Metabolic Characterization of Hypertrophic Cardiomyopathy in Human Heart

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Abstract

Hypertrophic cardiomyopathy (HCM) is a common inherited cardiovascular disease with heterogeneous clinical presentations, governed by multiple molecular mechanisms. Metabolic perturbations underlie most cardiovascular diseases, however, the metabolic alterations and their function in HCM are unknown. Here, we described the metabolome and lipidome of heart and plasma samples from HCM patients and individuals without HCM. Correlation analyses showed strong association between metabolic alterations and HCM patients' cardiac function and prognosis. Using machine learning we identified metabolite panels as potential HCM diagnostic markers or predictors of survival. Clustering based on metabolome and lipidome of heart enabled stratification of HCM patients into three subgroups with distinct cardiac function and survival. Integration of metabolomics and proteomics data identified metabolic pathways significantly altered in HCM patients, with the pentose phosphate pathway (PPP) and oxidative stress being particularly upregulated. Thus, targeting the PPP and oxidative stress may serve as potential therapeutic strategies for HCM.

Introduction

Hypertrophic cardiomyopathy (HCM), one of the most common inherited cardiovascular diseases, has a prevalence of up to 1:500 in the general population and affects approximately 20 million people globally[1-3]. HCM has emerged as an important cause of sudden cardiac death (particularly in adolescents and athletes), heart failure, and atrial fibrillation[4,5], thus exerting a large burden on healthcare system and economy in modern society[3]. HCM is characterized clinically by left ventricular hypertrophy (left ventricular wall thickness ≥15 mm), nondilated left ventricle and preserved or increased ejection fraction, and pathologically by marked myocyte enlargement and disarray as well as interstitial fibrosis[6].

Over the last three decades, HCM is widely considered as an autosomal dominant disease mainly caused by sarcomere gene mutations and approximately 60% of HCM patients carry pathogenic genes[6,7]. Presently, most of the reported potential pathogenic mechanisms of HCM are associated with gene mutations. For example, sarcomere associated gene mutations impair myofibrillar contractile function and disorder calcium cycling or sensitivity, resulting in compensatory hypertrophy, interstitial fibrosis and diastolic dysfunction[8,9]. However, gene mutations alone are insufficient to interpret overall clinical and pathological features of HCM[10]. Moreover, known pathogenic genes are absent in about 40% of HCM patients, and some pathogenic genes carriers live to advanced ages without developing HCM[11,12]. Therefore, it is particularly important to investigate the pathogenesis of HCM more comprehensively from new perspectives beyond gene mutations, which may promote the development of novel

therapeutic strategies.

Metabolism responds to multiple pathogenic factors, including genetic mutations, gut microbes and environment[13,14]. Study of metabolites in an omics manner (metabolomics and lipidomics) shows great potential in the identification of therapeutic targets, diagnosis of diseases, and patient stratification in a heterogeneous patient population[15-17]. Systemic and myocardial metabolism disturbances are associated with numerous cardiovascular diseases, such as coronary artery disease, heart failure and hypertension[18,19]. However, our understanding on the global metabolic alterations in HCM is still limited.

Herein, by using targeted metabolomics and lipidomics, we identify the metabolic landscape of heart tissue and plasma samples from HCM patients. Correlation analyses reveal that a substantial number of metabolites exhibit significant correlations with cardiac function and survival outcomes. We identify metabolite panels that may serve as diagnostic tools for HCM and predictors of survival. Consensus clustering of metabolomics and lipidomics identifies three distinct metabolic subgroups, which show distinct New York Heart Association (NYHA) class and survival outcomes. By proteomics and metabolomics integrative analysis, we reveal significantly altered metabolic pathways and suggest that intervening with PPP and oxidative stress may serve as potential therapeutic strategies for HCM. Overall, this study provides an valuable resource that expands our understanding of the metabolic alterations in HCM patients and suggests that metabolic changes may facilitate the diagnosis, prognosis, prediction and

91 stratification of HCM patients. In addition, the metabolic interventions may be exploited as
92 potential strategies for HCM treatment.

Results

Cohort characteristics and metabolic landscape of HCM patients

To explore the global metabolic alterations in the heart and plasma samples from HCM patients, we performed targeted metabolomics and lipidomics analyses on the human left ventricular cardiac tissues collected from 349 HCM patients and 16 non-HCM controls, as well as metabolomics analysis on the plasma collected from 143 HCM patients and 60 non-HCM controls (Fig. 1a). Whole exome sequencing (WES) was carried out to detect genetic variants in eight sarcomere pathogenic genes of HCM. Correlation analyses between metabolites and clinical characteristics were applied to investigate the potential regulatory role of metabolism in HCM. In addition, machine learning algorithms were applied to select metabolites that can precisely diagnose HCM and predict the survival outcomes of HCM patients. Consensus clustering was performed to determine if metabolism is a viable framework for patient classification in HCM. Proteomics was also performed to facilitate the understanding of the metabolic alterations in HCM patients from protein level (Fig. 1a). In our cohort, all patients were diagnosed with cardiac hypertrophy by echocardiography and/or cardiovascular magnetic resonance (Extended Data Fig. 1a). WES data showed that 202 (58%) HCM patients carried pathogenic mutations, with myosin heavy chain 7 (*MYH7*) and myosin-binding protein C 3 (*MYBPC3*) the most common pathogenic genes as expected (Fig. 1b)[6,7]. Targeted metabolomics detected 154 metabolites in heart tissues and 142 metabolites in plasma, and detected metabolites were mainly composed of amino acids, organic acids, nucleosides, nucleotides and acylcarnitines (Fig. 1c, d), which are essential for cell growth and energy

metabolism[20]. Lipidomics detected a total of 768 lipids, which belong to 13 distinct lipid classes (Fig. 1e). The high quality of metabolomics and lipidomics data was confirmed by the absence of batch effects and consistency of quality control samples (QCs) (Extended Data Fig. 1b-e). 98% of metabolites in heart tissues and 100% metabolites in plasma detected by targeted metabolomics and 97% of lipids showed a coefficient of variation below 20% across QCs (Extended Data Fig. 1c-e).

Metabolomics perturbations in the heart tissues from HCM patients

To identify the metabolic disturbances of HCM patients and determine whether they share the alterations with the patients of dilated cardiomyopathy (DCM), another inherited cardiomyopathy defined by an enlarged left ventricular chamber[21], we also collected heart tissue samples from 46 DCM patients and performed a targeted metabolomics analysis. Heatmap and principal component analysis (PCA) clearly distinguished HCM from non-HCM controls and DCM patients (Extended Data Fig. 2a, b). A clear segregation between non-HCM controls and DCM patients was also observed (Extended Data Fig. 2b). We found that there were 18 common upregulated (false-discovery rate (FDR)-corrected P value < 0.05, fold change > 1.5) and 13 common downregulated (FDR-corrected P value < 0.05, fold change < 0.67) metabolites between HCM and DCM patients compared with non-HCM controls (Extended Data Fig. 2c). Volcano plot highlighted 60 differential metabolites (36 upregulated and 24 downregulated) between HCM patients and non-HCM controls (Fig. 2a). Strikingly, we noticed that most detected metabolites related to carnitine synthesis were significantly

downregulated both in HCM and DCM patients (Fig. 2b), indicating that fewer fatty acyls entered into the mitochondria for beta-oxidation[22].

To characterize the dysregulated metabolic pathways in HCM patients, we performed pathway enrichment analysis of the differential metabolites and observed that purine and pyrimidine metabolism, cysteine and methionine metabolism, vitamin B6 metabolism and glucose-related metabolic pathways were significantly perturbed in HCM patients (Fig. 2c). Specifically, the level of S-adenosylmethionine (SAM), a donor for DNA and protein methylation[23], was reduced and SAM/S-adenosylhomocysteine (SAH) ratio was also significantly downregulated, which indicates the methylation levels were disturbed in HCM patients (Fig. 2d). Notably, pyridoxal 5'-phosphate (PLP) and pyridoxamine that were involved in vitamin B6 metabolism displayed significant upregulation in HCM patients (Fig. 2e). The upregulation of PLP, serving as a cofactor involved in more than 150 enzymatic reactions, may support the enlargement of cardiomyocytes[24]. Previous findings suggested that the oxidative stress was enhanced in HCM and the ratio of glutathione (GSH) to oxidized glutathione (GSSG) was significantly decreased in HCM animal models[25,26]. Our results showed that the level of GSSG was significantly upregulated both in HCM and DCM patients whereas the ratio of GSH/GSSG was only significantly downregulated in DCM patients but not in HCM (Fig. 2f), indicating HCM and DCM patients experienced various degrees of oxidative stress.

