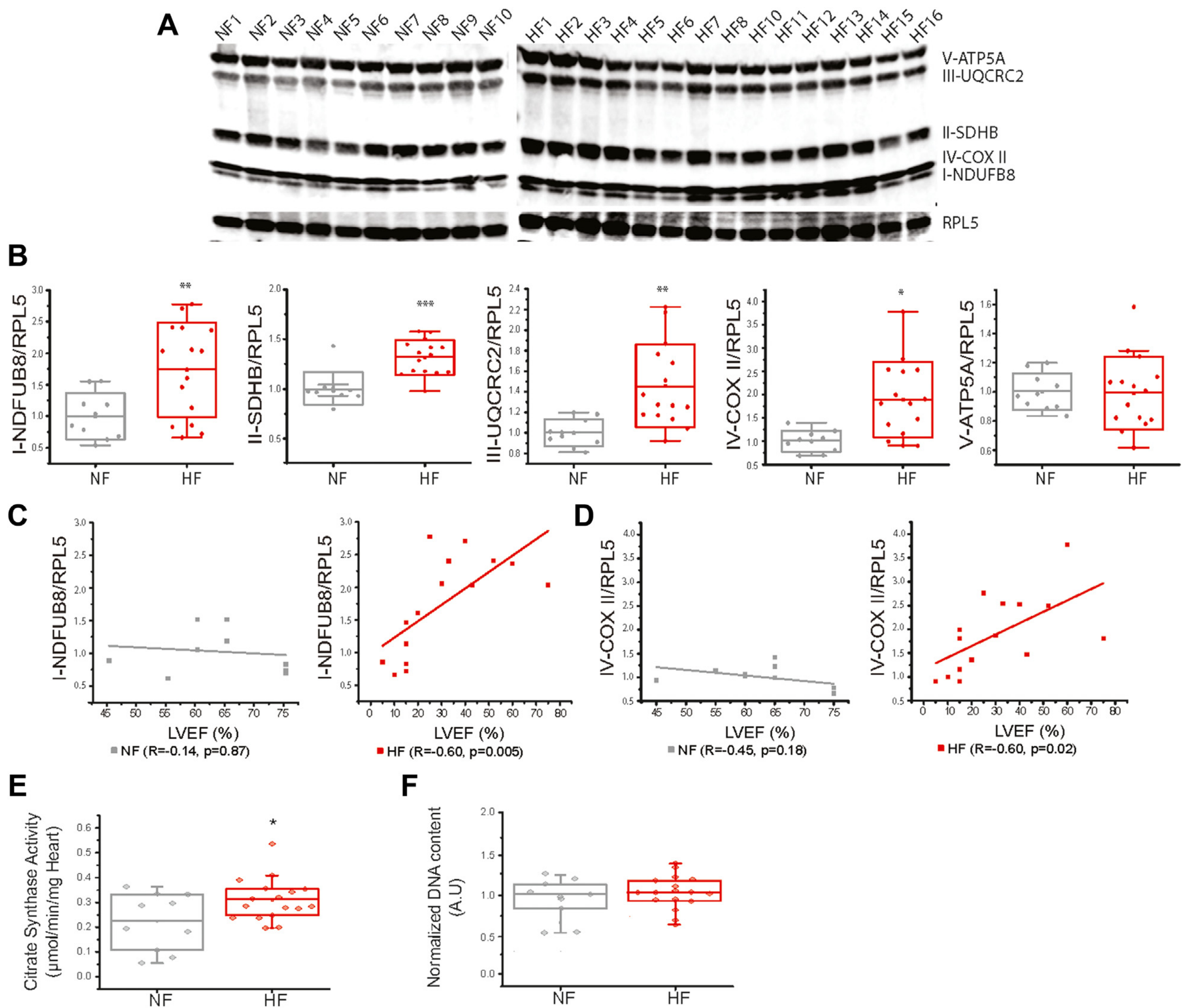
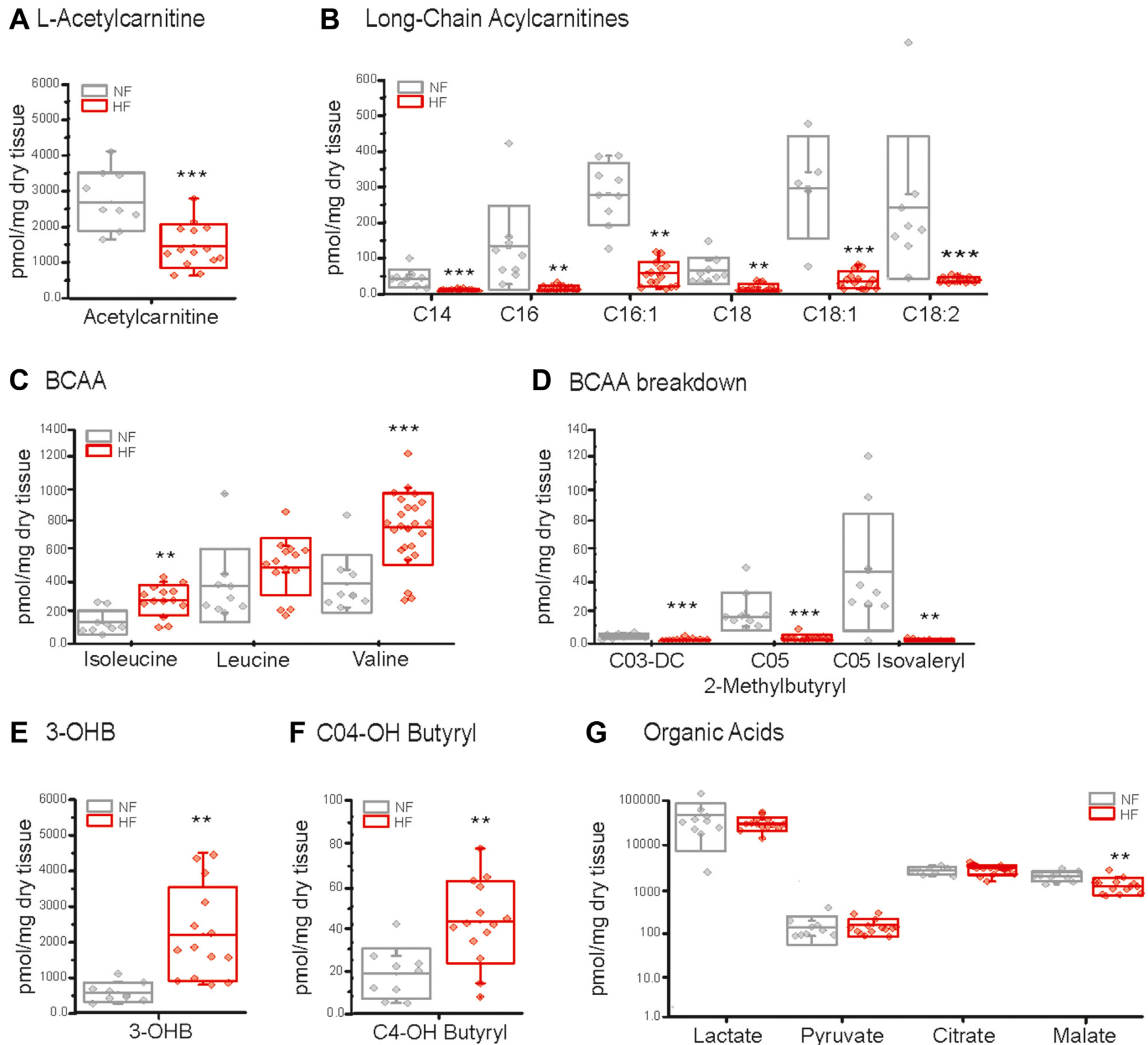
**FIGURE 4** **Assessments of Mitochondrial Characteristics in NF and HF**




(A) Representative immunoblot of OXPHOS electron transport chain (ETC) proteins in left ventricular (LV) tissue extracts and (B) protein expression analysis of each ETC subunit in NFs (gray; n = 10) or HFs (red; n = 15) hearts. (C) For complex I and IV, the correlation analysis with each NF, ie, *R* value, under each graph represents Pearson's correlation coefficient, and (D) HF patient's LVEF has been included. (E) Citrate synthase activity and (F) mitochondrial DNA measured in tissues from NFs (gray; n = 10) and HFs (red; n = 16). Box and whiskers indicate mean ± SD and range. $^{*}P < 0.05$; $^{**}P < 0.01$ (Student's *t*-test). AU = arbitrary units; other abbreviations as in Figures 1 to 3.

To explore potential contributors to the altered metabolic fuel responses in HF cardiomyocytes, we first quantified the abundance of electron transport chain proteins with the use of an OXPHOS antibody cocktail. As shown in Figures 4A and 4B, expression analysis revealed a significant increase of complex I-IV proteins, and no up-regulation of complex V (ATP synthase) in LV tissue lysates derived from HF subjects compared with NF subjects when RPL5 was used as loading control (the same trend was found when whole Ponceau was used as the loading control). Moreover, the abundance of complex I and IV proteins in HF subjects was positively correlated with the LVEF in HF patients with better preserved LVEF, and normal values were observed in patients with LVEF <20% (Figures 4C and 4D). These correlations were not significant for complexes II and III. As shown in Figure 4E, citrate synthase activity (CSA), commonly used as a marker of overall mitochondrial oxidative capacity, was slightly increased in the HF

**FIGURE 5** **Metabolomic Profiling of Selected Pathways and Intermediates**

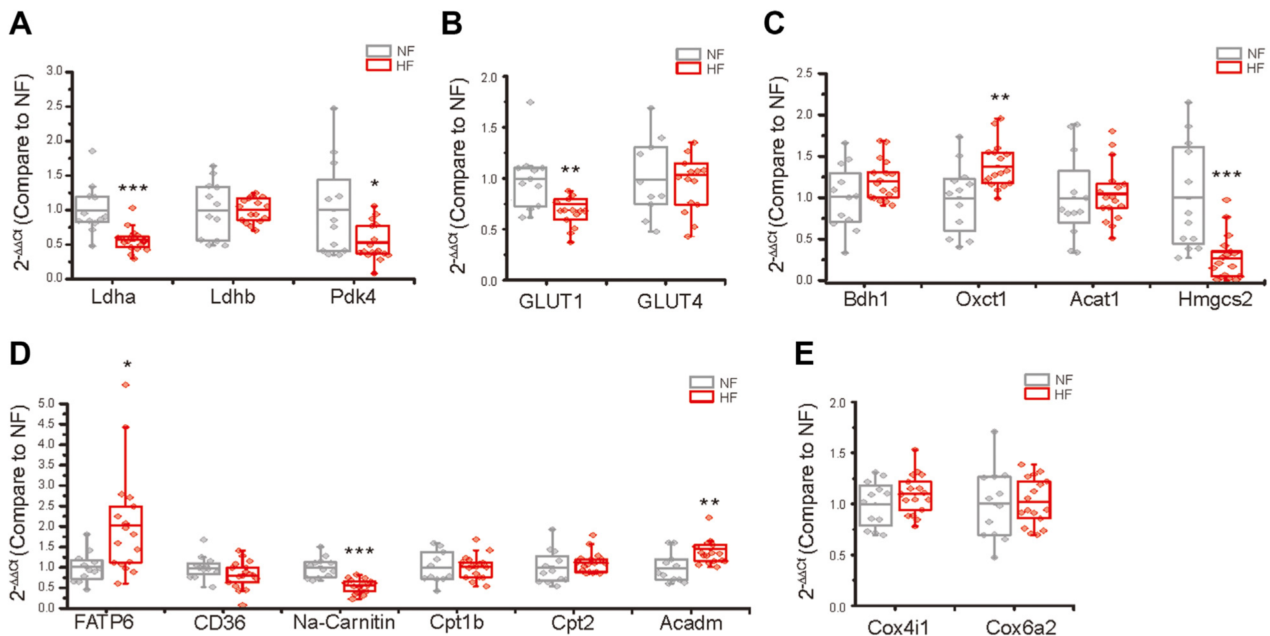



Liquid chromatography–mass spectrometry analysis of LV tissues from NFs (gray; n = 9) and HFs (red; n = 14). The box plots depict the abundance of (A) L-acetylcarnitine, (B) long-chain acylcarnitines, (C) branched chain amino acids (BCAA), (D) BCAA breakdown products, (E) 3-OHB, (F) C4-OH butyryl, and (G) organic acids. Data are represented mean ± SD. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. Abbreviations as in Figures 1, 2, and 4.