Previous studies have identified that the hypertrophic heart gradually switches the source of

bioenergy metabolism from fatty acid oxidation to glycolysis[27,28]. To comprehensively detect the metabolites in glycolysis and precisely discriminate isomers, such as glucose 6-phosphate (G6P) – fructose 6-phosphate (F6P), we selected 39 HCM patients that matched with 8 non-HCM controls in age and measured the related metabolites using a derivatization method. We observed that metabolites in upper glycolysis, including glucose, G6P and F6P were significantly decreased, whereas metabolites in lower glycolysis, such as 3-phosphoglycerate (3PG) and phosphopyruvate (PEP), were significantly increased in HCM patients (Fig. 2g). We detected most of TCA cycle metabolites and observed that citrate and isocitrate were significantly elevated, while succinate level was greatly reduced (> 5-fold) in HCM patients (Fig. 2g). Interestingly, most of TCA cycle metabolites (e.g. cis-Aconitic acid, isocitrate and succinate) were significantly decreased in DCM patients compared to non-HCM controls (Extended Data Fig. 2d, e). Of note, the level of gluconate, a key metabolite in PPP, was dramatically increased in HCM patients (> 10-fold), suggesting that dysregulated amount of glucose entered into the PPP (Fig. 2g), whereas not significantly changed in DCM patients (Extended Data Fig. 2d). Moreover, glucose may enter into amino sugar and nucleotide sugar metabolism pathway, evidenced by the increased levels of UDP-N-acetylglucosamine (UDP-GlcNAc) and UDP-galactose (UDP-Gal) (Fig. 2g). In addition, numerous purine and pyrimidine nucleotides, such as xanthosine 5-monophosphate (XMP), deoxyadenosine monophosphate (dAMP), uridine monophosphate (UMP) and cytidine monophosphate (CMP), were significantly increased (>1.5 fold) in HCM patients (Fig. 2g). We further observed that aspartate showed >3.5-fold increases in abundance (Fig. 2g), consistent with the previous report that

aspartate synthesis is elevated to support biomass by enhancing glucose consumption during cardiac hypertrophy[29]. Taken together, these results suggest a dramatic and programmed change of metabolites in the heart of HCM patients.

Strong correlations among metabolites may suggest that these metabolites share common functions and lie in related metabolic network[30]. To identify potential co-regulated relationships of the differential metabolites mentioned above, we performed correlation analysis and constructed correlation network for these metabolites. We discovered that 58% of differential metabolites displayed significant correlations with each other (Extended Data Fig. 2f). Of particular interest, we noticed that almost all the differential metabolites showed strong positive correlations (Extended Data Fig. 2g). As expected, metabolites in the same metabolic pathway (e.g. carnitine synthesis) showed a strong correlation (Extended Data Fig. 2g). Succinate, which acts as an electron carrier in the mitochondrial electron transport chain[31], showed a significant positive correlation with multiple carnitines (Extended Data Fig. 2f, g) , suggesting the mutual regulation between fatty acid oxidation and succinate[22]. Collectively, our findings highlight that the metabolic alterations are highly coordinated in HCM patients.

Lipidomics alterations in the heart tissues from HCM patients

Given the essential roles of lipids, such as ceramides and triacylglycerols (TAGs), in underlying the pathogenesis and predicting the risk of cardiovascular diseases[32,33], we further conducted lipidomics analysis to characterize the lipid profile of HCM patients. Partial least squares

198 discrimination analysis (PLS-DA) showed that HCM patients had a distinct lipid profile
199 compared to non-HCM controls (Extended Data Fig. 3a). All the detected lipids were showed
200 in a bubble plot according to lipid classes, with 301 differential lipids (73 upregulated and 228
201 downregulated) between HCM patients and non-HCM controls (Fig. 3a). Of note, the
202 subclasses of sphingolipids including hexosylceramide (HexCer) and sphingomyelin (SM)
203 were significantly upregulated in HCM patients (Fig. 3b), among which 50% of detected
204 individual sphingolipids were significantly increased in HCM patients (Extended Data Fig. 3b).
205 Given that previous study has demonstrated the *de novo* synthesis of sphingolipids as a
206 requirement for pathogenesis of lipotoxic cardiomyopathy and hypertrophy[34], our data suggest
207 that intervening with sphingolipids metabolism might serve as a potential target for HCM
208 treatment. Glycerophospholipids, including lysophosphatidylethanolamine (LPE) and
209 phosphatidylinositol (PI), were significantly reduced and most of detected individual LPE and
210 lysophosphatidylcholine (LPC) were significantly decreased in HCM patients (Fig. 3b, c and
211 Extended Data Fig. 3c, e), indicating the cytosolic phospholipase A_2 (PLA_2), an enzyme that
212 generates fatty acids and lysophospholipids from phospholipids, was dysregulated in HCM
213 patients. Previous study has reported that PLA_2 deficiency induces hypertrophy growth of heart
214 and exaggerates pathologic stress-induced cardiac hypertrophy[35]. The remaining
215 glycerophosholipids, including phosphatidylethanolamine (PE), phosphatidylcholine (PC) and
216 phosphatidylglycerol (PG), showed no difference between HCM patients and non-HCM
217 controls (Fig. 3b and Extended Data Fig. 3e). Nevertheless, individual lipids in PE, PC and PG,
218 such as PE34:3(16:1/18:2), PC36:3(18:0/18:3) and PG36:3(18:0/18:3), were significantly

upregulated in HCM patients (Extended Data Fig. 3c). Among neutral lipids, diacylglycerol (DAG) class was significantly increased with no change of TAG and free fatty acid (FFA) (Fig. 3b), whereas 40% detected individual TAGs were significantly decreased in HCM patients (Fig. 3c and Extended Data Fig. 3d). A closer examination of fatty acid composition of TAGs revealed that the TAGs containing lower double-bond content showed a more obvious downregulation in HCM patients (Fig. 3d). Moreover, the proportion of significantly downregulated TAGs was elevated with the decrease of double-bond content in HCM patients (Fig. 3d), whereas TAGs showed no constant changing trend along with their carbon number (Extended Data Fig. 3f). Taken together, lipidomics data indicate significant alterations of complex lipids between HCM patients and non-HCM controls.

Metabolic disturbances in plasma from HCM patients

To further determine the plasma metabolic perturbations and compare the metabolic alteration between the plasma and cardiac tissues from HCM patients, we profiled the plasma samples from non-HCM controls and HCM patients by using metabolomics analyses. A clear segregation between HCM patients and non-HCM controls was observed (Extended Data Fig. 4a, b). We totally identified 34 differential metabolites (22 upregulated and 12 downregulated) in the circulating plasma between HCM patients and non-HCM controls (Fig. 4a). Comparing the metabolic alteration in the plasma and cardiac tissues from HCM patients, we observed that there were 12 common differential metabolites in the plasma and cardiac tissues (Extended Data Fig. 4c) and most of them exhibited opposite alteration trend in these two

types of samples (Fig. 4b). To compare the differences in dysregulated metabolic pathways between plasma and cardiac tissues of HCM patients, we performed pathway enrichment analysis individually for the differential metabolites in these two types of samples, respectively. We observed that purine and pyrimidine metabolism pathway were significantly perturbated in both plasma and cardiac tissues, whereas glutathione metabolism pathway and related metabolites were only significantly perturbated in plasma (Fig. 4c, d). Specifically, most of the metabolites in purine metabolism pathway displayed opposite alteration trend in the plasma and cardiac tissues of HCM patients (Fig. 4d). Most of metabolites in glucose-related metabolism pathway (i.e. glycolysis, PPP and TCA cycle) significantly altered only in the cardiac tissues of HCM patients (Fig. 4d). Of note, short-chain carnitines (e.g., 6:0-Carnitine, 5:0-Carnitine and 3:0-Carnitine) were significantly decreased both in the plasma and cardiac tissues of HCM patients (Fig. 4d). Conversely, long-chain carnitines (i.e., 18:0-Carnitine and 14:0-Carnitine) were significantly downregulated in the cardiac tissues, whereas significantly upregulated in the plasma (Fig. 4d).