group compared with NF ($CSA_{NF}$ 0.223 ± 0.11 μm/min per mg vs $CSA_{HF}$ 0.309 ± 0.31 μm/min per mg; $P = 0.034$). There were no intergroup differences in mitochondrial DNA content (Figure 4F), suggesting preserved bulk mitochondrial TCA activity and content within the failing myocardium. We observed no correlation between CSA and patient's LVEF.

We then sought to investigate metabolic pool sizes in ventricular tissue from the patient cohort. Using a targeted quantitative metabolomics platform, we looked at a total of 75 metabolites (Figure 5, Supplemental Figure 5). As shown in Figures 5A and 5B, we observed a significant reduction in mitochondrial FA oxidation intermediates, including profound reductions in long-chain acylcarnitines, in the HF cohort compared with NF. In contrast, the 3 branched chain amino acids (BCAAs) were all significantly increased in HF hearts (Figure 5C) together with reductions in several BCAA breakdown products (C3-DC, C5 methylbutyryl, and C5 isovaleryl),

**FIGURE 6 Quantitative Polymerase Chain Reaction Analysis Reveals Switch in Metabolic Pathways in HFs**




Results are shown for NF (gray; n = 12) and HF (red; n = 17) tissues for (A) *Ldha*, *Ldhb*, and *Pdk4*; (B) *Glut1* and *Glut4*, (C) *Bdh1*, *Oxct1*, *Acat1*, and *Hmgcs2*, (D) *FATP6*, *CD36*, *Na-carnitine*, *Cpt1b*, *Cpt2*, and *Acadm*, and (E) *Cox4i1* and *Cox6a2*. Box and whiskers indicate mean ± SD and range. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ (Student's *t*-test). Abbreviations as Figure 1.

suggesting disruption of the BCAA degradation pathway (Figure 5D). In addition, the abundance of the ketone body 3-OHB (Figure 5E) and the associated acylcarnitine C4-OH butyryl (Figure 5F) were increased in HF, consistent with the known increase in ketone oxidation in the failing heart.[7,11] Beyond these differences in fuel metabolites, citrate was not different among the groups, and malate was slightly decreased in the HF group compared with NF (Figure 5G). Taken together, these results are consistent with known substrate utilization shifts in HF, including reduced FA and BCAA degradation and an increased reliance on ketone bodies.

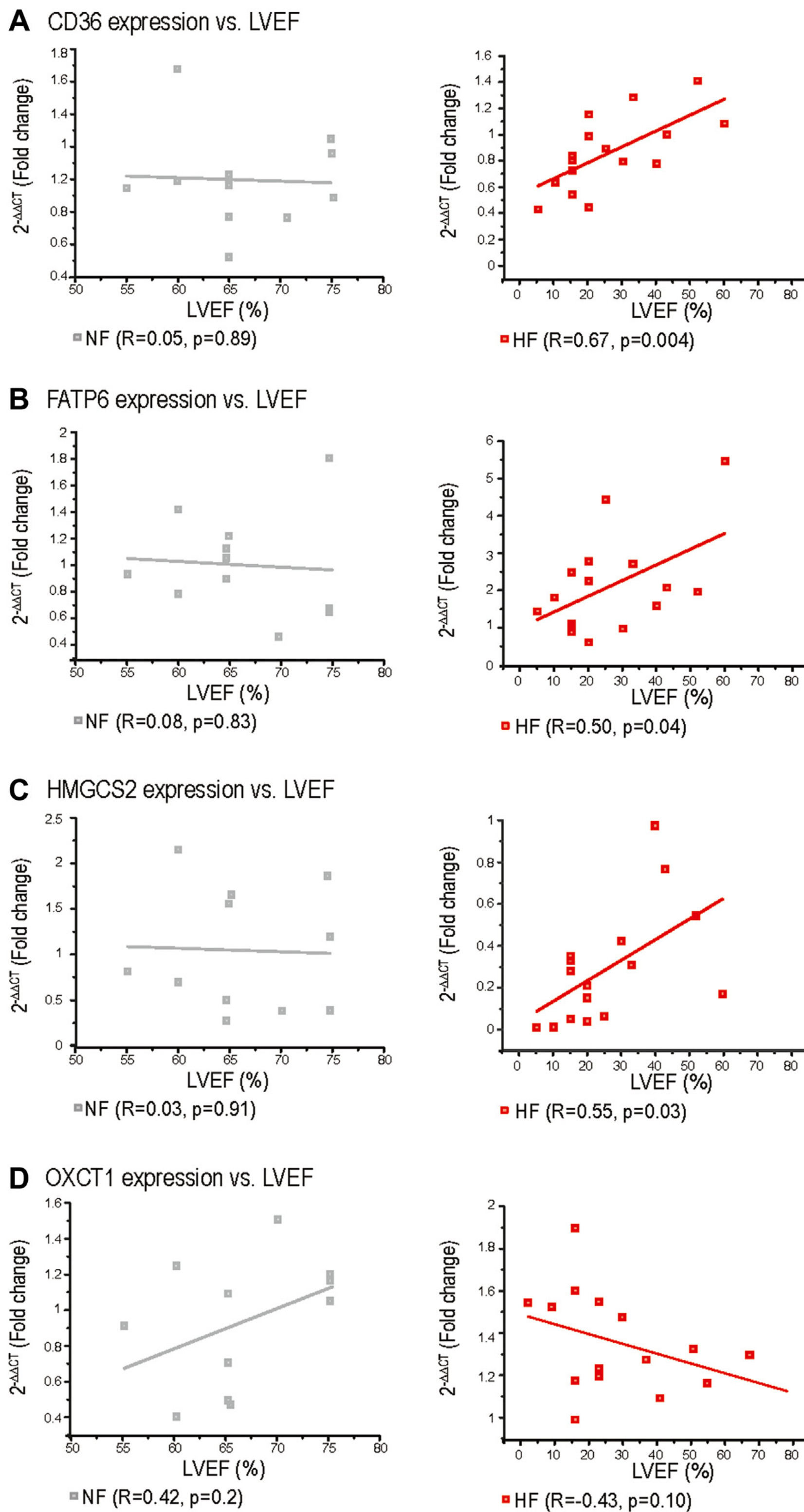
Targeted transcriptional profiling of LV tissue of our NF and HF patients (Figure 6) revealed significant reductions in the glucose transporter *GLUT1* in addition to pyruvate dehydrogenase kinase 4 (*PDK4*) and lactate dehydrogenase (*LDHA*), which contribute to regulation of glycolysis and glucose oxidation. In the HF patients, we also observed a significant increase of the long-chain fatty acid transport protein 6 (*FATP6*), but also 3-oxoacid CoA-transferase 1 (*OXCT1*), a key enzyme of the ketone catabolism, as well as a decrease in *HMGCS2*, a gene that regulates ketone body production. Protein expression for GLUT1, OXCT1, and BDH1 was measured via Western blot, but no significant changes were found (Supplemental Figure 6).

Finally, patient level explorations revealed that 3 genes involved in FA transport or ketone oxidation and synthesis pathways were shown to correlate with LVEF within the HF cohort: *CD36*, *FATP6*, and *HMGCS2*. Patients with cardiomyocytes responsive to ketones and FFAs to improve their cardiac performance had up-regulation of these transcripts, as shown in Figure 7. Interestingly, although *CD36* abundance was equivalent in the overall NF and HF comparison (Figure 7A), its abundance was highly correlated with functional responses to Mix supplementation within the HF cohort (data not shown).