To identify diagnostic biomarkers for distinguishing non-HCM controls and HCM patients, we performed feature selection with 142 metabolites in plasma and used the selected features to build a random forests classifier on the training dataset. We then ran the established classifier in testing dataset to measure the independent performance of our model (Fig. 4e). The random forests model by a biomarker panel of five metabolites (i.e. 8:0-carnitine, hypoxanthine, creatine, phenylalanine and tryptophan) enabled the discrimination between non-HCM and

HCM groups with area under the receiver operating characteristic curve (AUROC) of 0.976 (0.947-0.998) and area under the precision-recall curve (AUPRC) of 0.991 (0.98-0.999) (Fig. 4f, g). Collectively, these findings reveal the differences of metabolic alteration between plasma and cardiac tissues of HCM patients and demonstrate the power of metabolomics for biomarker discovery, which may facilitate the diagnosis of HCM more accessible.

Metabolic association with the genotypes and cardiac function in HCM patients

The myriad of diverse mutations within each of these genes produces remarkable genetic heterogeneity for HCM[6,7], we thus next explored the metabolic profiles of HCM patients with different genotypes. PCA showed that similar metabolic profiles were observed between patients with or without gene mutations and among patients with diverse pathogenic genes (Fig. 5a, b). Notably, there was no differential metabolite (*P* value < 0.05, fold change > 1.25 or < 0.8) between patients with *MYH7* (n=98) and *MYBPC3* (n=67) mutations, the two most common pathogenic genes (Fig. 5c). Thus, these data suggest that myocardial metabolism is not significantly affected by genetic heterogeneity in HCM patients.

The NYHA class is regarded as an indicator of cardiac function for HCM patients, and higher NYHA class indicates worse cardiac function[12,36]. Odd ratios (OR) of NYHA class were estimated by using univariate and multivariate logistic regression analyses in order to determine the correlation between metabolites and NYHA class. Univariate analysis revealed that galactose 1-phosphate and UDP-Gal, the metabolites in galactose metabolism, were

positively and inversely associated with NYHA class, respectively (Fig. 5d). Strikingly, almost all the purine and pyrimidine metabolism related metabolites were significantly related to higher NYHA class, indicating that excess biomass supplementation may lead to worse cardiac function of HCM (Fig. 5d). Interestingly, metabolites in glucose-related metabolic pathways, including glycolysis and PPP, also exhibited significantly positive association with NYHA class (Fig. 5d). In addition, medium- and long- chain carnitines were inversely associated with NYHA class (Fig. 5d). Of note, significant correlations with increased NYHA class were observed for most TAGs (Fig. 5e). Multivariate analysis indicated that galactose 1-phosphate, UDP-Gal, G6P-F6P and GSSG were major factors that associated with NYHA class (Fig. 5f). Taken together, these findings showed significant correlation of the level of metabolites with the cardiac function, suggesting a potential role of metabolic profiling for the evaluation of cardiac function.

Metabolic subtyping of the heart tissues from HCM patients

Given the lack of effective molecular typing in HCM[12,36], we evaluated whether metabolic profiles could be harnessed for molecularly subtyping HCM patients. Consensus clustering based on metabolomics data identified three subgroups with distinct metabolic profiles (Fig. 6a, b and Extended Data Fig. 5a-d). We then examined the survival outcomes among the three subtypes patients, which revealed a clear separation between subtype I (S-I) and S-II or S-III (*P* value = 0.25, Log-rank test) (Fig. 6c). Moreover, patients with NYHA class I and II were more prominent in S-I over other two subtypes (*P* value = 2.8×10^{-8}, two-sided Fisher's exact

303 test), whereas maximum left ventricular wall thickness (MWT) (*P* value = 0.34) showed no
304 significant difference among the three subtypes (Extended Data Fig. 5e, f).
305
306 To further investigate the metabolic differences in each distinct subtype, we performed KEGG
307 pathway enrichment analyses and observed that glutathione metabolism and TCA cycle were
308 only significantly enriched between S-I and S-II, and S-I and S-III (Fig. 6d). Interestingly, the
309 differential metabolites (FDR-corrected *P* value < 0.05, fold change > 1.25 or < 0.8), involved
310 in glutathione metabolism and TCA cycle, displayed consistent upward or downward trend
311 among different subtypes (Fig. 6e). Consistently, patients with higher levels of TCA cycle
312 metabolites (i.e., isocitrate and fumarate) had better survival outcomes, suggesting an
313 energetic compensation may eventually impose beneficial effects on patient outcomes[37,38]
314 (Extended Data Fig. 5g). In addition, purine metabolism was identified as the most significantly
315 changed pathway among the three subtypes and most of the purine and pyrimidine
316 metabolism related metabolites were increased in S-II and S-III compared with S-I (Fig. 6e).
317 Moreover, patients with high levels of purine and pyrimidine related metabolites exhibited poor
318 survival outcomes (Extended Data Fig. 5h). Of note, medium- and long- chain carnitines were
319 significantly downregulated in S-II and S-III. On the contrary, short-chain carnitines were
320 significantly upregulated in S-II and S-III (Fig. 6e).
321
322 Consensus clustering analysis on lipidomics data also identified three subgroups (Fig. 6f and
323 Extended Data Fig. 5i-l). The distribution of patients with different NYHA class (*P* value = 0.99)

had no significant difference among the three subtypes (Extended Data Fig. 5m). However, MWT (*P* value = 0.016) and survival outcomes (*P* value = 0.0084) were significantly differed among the three subtypes (Fig. 6g and Extended Data Fig. 5n), and patients in S-I had the best clinical prognosis, whereas patients in S-II had the poorest prognosis (*P* value = 0.004) (Fig. 6g). Comparing the differential metabolites between S-I and S-II, we found that most of differential metabolites belong to TAGs. Notably, the proportion of TAGs with low carbon number (< 54) were significantly increased in S-II, whereas those with high carbon number (≥ 54) were significantly increased in S-I (Fig. 6h). Consistently, we found that the high proportion of TAGs with low carbon number (< 54) accompanied with poor survival outcomes, whereas the low proportion of TAGs with high carbon number (⩾ 54) accompanied with poor survival outcomes (Extended Data Fig. 5o). TAGs with longer chain may produce more long-chain fatty acids, that enhances energy supply for cardiomyocytes[39], and maintains normal cytosolic Ca^{2+} cycling[40], which are beneficial for improving the pathological and clinical features of HCM[9]. Notably, the level of HexCer d18:1/22:2, a hexosylceramide, was significantly elevated in HCM patients and patients with high level of HexCer d18:1/22:2 presented poor survival outcomes (Extended Data Fig. 3b, 5p). Taken together, our results indicate that HCM are molecularly diversified, which may benefit the potential personalized therapies of HCM patients.

Predicting survival outcomes of HCM using machine learning

To determine whether a panel of metabolites could be identified to predict the survival outcomes for HCM patients, we performed the feature selection with all the 922 detected

metabolites and built a prediction model by using random survival forests on the training dataset. Then, we applied the model to predict the survival outcomes in testing dataset to measure the performance of our model (Fig. 6i). Finally, we selected a panel of twelve metabolites (including Dimethylglycine, N-acetyl-L-glutamine, γ-aminobutyric acid, XMP, 18:0-Carnitine, GMP, UDP-galactose, PC38:6p(16:0/22:6), PE32:0(16:0/16:0), PS34:3(16:1/18:2), PG38:6(18:2/20:4), TAG52:2(C18:0)), which showed remarkable predictive power (C-index = 0.916 (0.814-0.978)) for predicting the survival outcomes of HCM patients (Fig. 6j). Thus, our findings identify a potential metabolite panel as prognostic predictor for HCM.

Metabolomics and proteomics analyses reveal potential therapeutic strategies for HCM

Given that proteomics analysis can provide an orthogonal perspective of metabolism by profiling the metabolic proteins, thereby facilitate the understanding of investigate HCM metabolism, we thus conducted a global proteomics study on HCM patients and non-HCM control samples. In total, 643 proteins were identified as differential proteins between the two groups (FDR-corrected *P* value < 0.05), with 403 upregulated and 240 downregulated in HCM patients from 3737 quantified proteins. Notably, KEGG pathway enrichment analysis of differential proteins revealed that multiple metabolic pathways, such as oxidative phosphorylation, PPP and glycolysis, were significantly altered in HCM patients (Fig. 7a). Strikingly, oxidative phosphorylation was identified as the most significantly changed metabolism pathway with decrease in substantial subunits of mitochondrial respiration chain complexes, suggesting a dramatic downregulation of ATP generation in HCM patients (Fig.