## DISCUSSION

This study demonstrates, for the first time, the functional significance of reprogrammed fuel utilization in isolated failing human cardiomyocytes. We found that HF cardiomyocytes exhibit reduced contractility as well as slower rates of contraction and relaxation compared with NF cardiomyocytes when assessed with carbohydrates (G/L/P) as the sole metabolic fuels. This response was consistent in each HF patient and independent from their etiology or

**FIGURE 7** Relationship Between LVEF and Expression of Selected Metabolic Regulators




Scatterplots show the relationship between NFs (gray) and HFs (red) and the expression of (A) *CD36*, (B) *FATP6*, (C) *HMGCS2*, and (D) *OXCT1*. *R* value under each graph represents Pearson's correlation coefficient. Abbreviations as in Figures 1 and 3.

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in vivo LVEF. However, supplementing the G/L/P with physiologic concentrations of FFAs, 3-OHB, or both significantly improved the contractile performance of the HF cardiomyocytes, reaching levels similar to NF in many cases when all substrates are present. This finding was mainly true in cardiomyocytes isolated from patients with relatively preserved LVEF. Cardiomyocytes from HFs with lower LVEF (<25%), mainly composed of patients with nonischemic cardiomyopathy, exhibited poor contractility with any substrate. These findings indicate that carbohydrates as the sole source of energy are sufficient to meet the needs of unstressed cardiomyocytes isolated from NFs, but they are not sufficient to do so in cardiomyocytes from HFs. The data thus provide strong evidence of metabolic inflexibility in cardiomyocytes from human HFs, ie, an inability to rely solely on carbohydrates.

Despite the dependence on hearts from transplant recipients to provide the human HF cardiomyocytes for these studies, the diversity of etiologies and variability of in vivo function represents a strength of our study design. In particular, the variability of in vivo function allowed us to identify key relationships between cardiomyocyte fuel responses and the severity of cardiac systolic dysfunction. Importantly, when presented with a physiologic mix of substrates reflecting those present in the circulation, there was a remarkably strong correlation between the ex vivo isolated LV cardiomyocyte shortening and the in vivo LVEF ($r = 0.82$; $P < 0.001$). This unprecedented correlation of ex vivo cardiomyocyte function with in vivo function in human hearts affirms the validity of the isolated cardiomyocyte preparation as a means of defining intrinsic myocardial contractile dynamics independently from circulating catecholamines or other neurohumoral modulators of contractility. At the same time, the much weaker correlation observed when isolated cardiomyocytes were presented with G/L/P as the only metabolic fuel ($r = 0.42$; $P = 0.12$) underscores how this pathologic condition can distort and dampen the assessment of intrinsic contractile performance. Such findings support a broader use of mixed fuel substrates in future studies examining isolated cardiomyocyte studies in HFs.

Metabolomic analyses of LV myocardium reveals a variety of metabolic changes, including reductions in FA oxidation intermediates and a "bottleneck" in the BCAA pathway, both of which have been shown to correlate with reduced FA and BCAA oxidation flux in the failing heart.[17] Changes in metabolic fuel utilization in the failing myocardium has received considerable past attention. However, few studies have directly examined the functional impacts of alternative fuels in HFs and none have done so in human cardiomyocytes. One previous study reported that metabolic fuel supplementation of glucose with FFAs increased cardiomyocyte shortening and relaxation rates in rat NF cardiomyocytes.[13] Our findings in human NF cardiomyocytes confirm and extend those findings and provide first evidence in human cardiomyocytes.

Cardiomyocytes from NFs displayed excellent contractile function when provided exclusively with G/L/P or supplemented with fats or ketones. This is consistent with "metabolic flexibility" of the normal heart as highlighted in recent reviews.[18] In contrast, provision of G/L/P alone was consistently insufficient for normal cardiomyocyte shortening and relaxation kinetics in HF cardiomyocytes. Because supplementation with additional fuels is often capable of eliminating the difference between NF and HF cardiomyocyte contractility, these findings strongly suggest defects in glucose utilization and a consequent loss of metabolic flexibility in HF cardiomyocytes.