7b).

Combining metabolomics and proteomics datasets, we found that PPP was altered both in metabolite and protein levels (Fig. 2c, 7a). PPP produces precursors for nucleotide biosynthesis by using the glycolysis intermediates[41]. Moreover, our study revealed that purine and pyrimidine metabolism pathways were significantly altered in HCM patients (Fig. 2c) and the related metabolites were highly correlated with NYHA class and survival outcomes (Fig. 5d and Extended Data Fig. 5h). Therefore, it is reasonable to speculate that the upregulation of PPP may result in the increase of purine and pyrimidine metabolism related metabolites and thus support the expansion of biomass, ultimately leading to pathogenesis of cardiac hypertrophy. This prompted us to further explore the roles of PPP and purine and pyrimidine metabolism by integrating the metabolomics and proteomics data. We observed that the rate-limiting enzyme of PPP, glucose-6-phosphate dehydrogenase (G6PD), was increased in HCM patients (> 1.2-fold), although not significantly, in agreement with the decreased G6P and increased 6-phosphpogluconate (6PG) (Fig. 7c). To validate the proteomics data, we performed western blotting and observed the significantly increased protein levels of G6PD in HCM patients (Fig. 7c). In addition, PPP is a major source generating nicotinamide adenine dinucleotide phosphate (NADPH), which plays a crucial role in the maintenance of cellular redox status[41]. Previous studies have demonstrated that oxidative stress is enhanced in HCM[42], and the antioxidant glutathione precursor N-acetylcysteine effectively reversed myocyte hypertrophy, interstitial fibrosis and cardiac function in animal models of HCM[25,26].

Consistent with these reports, our data indicated that hearts in HCM patients may experience higher oxidative stress and the subsequential redox imbalance. It can be proposed that to maintain the redox homeostasis, the expression of G6PD in the hearts with HCM was upregulated to produce more NADPH thereby enhance their capacity for regenerating GSH. In addition, the mitochondrial activities were downregulated, evidenced by the overall downregulation of mitochondrial respiration chain complexes and the dramatic reduction of succinate, to reduce the reactive oxygen species generation as a compensation (Fig. 7d). Together, our metabolomics and proteomics data highlight that intervening with the PPP and oxidative stress might serve as potential therapeutic targets for HCM.

Discussion

HCM is highly heterogeneous and governed by multiple molecular mechanisms[5,8,10]. Disturbances in cardiac metabolism underlie most cardiovascular diseases[43,44]. However, the underlying metabolic alterations and their potential roles in HCM are largely unknown. Here, we presented a comprehensive metabolic characterization of human heart tissues and plasma from HCM patients and non-HCM controls by using targeted metabolomics and lipidomics, which revealed the distinct metabolic profiles between HCM patients and non-HCM controls. Beyond that, we identified a large number of metabolites that highly correlated with the heart function and prognosis of HCM patients. Moreover, consensus clustering of metabolomics and lipidomics data identified three distinct metabolic subgroups of HCM. Importantly, by proteomics and metabolomics integrative analysis, we nominated that intervening the PPP and oxidative stress might be potential therapeutic strategies for HCM.

Fatty acid oxidation (FAO) is the major resource for energy production in the heart[22,45], and previous studies have demonstrated that the hypertrophic and failing hearts reduce the utilization of FAO while increase that of the glucose or other alternative substrates[18,28,46]. Fatty acids are produced by TAG hydrolysis or via the *de novo* synthesis. Medium- and long- chain fatty acids conjugate with carnitine via carnitine palmitoyltransferase 1 (CPT1), and then translocate into mitochondria for beta-oxidation[47]. Indeed, the CPT1 inhibitor-perhexiline significantly improves diastolic function, exercise capacity and cardiac energetics in patients with HCM[48]. Our data revealed that numerous carnitines were dramatically decreased in HCM

418 patients, indicating reduced mitochondrial fatty acid uptake. Moreover, our proteomics analysis
419 identified that mitochondrial respiration chain complexes exhibited overall downregulation in
420 HCM patients. Thus, our data demonstrated that both substrates and “machines” for the
421 energy generation in FAO were dysregulated in HCM patients.
422
423 Enhanced glucose utilization partially improves cardiac energetics, whereas does not mitigate
424 cardiac hypertrophy[49,50]. In addition, recent studies showed that glucose is preferentially
425 utilized for the biosynthesis of macromolecule rather than ATP generation during cardiac
426 hypertrophy[29,51]. Our data suggested that glucose consumption not only supported glycolysis,
427 but also entered into ancillary pathways such as PPP in HCM patients. Previous study has
428 demonstrated that the activity of the PPP rate-limiting enzyme-G6PD is dysregulated in
429 desmin-related cardiomyopathy (DRM) mouse model and downregulation of G6PD abrogates
430 the manifestations of DRM by reversing the redox imbalance[52]. Our data showed that both the
431 protein level of G6PD and the intermediates of PPP, such as gluconate, were increased in
432 HCM patients. The upregulation of PPP may not be sufficient to reverse the imbalanced
433 oxidative stress in HCM patients, evidenced by the increase of GSSG. However, the
434 upregulated PPP may consequentially promote the hypertrophy of cardiomyocytes by
435 increasing purine and pyrimidine nucleotides.
436
437 Raised concentration of TAGs has been regarded as a causal risk factor for cardiovascular
438 diseases[33]. Previous study indicated that TAGs with saturated fatty acids are more likely used

for energy production[53]. In our study, we observed that most of TAGs were decreased in HCM patients and TAGs with lower double-bond content were more significantly decreased, suggesting a deprivation of TAGs applicable for energy production in HCM patients. In addition, we observed that HCM patients showing high proportion of TAGs with low carbon number had poor survival outcomes, whereas patients showing high proportion of TAGs with high carbon number had better survival outcomes. Previous studies showed that TAGs with high carbon number were associated with a decreased risk of type 2 diabetes, whereas TAGs with low carbon number were associated with an increased risk of cardiovascular disease and type 2 diabetes[54,55], which indicated that the level/proportion of TAGs with different carbon number may serve as a good biomarker for the risk of different diseases.

Risk stratification effectively guides disease treatment decisions and surveillance[56]. Current risk stratification of patients with HCM, such as MWT[12,36], do not consider subtle pathophysiologic differences between HCM patients. In contrast, metabolic clustering has been broadly applied to the risk stratification of cancers such as triple-negative breast cancer and clear cell renal cell carcinoma[57,58]. In this study, by using targeted metabolomics and lipidomics data, we performed a metabolic classification of HCM patients and identified three metabolic subtypes of HCM patients that feature distinct clinical characteristics including NYHA class and survival outcomes. Moreover, given that the relation between gene mutations and clinical outcomes has proved unreliable and there is no prognostic utility of a specific mutant gene[4,59], and according to our metabolomics and lipidomics data, myocardial

460 metabolism was not significantly affected by genetic heterogeneity. We suggest that study of
461 metabolites in an omics manner provides a perspective beyond gene mutations for
462 understanding the complex heterogeneous phenotypes of HCM, which may facilitate the risk
463 stratification and thereby shed light on precision treatment of the disease.
464
465 There are several limitations of our study. First, owing to the limited sample availability, only
466 16 non-HCM control samples were collected for metabolic study. Second, the patient cohort
467 used in our study represents only a sub-class of all HCM patients, and it is characterized by
468 the indications of the myectomy for symptomatic left ventricular outflow tract gradient (LVOT)
469 obstruction. Finally, given the high overall 5-year survival rate (95%[60]) of HCM patients after
470 surgery and our relatively short follow-up after surgery (4.5 ± 2.1 years), only 16 patients who
471 died of HCM-related cardiovascular diseases were included in our study. Longer term follow-
472 up may help understand the relationship between metabolism and prognosis more accurately
473 and build a more effective machine learning model for the prediction of survival outcomes.
474
475 In summary, our study delineated the global metabolic alterations in the heart tissues and
476 plasma of HCM, revealed two panel of metabolites that may serve as diagnostic for HCM and
477 predictor for the survival outcomes of HCM patients, respectively, identified three metabolic
478 subtypes of HCM patients and highlighted potential therapeutic targets for HCM. Our work
479 thus provides novel insights into the metabolic heterogeneity and pathogenesis of HCM that
480 may potentially help understand the pathology, facilitate the development of rational

481 therapeutic interventions and enable precision treatment for HCM.

482

Methods

Clinical sample acquisition

Left ventricular myocardium samples (i.e. septum) used for this study were collected from obstructive HCM patients who underwent a Morrow Septal Myectomy at Fuwai Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China. Human DCM left ventricular myocardium samples (i.e. septum) were obtained from patients with dilated cardiomyopathy undergoing cardiac transplantation at Tongji Hospital, Tongji Medical College, Wuhan, China. The HCM diagnosis was based on the following criteria: echocardiographic evidence of maximum left ventricular wall thickness of ≥ 15 mm, excluding patients with significant concomitant disease, HCM phenocopies and secondary hypertrophy (i.e. aortic stenosis, systemic hypertension)[61]. Left ventricular myocardium samples (i.e. septum) of eleven non-hypertrophic cardiomyopathy (non-HCM) were collected from healthy donor hearts that were obtained from explanted normal hearts but not used for transplantation and five were collected from human who donated organs. All of the non-HCM controls had no history of cardiac diseases and therefore were used as control for HCM and DCM. The excised myocardium was immediately flash frozen in liquid nitrogen and stored in liquid nitrogen or -80 °C until used. Plasma samples of HCM were collected before surgery and plasma of non-HCM controls were collected from the physical examination population. Blood was drawn using BD Vacutainer EDTA tubes. The collected blood was centrifuged at 1000 × g for 10 min at 4 °C and the supernatant as plasma. After aliquoting, plasma was frozen at −80 °C until metabolite extraction. This study was approved by the Ethics Committee of Fuwai Hospital, Chinese

Academy of Medical Science and Peking Union Medical College, Beijing, China and Tongji Hospital, Tongji Medical College, Wuhan, China. Written informed consent was obtained from all the participants or their relatives and they agree that the data and information obtained from patients could be used in a publication. The baseline clinical characteristics of patients with HCM were collected at enrollment. Left ventricle wall thickness, left ventricular outflow tract gradient and left ventricular ejection fraction were all assessed by echocardiography, which was performed before myectomy therapy. Meanwhile, all patients were followed-up annually until July 2020 by a clinic visit or telephone interview. The follow-up time extended from the first evaluation until death from any cause or the last known contact date. The end point of the study was cardiovascular deaths, including sudden cardiac death and deaths induced by heart failure and stroke. Resuscitation from cardiac arrest and appropriate implantable cardioverter-defibrillator shock therapy for ventricular tachycardia or fibrillation were considered to be equivalent to sudden cardiac death.

Genotyping

The sarcomere pathogenic genes of HCM, including *MYH7*, *MYBPC3*, *TNNT2*, *TNNI3*, *MYL2*, *MYL3*, *TPM1*, and *ACTC1* were genotyped by WES or panel sequencing as previously reported[62,63]. Briefly, WES was performed on Illumina NovaSeq platform as the manufacturer's protocols using the Agilent Sure SelectXT Human All Exon V6 kit[62]. For panel sequencing, genomic DNA library were constructed and targeted-exonic regions were enriched using a custom-designed probe library, followed by sequencing on Illumina GAIIx[63]. The obtained

reads were aligned to the human reference genome and variants were called using Genome Analysis Toolkit (v.3.7). The SnpEff software (v.4.3) was used to annotate variants to canonical transcripts. The pathogenicity of variants detected in eight sarcomere pathogenic genes were classified according to the criteria of American College of Medical Genetics and Genomics[64].

Metabolite extraction

Samples extraction for targeted metabolomics analysis: each tissue sample was accurately weighed and homogenized in ice-cold 80% methanol aqueous solution (50 mg tissue/mL). 100 μL of tissue homogenate was mixed with ice-cold 900 μL 80% methanol aqueous solution. The mixture was then vortexed and centrifuged at 20,000 × g for 15 min at 4 °C, 900 μL supernatant was transferred to a new tube. The pellet was mixed with 500 μL ice-cold 80% methanol and re-centrifuged at 20,000 × g for 15 min at 4 °C, 500 μL supernatant was collected and combined with the previous supernatant. The mixed supernatant was vortexed and divided into two new tubes with equal volume. The supernatant was dried with SpeedVac (Thermo Scientific) and the dried metabolite extracts were stored at -80 °C until LC-MS analysis. Quality control (QC) samples were prepared by pooling 20 μL homogenate of each tissue sample. Pretreatment of QC samples was paralleled and same to the study samples.

For metabolite extraction of plasma samples, 60 μL plasma of each person was mixed with 240 μL ice cold methanol. The mixture was then vortexed and centrifuged at 20,000 × g for 15 min at 4 °C. The supernatant was divided into 2 replicates and evaporated to dryness with SpeedVac. For QC sample preparation, 20 μL plasma of each person was mixed and then

processed the same as that of the study plasma samples.

Samples extraction for lipidomics analysis was performed as previously described with some modification[65]. Briefly, for polar lipids (FFA, PC, PE et al) extraction, 10 μL tissue homogenate was mixed with 50 μL ice-cold isopropanol (IPA). Samples were vortexed briefly and then incubated for 2 hours at 4 °C. After incubation, samples were centrifuged at 20,000 × g for 10 minutes at 4 °C, 50 μL supernatant was transferred to glass vials for LC-MS analysis. For TAG extraction, 10 μL tissue homogenate was mixed with 40 μL ice-cold methanol. After vortexed briefly and incubated for 10 minutes at 4 °C, samples were centrifuged at 20,000 × g for 10 minutes at 4 °C, 40 μL supernatant was diluted with 40 μL of deionized water and mixed for LC-MS analysis. QC samples were prepared by pooling 20 μL homogenate of each tissue sample. Pretreatment of QC samples was paralleled and same to the study samples.

Targeted metabolomics analysis

The dried metabolites were reconstituted in 50 μL of 0.03% formic acid in water, vortexed, centrifuged at 20,000 × g for 15 min at 4 °C and the supernatant was analyzed using liquid chromatography–tandem mass spectrometry (LC–MS/MS). An Ultra High Performance Liquid Chromatograph (UHPLC) system (Nexera×2 LC-30A, Shimadzu) was used for liquid chromatography, with an ACQUITY UPLC HSS-T3 UPLC column (150 × 2.1 mm, 1.8 μm, Waters) and the following gradient: 0–3 min 1% mobile phase B; 3–15 min 1–99% B; 15–17 min 99% B; 17–17.1 min 99–1% B; 17.1–20 min 1% B. Mobile phase A was 0.03% formic acid

in water and mobile phase B was 0.03% formic acid in acetonitrile (ACN). The flow rate was 0.25 mL/min, the column was at 35 °C and the samples in the auto sampler were at 4 °C. The injection volume was 10 µL. Mass spectrometry was performed with a triple quadrupole mass spectrometer (Qtrap 6500+, Sciex) in multiple reaction monitoring (MRM) mode. Sample analysis was performed under a positive/negative switching mode, a total of 251 metabolites were monitored with 158 ion transitions in positive mode and 93 ion transitions in negative mode as previously described[66], with some modifications. Samples were analyzed in a randomized order, with QC samples evenly inserted in each batch of the acquisition sequence per 10-20 samples to monitor the stability of the large-scale metabolomics analysis. Chromatogram review and peak area integration were performed using MultiQuant software v.3.0 (Sciex).

Lipidomics analysis

Lipidomics were analyzed using the LipidQuan platform (Waters) that comprises a Xevo TQ-XS Mass spectrometer (Waters) and an ACQUITY UPLC I-Class system (Waters). For polar lipids analysis, the UPLC separation was performed on an ACQUITY BEH Amide column (100 × 2.1 mm, 1.7 µm, Waters) at 45 °C. 95% ACN in water with 10 mM ammonium acetate (mobile phase A) and 50% ACN in water with 10 mM ammonium acetate (mobile phase B) were employed as the mobile phase. The gradient of 0-2 min 0.1-20.0% B, 2-5 min 20.0-80.0% B, 5-5.1 min 80.0-0.1% B and 5.1-8 min 0.1% B. The flow rate was set at 0.6 mL/min and the injection volume was 1 µL. Mass spectrometry was performed using a MRM mode with positive

(CE, SM, LPC, LPE, Cer, Hexcer, PC, DAG) or negative (FFA, PE, PG, PS, PI, PC) ESI mode. The ion source temperature and desolvation temperature were set as 150 °C and 650 °C respectively, and the capillary voltage was kept at 2.8 kV for positive mode and 1.9 kV for negative mode. For TAG analysis, samples were separated with a CORTECS T3 column (30 × 2.1 mm, 2.7 μm, Waters). 2 μL sample was injected and the flow rate was 0.25 mL/min. Mobile phase A was 0.01% formic acid containing 0.2 mM ammonium formate and mobile phase B was 50% IPA in ACN containing 0.01% formic acid and 0.2 mM ammonium formate. The gradient was set as: 0-2 min 90% B, 2-6 min 90-98% B, 6-8 min 98% B, 8-8.1 min 98-90% B and 8.1-10 min 90% B. All TAGs were performed in positive mode with MRM mode. The ion source temperature and capillary voltage were kept constant at 150 °C and 2.0 kV respectively and the desolvation temperature was 650 °C. All samples were acquired in random orders with QC samples evenly inserted in each batch of the acquisition sequence per 12-20 samples to monitor the stability of the large-scale lipidomics analysis. Chromatogram review and peak area integration were performed using Skyline software (MacCoss Lab).