Defects in carbohydrate utilization in HF cardiomyocytes likely reflect reductions in the capacity for glucose uptake and a reduced ability to oxidize glucose in the failing heart. Our finding of reduced *GLUT1* RNA expression, similarly to previous reports in explanted failing human hearts,[19] is of uncertain significance given that GLUT1 protein expression was not reduced. Reduction of other transporters (such as FATP6) could also play a role in the overall decrease in metabolic fuel uptake. Alternatively, or in addition, decreased oxidation of pyruvate derived from glucose, as has been observed in several nonhuman models of HF,[3] could be contributing to the defects in contractility of HF cardiomyocytes when carbohydrates are the sole available energy substrate, although our previous work suggests that lactate and pyruvate oxidation is increased, rather than decreased, in human HFs.[8,9] From this perspective, the ability of 3-OHB and FFAs to overcome this defect in glucose utilization supports a functionally significant role for impaired glucose utilization in HFs. Future analyses of metabolic substrate flux with isolated cardiomyocytes or tissues would help clarify these issues.

The improvement in contractility with the addition of FFAs in most of the HFs indicates that FA oxidation is essential for normal contractility in human HF cardiomyocytes. It should be noted that despite a reduction in FA oxidation capacity and flux in HFs,[20] this substrate is still important for energy transduction in the context of altered glucose utilization, such as occurs in HFs, as supported by our data.

These findings are consistent with previous studies demonstrating that decreased FA oxidation is associated with decreases in cardiac work in humans with dilated cardiomyopathy.[21] Decreased FA oxidation is often, though not always, associated with progressive heart failure in humans and in animal models.[3] Thus, fat oxidation remains essential for supporting contractile function to compensate for the loss of ability of HF cardiomyocytes to utilize carbohydrates. However, our data suggest that mild or moderate heart failure does not necessarily cause reduced capacity of cardiomyocytes to use FFAs. Improved contractility of HF cardiomyocytes with 3-OHB also reinforces recent studies suggesting that up-regulation of ketone utilization is an adaptive feature of metabolic remodeling in HFs.[22] The increase in the abundance of proteins representing complex I-IV of the electron transport chain in HF tissues from patients with preserved LVEF suggests a potential compensatory response that might support respiratory capacity and contractility in these hearts.

Several lines of evidence support the conclusion that addition of substrates in addition to carbohydrates improves cardiomyocyte contractility in HF cardiomyocytes via enhanced direct bioenergetics rather than effects mediated through independent signaling pathways. First, addition of either ketones or FFAs elicited improved cardiomyocyte contractility with little qualitative or quantitative difference between their effects. Second, responses were quite fast, occurring within 15 minutes of whichever additional substrate was added. Finally, because diastolic $[Ca^{2+}]_i$ uptake is an energy-dependent active transport process, the increases in resting SL observed with addition of supplemental fuel substrates also suggest a direct contribution of additional substrates to ATP generation. Accordingly, these observations provide a foundation for further investigations exploring the effects of alternative substrates on key regulators of cardiomyocyte contractility, including intracellular $[Ca^{2+}]_i$ cycling and other ATP-dependent processes that directly affect contractility.

One observation that emerged from the correlation of ex vivo cardiomyocyte function with in vivo LVEF is that hearts with the most severely depressed in vivo function are relatively unresponsive to the addition of supplemental fuel substrates. In hearts with an in vivo LVEF $\leq$25%, most of which were receiving intravenous inotropes at the time of echocardiography, supplementation of ketones, FFAs, or both had relatively little impact on the contractility of their isolated cardiomyocytes. The basis for this consistent unresponsiveness to alternative fuel sources is not clear but could reflect defects in downstream respiratory capacity or alterations in excitation-contraction coupling. However, neither these severely underperforming hearts nor our overall HF cohort exhibited reductions in electron transport chain proteins, citrate synthase activity. or mitochondrial DNA abundance that would indicate a global reduction in mitochondrial content. Nevertheless, unexplored abnormalities at the level of the mitochondria in these more refractory HFs cannot be excluded. Of the pathways explored in this study, dysregulation of the FA transporter CD36 was most closely correlated with both in vivo cardiac systolic function and ex vivo cardiomyocyte contractility with the MIX substrate. This result is supported by recent studies demonstrating the importance of CD36 and FA uptake to prevent progression of pressure overload-induced heart failure.[23]