Targeted metabolomics and lipidomics data processing

Data processing was performed as previously described[67]. Briefly, the mean peak area of each metabolite from all the QC samples in all given batches (QCall), as well as the mean peak area of each metabolite from the QC samples that are the most adjacent to a given group of test samples (QCadj) were first calculated. The ratio between these two mean peak areas for each metabolite was computed by dividing the same QCall by each QCadj and used as the

normalization factor for each given group of test samples. The peak area of each metabolite from each test sample was normalized by multiplying their corresponding normalization ratio to obtain the normalized peak areas to remove potential batch variations. In addition, to effectively correct the sample-to-sample variation in biomass that may contribute to systematic differences in metabolites abundance detected by LC-MS, we generated the scaled data by comparing the normalized peak area of each metabolite to the sum of the normalized peak area from all the detected metabolites in that given sample (excluding TAGs).

Detection of metabolites in glycolysis pathway by 3-NPZ derivatization

Measurement of key metabolites in glycolysis was performed as previously described[68,69]. Briefly, the dried metabolite extracts were derivatization with 3-nitrophenylhydrazine (3-NPZ) and injected for chromatographic separation on an ACQUITY UPLC HSS-T3 UPLC column (150 × 2.1 mm, 1.8 μm, Waters) followed by detection and measurement using a Qtrap 6500^{+} mass spectrometer equipped with an ESI source.

Proteomics analysis

Seven male non-HCM control samples were separated into 4 control groups, including one group with one sample and three groups with 2-pooled samples per group. Sixty-three male HCM samples were used to pool into 6 HCM groups with 10-12 samples per group. Tissue samples were ground to a powder in liquid nitrogen and then lysed in 8 M urea containing 1% protease inhibitor cocktail, and then sonicated using a high-intensity ultrasonic processor

(Scientz, Ningbo, China) three times and centrifuged at 12,000 × g at 4 °C for 10 min. The supernatant was collected and protein content was quantified using a BCA Protein Assay Kit (Beyotime). The proteins were then reduced and alkylated by mixing sequentially with 5 mM dithiothreitol at 56 °C for 30 min and with 11 mM iodoacetamide at room temperature for 15 min in the dark. The protein sample was diluted by addition of 100 mM tetraethylammonium bromide (TEAB) to give a final urea concentration of < 2 M. Trypsin was added at a 1:50 trypsin-to-protein mass ratio overnight followed by a second digestion for 4 h at a 1:100 ratio. Peptides were desalted using a Strata X C18 SPE column from Phenomenex® (Torrance, CA, USA) and then vacuum-dried. The peptides were reconstituted in 0.5 M TEAB and processed using a TMT 10 plex kit (ThermoFisher-90406LCS). The peptides were then fractionated using high pH RP-HPLC on an Agilent 300Extend C18 column (5 μm particle size, 4.6 mm id, 250 mm length), combined into 18 fractions, and dried by vacuum centrifugation. Peptides from each fraction were dissolved in 0.1% formic acid (solvent A) and loaded onto a home-made RP-HPLC analytical column (75 μm id, 150 mm length). The peptides were separated using an EASY-nLC 1000 UPLC system with the following gradient: 6% to 23% solvent B (0.1% formic acid in 98% acetonitrile) over 26 min, 23% to 35% over 8 min, and 35% to 80% over 3 min, followed by a hold at 80% for 3 min. The flow rate was constant at 400 nL/min. The peptides were analyzed using an Q ExactiveTM Plus MS/MS system (Thermo Scientific-Pierce Protein Biology Products) coupled online to the UPLC. The peptides were selected for MS/MS using a normalized collision energy setting of 28 and the fragments were detected in the Orbitrap at a resolution of 17,500 with a fixed first mass set at 100 m/z. The TMT reporter ions

were measured in high-resolution MS2 mode and the interference was controlled by FDR and score cutoff. The cutoffs for modified peptides, score for recalibration and the FDR of peptide level were 40, 70, and 0.01, respectively. The raw MS/MS data were analyzed using the Maxquant search engine (v.1.5.2.8). Tandem mass spectra were searched against the SwissProt Human database (20,317 sequences) concatenated with a reverse decoy database. Trypsin/P was specified as the cleavage enzyme, allowing up to two missing cleavages. In the first and main searches, the mass tolerance of precursor ions was set at 5 ppm and 20 ppm, respectively, and the mass tolerance for fragment ions was set at 0.02 Da.

Identification of differential metabolites and proteins in HCM patients

Statistical differences of the metabolites were analyzed by R stat (v3.6.3) package using Mann-Whitney U test (two-sided) and followed by Benjamini-Hochberg (BH) multiple comparisons test (FDR). Statistical differences of proteins were analyzed by Excel (version 2016) using Student's t-test (two-sided) and followed by BH multiple comparisons test. Metabolites with FDR-corrected *P* value < 0.05, fold change > 1.5 or < 0.67, were considered differential metabolites and used for subsequent analysis. Proteins with FDR-corrected *P* value < 0.05 were considered differential protein and used for subsequent analysis.

KEGG pathway analysis

KEGG metabolic pathways and involved metabolites were downloaded from KEGG API (https://www.kegg.jp/kegg/rest/keggapi.html). Significant enriched KEGG pathways were

672 determined by R clusterProfiler (v3.12.0) package[70] with Fisher's exact test followed by BH
673 multiple comparison test as FDR-corrected *P* value < 0.05 and enriched for at least 2
674 metabolites (for differential metabolites). Significant enriched KEGG pathways for proteins
675 were performed in Database for Annotation, Visualization and Integrated Discovery (v6.8)
676 (https://david.ncifcrf.gov) with Fisher's exact test followed by BH multiple comparison test as
677 FDR-corrected *P* value < 0.05 and enriched for at least 5 proteins (for differential proteins).
678
679 **PCA and PLS-DA for metabolomics and lipidomics data**
680 PCA and PLS-DA were performed by the prcomp and plsda function under R stat (v3.6.3) and
681 mixOmics (v6.8.5) package using the log2 transformed abundance matrixes of global
682 metabolites, respectively. To evaluate the robustness of the results, we evaluated the PLS-DA
683 model by the perf function under R mixOmics packages[71]. 95% confidence coverage was
684 represented by ellipses, which was calculated based on the mean and covariance of points in
685 each group.
686
687 **Correlation analysis of differential metabolites**
688 Correlation between differential metabolites abundance for each metabolite-metabolite pair
689 across all 349 HCM patients were calculated by spearman correlation method using the cor
690 function under R stat (v3.6.3) package. In addition, *P* value corresponding to the correlation
691 coefficient was computed by the cor_pmat function under R stat (v3.6.3) package. Differential
692 metabolites with a correlation coefficient > 0.5 or < -0.5 were imported into Cytoscape (version

693 3.8.0) software to create co-expression networks[72].

694

695 **Logistic regression analysis**

696 Logistic regression model with covariates BMI, age and sex were built with each metabolite to
697 search for significant metabolites that associated with the NYHA class. The odds ratios were
698 estimated by the glm function under R stat (v3.6.3) package. *P* value of the variable estimate
699 in the model were computed by the Wald test and adjusted by the FDR correction.

700

701 **Association between metabolites and clinical outcome**

702 Survival analysis was performed using R survival (v3.1-11) package. The median of metabolite
703 abundances as the cutoff for expression dichotomization. For the association of metabolites
704 level with survival, Log-rank test was used to compare overall survival outcomes between two
705 groups and among three clusters generated by metabolomics and lipidomics. For all analyses,
706 significant association with survival was achieved for P value < 0.05. Kaplan-Meier survival
707 curves were plotted by function ggsurvplot in R survminer (v0.4.6) package.

708

709 **Machine learning for the diagnosis of HCM and prediction of survival outcomes**

710 For the diagnosis of HCM, we first performed least absolute shrinkage and selection operator
711 (LASSO) to select a reduced number of features which were diagnosis enough for the HCM
712 on the training dataset, and then we conducted 5-fold cross validation on the training set for
713 10 times with different random seeds to obtain stable feature selection results. We set the

coefficient of L1 constraint to 0.001 and selected the features which had a non-zero coefficient[73]. For the prediction of survival outcomes, 302 patients (286 participants survived after surgery and 16 participants deceased after surgery) were divided into the training set and testing set according to the chronological order of sample collection. The features were selected based on their variable importance (VIMP). Once the feature selection was finished, we built a diagnosis/prediction model by using random forests (RF) /random survival forests (RSF) method based on the training dataset. Then diagnosis and prediction model was applied to diagnose HCM and predict the survival outcomes in the testing set[74,75]. One hundred trees were built using the criterion of Gini impurity for RF analysis and one thousand trees were built using log-rank test for RSF analysis. LASSO and RF were performed via the scikit-learn package (v0.24.1) and RSF was performed via the scikit-survival package (v0.17.1) in Python (3.7.4).

Metabolomics and lipidomics consensus clustering

K-means consensus clustering was performed on metabolomics and lipidomics data with no missing values to generate subgroups using R ConsensusClusterPlus (v1.48.0) package[76], and the following detail settings were used for clustering: number of repetitions = 1,000 bootstraps; pItem = 0.8; pFeature = 0.8. K-means clustering range from k=2 to 6. The optimizing K-means clustering was ascertained by the average pairwise consensus matrix within consensus clusters and the delta plot of the relative change in the area under the cumulative distribution function (CDF) curve. Based on the evidence mentioned above, the

metabolomics and lipidomics data of HCM patients were clustered into three subtypes.

Cluster related metabolic changes

Kruskal-Wallis tests with Dunn's multiple comparison test by R dunn.test (v1.3.5) package was used to obtain the significantly altered metabolites in each cluster, relative to other two clusters. Metabolites with FDR-corrected $P < 0.05$, fold change > 1.25 or < 0.8, were considered as differential metabolites and used for subsequent analysis. The average absolute log2 fold change of differential metabolites enriched in each KEGG pathway was calculated.

Immunoblot analysis

Human cardiac tissues of 10-15 mg were homogenized in 500 μL RIPA (Solarbio) supplemented with protease and phosphate inhibitor (Biomake). Tissue lysate samples were centrifuged at 15,000 × g for 10 minutes at 4 °C and protein concentration was measured using a BCA protein assay kit (Beyotime). Samples were heated at 95 °C for 20 min in loading buffer. Equal amount of total proteins were subject to SDS–PAGE gels and transferred to PVDF membranes (GE Amersham). Membranes were blocked with 5% non-fat milk (Bioruler) in PBS-Tween for 1 h at room temperature, and then probed with the appropriate antibodies at 4 °C overnight. Primary antibodies used for western blot analysis were as follows: Anti-GAPDH (Proteintech, 60004-1-lg, 1:5000), Anti-G6PD (Sigma, HPA000834, 1:1000). After that, the appropriate secondary antibody (CST, anti-mouse: 7076S, anti-rabbit: 7074S, 1:5000) conjugated with horseradish peroxidase was added to incubate for 1 h at room temperature.

Chemiluminescence blotting system (Pierce) was used for detection. The western blot gel image was obtained with a chemiluminescent imager (Tanon5200).

Statistical analysis

All statistical methods used were listed in the figure legends or corresponding methods. Mann-Whitney U test and Student's t-test were used to compare two groups of independent samples. To account for multiple-testing, the *P* values were adjusted using the FDR correction. For categorical variables versus categorical variables, fisher's exact test was used. Kruskal-Wallis tests with Dunn's multiple comparison test was used to test if any of the differences between the subgroups were statistically significant. No statistical methods were used to pre-determine sample sizes but our sample sizes are similar to those reported in previous publications[77,78]. All the analyses of clinical data were performed in R (version 3.6.3), Excel (version 2016) and GraphPad Prism (v.8.0).

Data availability

The clinical information of each HCM patient is provided in Supplementary Table 1. The baseline clinical characteristics for DCM and non-HCM controls are included in Supplementary Table 2. As public sharing of the raw genomic data is restricted by the regulation of the Human Genetic Resources Administration of China, detailed results of whole exome sequencing are included in Supplementary Table 3. Raw metabolomics and lipidomics data are included in Supplementary Table 4, 5 and 6. The MS proteomics raw data is deposited in the

ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the iProX partner repository with dataset identifier PXD032097. Source data are provided with this paper.

Code availability

Codes for data analysis are available at https://github.com/WenminWang15/HCM_Nat-Cardiovasc-Res.

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Author contributions

W.W. and Z.H. designed the study and wrote the manuscript. W.W. performed data analyses, data integration and western blot experiments. K.Y. and W.W. performed targeted metabolomics experiments and data processing. K.Y. and J.X. performed lipidomics

experiments and data processing. J.W., G.W. and M.L. performed whole exome sequencing and proteomics experiments. M.N., Y.Z., B.W., H.P. and P.L. assisted in data interpretation and manuscript editing. N.T., C.Q., Y.L., Q.S., X.W., D.J., J.W., G.W., S.W. and L.S. provided clinical samples and information. Y.Z., H.P., X,L., D.L. and T.Y. performed machine learning. Z.H. conceived and supervised the project.

Competing interests

The authors declare no competing interests.

Figure legends

Fig. 1 | Genomic and metabolic landscape of the HCM cohort. a, Overview of the study design. The illustration was created using https://biorender.com/. **b**, Genetic profile of HCM patients (left). Mutation types and their frequencies were showed in the right panel. *MYH7*: myosin heavy chain 7, *MYBPC3*: myosin-binding protein C 3, *TNNT2*: cardiac troponin I 2, *TNNT3*: cardiac troponin I 3, *MYL2*, myosin light chain 2, *TPM1*: α-tropomyosin 1, *ACTC1*: cardiac α-actin 1. **c-e**, Counts and class of metabolites detected by targeted metabolomics in cardiac tissues (**c**), plasma (**d**) and metabolites detected by lipidomics (**e**). TAG: triacylglycerol, DAG: diacylglycerol, FFA: free fatty acid, SM: sphingomyelin, HexCer: hexosylceramide, Cer: ceramide, PE: phospha-tidylethanolamine, PC: phosphatidylcholine, LPC: lysophosphatidylcholine, LPE: lysophosphatidylethanolamine, PG: phosphatidylglycerol, PI: phosphatidylinositol, PS: phosphatidylserine.

Fig. 2 | Metabolomics perturbations in the heart tissues from HCM patients. a, Volcano plot of metabolites detected by target metabolomics in HCM and non-HCM controls (Ctrl). Significantly upregulated, downregulated (FDR *P* value < 0.05, fold change > 1.5 or < 0.67), and unchanged metabolites were colored in red, blue, and gray, respectively. Top 10 significantly increased or decreased metabolites of fold change in each group are labeled. The horizontal line denotes a FDR cutoff of 0.05, and the vertical lines denote a fold change of 1.5 or 0.67. **b**, Relative intensity of carnitine related metabolites in Ctrl (n=16), HCM (n=349) and

DCM (n=46) patients. **c**, KEGG metabolic pathway enrichment of differential metabolites (FDR *P* value < 0.05, fold change > 1.5 or < 0.67) between HCM and non-HCM controls. Fisher's exact test (one-side) followed by FDR-corrected *P* value was used and only pathways with FDR-corrected *P* value < 0.05 were presented. **d**, **e**, Relative intensity of metabolites involved in cysteine and methionine metabolism (**d**) and vitamin B6 metabolism (**e**) in Ctrl (n=16) and HCM (n=349). **f**, Relative intensity of metabolites involved in glutathione metabolism in Ctrl (n=16), HCM (n=349) and DCM patients (n=46). **g**, Schema of metabolic pathways with select metabolites. Relative intensity of glucose, G6P, F6P, F1,6P2, G3P, 3PG, 2PG and pyruvate were measured by a derivatization method. Color corresponds to the log2 fold change between HCM patients and non-HCM controls. Gray nodes represent metabolites that were not detected, and borders are color-coded by statistical significance. GlcN-6P: N-Acetyl-D-glucosamine 6-phosphate, UDP-GlcNAc: UDP-N-acetylglucosamine, G1P: glucose 1-phosphate, G3P: glyceraldehyde 3-phosphate, Thpp: thiamine pyrophosphate, PRPP: 5-phosphoribosyl diphosphate, AICAR: acadesine, IMP: inosine monophosphate, GMP: guanosine monophosphate, NAD: nicotinamide adenine dinucleotide, NAM: nicotinamide, NMN: nicotinamide mononucleotide.