**STUDY LIMITATIONS.** Our study has several limitations. The 17 human HFs used for cell isolation were derived from patients with varied disease etiologies and durations and degrees of systolic dysfunction, as highlighted in Supplemental Table 2. For some analyses, this heterogeneity has been exploited, but for others the variability may have obscured significant differences between NFs and HFs or among substrate interventions that might have emerged in a more homogenous cohort. Also, although the relatively low yields of viable rod-shaped responsive cardiomyocytes obtained during cardiomyocyte isolation were sufficient for our functional studies, the yields were not sufficient for assessment of metabolic flux, which could have provided greater mechanistic insight. Finally, as with any study comparing a relatively small number of isolated cardiomyocytes derived from billions of cells in each heart, there are concerns about whether the cells we studied were representative of the population from which they were derived. In this regard, the overall consistency of many of the responses and the surprisingly strong correlation between in vivo and ex vivo contractility suggests that nonrepresentative sampling did not cause major distortions of our results.

## CONCLUSIONS

Our studies of freshly isolated human cardiomyocytes from cardiac transplant recipients and organ donors demonstrate a cell-autonomous impaired metabolic flexibility.[3] Our findings show marked deficits in HF cardiomyocyte contractility when fat and/or ketones were unavailable. Recognizing that the in vivo context includes a varied mix of metabolic substrates, these findings suggest that studies using carbohydrates alone may be exaggerating the basal

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contractile defects of failing cardiomyocytes and other ex vivo myocardial preparations compared with what might be observed with a physiologic mix of metabolic substrates. The results also demonstrate a defect in glucose utilization in HFs, as has been suggested by preclinical studies.[24,25] Our findings also indicate that failing cardiomyocytes isolated from patients with preserved LVEF exhibit improved contractility when FFAs are available. Further improvements observed with the availability of ketones underscore an adaptive feature of HF cardiomyocytes, whereas ketone availability has virtually no effect in NF cardiomyocytes. Collectively, our results suggest that restriction in metabolic supply leads to more severe cardiac dysfunction. These observations provide a foundation for further investigations exploring the effects of alternative substrates on key regulators of cardiomyocyte contractility and therapeutics that might restore metabolic flexibility in failing human hearts.

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## PERSPECTIVES

**COMPETENCY IN MEDICAL KNOWLEDGE:** The strong correlation between the in vivo LVEF and ex vivo contractility of isolated cardiomyocytes, when provided with a physiologic mix of metabolic fuels, affirms the validity of freshly isolated human cardiomyocytes as a clinically relevant model for probing myocardial pathophysiology. Though masked by the complex mix of substrates usually available in vivo, these studies demonstrate a loss of metabolic flexibility in myopathic hearts across a wide spectrum of disease etiologies and severities. In the most severely dysfunctional hearts, with a reduced ability to metabolize free fatty acids and ketones to compensate for impaired carbohydrate utilization, there is a need to better understand and target the underlying molecular mechanisms of defects in substrate utilization.

**TRANSLATIONAL OUTLOOK:** From a methodologic standpoint, our findings suggest that studying isolated failing cardiomyocytes and other ex vivo myocardial preparations with carbohydrates as the sole fuel substrate may be exaggerating their basal contractile defects compared with what might be observed with a physiologic mix of metabolic substrates. From a translational perspective, our results support the clinical relevance of defects in glucose utilization in the failing human heart, as has been suggested by preclinical studies. Future studies using methodologies to determine metabolic substrate flux across the myocardium are needed to clarify the mechanisms of contractile improvements observed with free fatty acid and ketone supplementation. Further investigations are also needed to examine the origins of metabolic inflexibility and identify therapeutics that might restore metabolic flexibility in failing hearts.

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**APPENDIX** For supplemental figures and tables, please see the online version of this paper.