The box plots visualized as median and 25th and 75th percentiles, with whiskers indicating maximal and minimal values (**b**, **d-f**). (exact *P* values are provided in the source data). Statistical analyses were performed by two-sided Mann-Whitney U test (**b**, **d-f**) and followed by Benjamini-Hochberg correction (**a**, **g**). Asterisks indicate significance as follows: ns $P \geq 0.05$, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

Fig. 3 | Lipidomics alterations in the heart tissues from HCM patients. a, Bubble plot of log2 fold change in abundance of lipid species in HCM relative to non-HCM controls (Ctrl). Values are shown as log2 fold change relative to non-HCM controls. Each dot represents a lipid species. Color coded per lipid class. Dot size indicates significance. The horizontal lines denote a fold change of 1.5 or 0.67. **b**, Relative intensity of lipid classes in HCM (n=349) and Ctrl (n=16). Illustration shows lipid classes that achieved statistically significant increase (red) and decrease (blue). The box plots visualized as median and 25th and 75th percentiles, with whiskers indicating maximal and minimal values. **c**, The percentage of significantly upregulated (red) and downregulated (blue) (FDR-corrected *P* value < 0.05, fold change > 1.5 or < 0.67) lipid species for each lipid class. **d**, Fold change of TAGs and percentage of significantly decreased TAGs with different numbers of double bonds between HCM and non-HCM controls. Statistical analyses were performed by two-sided Mann-Whitney U test (**b**) and followed by Benjamini-Hochberg correction (**a**, **c**).

Fig. 4 | Metabolic disturbances in plasma samples from HCM patients. a, Volcano plot of metabolites detected by target metabolomics in the plasma of HCM and non-HCM controls (Ctrl). Significantly upregulated, downregulated (FDR-corrected *P* value < 0.05, fold change > 1.5 or < 0.67), and unchanged metabolites were colored in purple, orange, and gray, respectively. Top 10 significantly increased or decreased metabolites of fold change in each group are labeled. The horizontal line denotes a FDR cutoff of 0.05, and the vertical lines

870 denote a fold change of 1.5 or 0.67. **b**, Log2 fold change of common metabolites in the tissue
871 (x axis) and plasma (y axis) of HCM patients compared to non-HCM controls. Differential
872 metabolites with opposite or consistent alteration were marked with different colors. **c**, KEGG
873 metabolic pathway enrichment of differential metabolites between HCM and non-HCM controls
874 in plasma and heart tissue. Fisher's exact test (one-side) followed by FDR-corrected *P* value
875 was used and only pathways with FDR-corrected *P* value < 0.05 were presented, mean
876 absolute (abs.) log2 fold change of metabolites enriched in each pathway was calculated. **d**,
877 Log2 fold change of significantly changed metabolites in glutathione metabolism, purine
878 metabolism, glucose-related metabolism pathway and carnitine synthesis pathway in plasma
879 or heart tissue of HCM patients. **e**, Schematic of the data set creation and analysis strategy
880 for the diagnosis of HCM. C: non-HCM controls, H: HCM. **f**, **g**, Receiver operating
881 characteristic (ROC) curve (**f**) and precision-recall curve (**g**) of the random forest model in the
882 testing set. Statistical analyses were performed by two-sided Mann-Whitney U test and
883 followed by Benjamini-Hochberg correction (**a**, **d**).
884
885 **Fig. 5 | Metabolic association with clinical characteristics in HCM patients. a**, PCA score
886 plot of metabolomics and lipidomics data on HCM patients with or without gene mutations. **b**,
887 PCA score plot of metabolomics and lipidomics data on HCM patients with different pathogenic
888 genes. **c**, Volcano plot of metabolites alterations between HCM patients with *MYBPC3* (n=67)
889 and *MYH7* (n=98). Unchanged metabolites were colored in gray. The horizontal line denotes
890 *P* value cutoff of 0.05, and the vertical lines denote a fold change of 1.25 or 0.8. Statistical

analyses were performed by two-sided Mann-Whitney U test. Fc: Fold change. **d**, Forest plot of odd ratios (OR) for the association between NYHA class and the relative abundance of metabolites detected by targeted metabolomics. Metabolites in different metabolic pathways were marked with different colors. OR were presented by the center of error bars and 95% confidence intervals were presented by the line widths. **e**, Forest plot of OR for the association between NYHA class and the relative abundance of TAGs, the abundance of TAGs was defined as the ratio of each TAG to total TAG abundance in each patient. OR were presented by the center of error bars and 95% confidence intervals were presented by the line widths. **f**, OR and 95% confidence intervals for the association between NYHA class and metabolites based on multivariate logistic regression analysis. OR were presented by the center of error bars and 95% confidence intervals were presented by the line widths.

Fig. 6 | Metabolic subtyping and prognostic prediction of HCM patients. a, Consensus clustering analysis based on metabolomics data of HCM patients identified three subtypes. **b**, Heatmap of metabolites relative abundance in each individual metabolic subtype. **c**, Kaplan-Meier curves for overall survival of HCM patients among the three subtypes (Log-rank test). **d**, KEGG metabolic pathways enriched by significantly changed metabolites (two-side Kruskal-Wallis tests, FDR-corrected *P* value < 0.05, fold change > 1.25 or < 0.8) in each individual metabolic subtype. Fisher's exact test (one-side) followed by FDR-corrected *P* value was used. Only pathways with FDR-corrected *P* value < 0.05 were presented. Mean absolute (abs.) log2 fold change of metabolites enriched in each pathway was calculated. **e**, Relative intensity of

significantly changed metabolites between S-II or S-III and S-I in glutathione metabolism, TCA cycle, purine and pyrimidine metabolism and carnitine synthesis pathway. Data were presented as mean ± sd. **f**, Consensus clustering analysis based on lipidomics data of HCM patients identified three subtypes. **g**, Kaplan-Meier curves for overall survival of HCM patients among three subtypes (pairwise Log-rank test). **h**, Bubble plot of log2 fold change in abundance of significantly changed TAGs (Kruskal-Wallis tests, FDR-corrected *P* value < 0.05, fold change > 1.25 or < 0.8) in S-II relative to S-I. Each dot represents a TAG species. Color coded per number of TAGs carbon. Dot size indicates significance. The horizontal lines denote a fold change of 1.25 or 0.8. The abundance of TAGs was defined as the ratio of each TAG to total TAG abundance in each patient. **i**, Schematic of the data set creation and analysis strategy for the prediction of survival outcomes. S: Survived, D: Deceased. **j**, C-index values of the level of metabolites for overall survival in the testing dataset. Error bars represent 95% confidence intervals.

Fig. 7 | Metabolomics and proteomics analyses reveal potential therapeutic strategies for HCM. a, KEGG pathways enriched by significantly changed proteins (FDR-corrected *P* value <0.05) in HCM patients. Fisher's exact test (one-side) followed by FDR-corrected *P* value was used. Only pathways with FDR-corrected *P* value < 0.05 and related with metabolism were presented. **b**, Schema (top) and heatmap of relative intensity of significantly changed protein (bottom) in the oxidative phosphorylation pathway. **c**, Schema of metabolic pathways (pentose phosphate pathway, purine and pyrimidine metabolism) with select

933 metabolites and proteins (top) and immunoblot analysis and protein quantification of HCM
934 (n=16) and non-HCM (Ctrl) (n=8) human cardiac tissue samples for G6PD (bottom). (All the
935 samples derive from the same experiment and that blots were processed in parallel.)
936 Metabolites or proteins with significantly upregulated, downregulated and unchanged were
937 colored in red, blue, and black, respectively. Gray nodes represent metabolites or proteins that
938 were not detected. Statistical analyses were performed by two-tailed unpaired Student's t test
939 and data were presented as mean ± sd. **d**, The schematic diagram illustrates the alterations
940 of redox related proteins and metabolites in HCM patients and the effects of N-acetylcysteine
941 (NAC) to HCM animal models.

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