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Article

Multi-omic analysis identifies mitochondrial dysfunction as a key feature of hypertrophic cardiomyopathy

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SUMMARY

Hypertrophic cardiomyopathy (HCM) is an inherited cardiovascular disorder marked by left ventricular hypertrophy, known to be caused by genetic mutations in sarcomere proteins, such as *MYH7* and *MYBPC3*. Despite advancements in our understanding of HCM genetics, the relationship between genetic variants and clinical outcomes in HCM remains complex, particularly due to phenotypic variability and overlapping features with other cardiomyopathies. We hypothesized that HCM would exhibit disease-specific gene expression and methylation patterns, primarily in non-sarcomeric pathways, in comparison to healthy controls and aortic stenosis (AS) patients. We performed a multi-omic analysis to investigate the gene expression and DNA methylation patterns of HCM individuals utilizing publicly available RNA sequencing and bisulfite sequencing datasets. We identified a gene set consistently downregulated in HCM relative to healthy controls, while upregulated relative to patients with AS. These results were corroborated by parallel methylome data, which allowed us to identify specific genes that were both differentially expressed and methylated. When analyzed for functional signatures, we found consistent, significant downregulation of genes involved in mitochondrial processes, NADH dehydrogenase complex assembly, and electron transport chain function in individuals with HCM. Conversely, sarcomeric genes were not differentially expressed or methylated to the same extent as mitochondrial and energy-related genes. Our results highlight the potentially critical role of mitochondrial dysfunction in HCM pathogenesis, linking energy deficits to cardiac dysfunction and increased oxidative stress. Addressing this dysfunction through therapeutic interventions that target mitochondrial pathways, such as gene therapy combined with individualized nutritional therapies, could mitigate disease progression and improve clinical outcomes.

INTRODUCTION

Hypertrophic cardiomyopathy (HCM) is a prevalent cardiomyopathy affecting 1 in 500 individuals globally and is characterized by left ventricle hypertrophy or thickening of the heart muscle (1). This is often due to mutations in genes that encode proteins involved with the sarcomere, which is the main contractile unit of muscle fibers, although a portion of cases remain genetically diverse (1). Hypertrophic cardiomyopathy presents as a wide range of clinical manifestations, from asymptomatic to severe symptoms such as heart failure, arrhythmias, and sudden cardiac death (SCD) (1).

HCM is typically diagnosed when left ventricular wall thickening exceeds 15 mm, independent of other causes of hypertrophy or overload conditions, such as hypertension and valvular disease (1). The thickened heart muscle in HCM impairs relaxation, preventing proper blood filling. This can lead to left atrium enlargement, diastolic dysfunction, and systolic dysfunction (1). However, the clinical course of HCM varies considerably among individuals, influenced by factors such as age of onset, extent of hypertrophy, and genetic predisposition (1). For example, women are often diagnosed later than men with more severe symptoms, exhibiting a higher prevalence of left ventricular outflow tract obstruction (LVOTO), which carries implications for disease management (1).

Hypertrophic cardiomyopathy is intricately linked with genetic variants, particularly those that affect sarcomeric proteins (2). The two most common mutated genes are *MYH7,* which encodes for B-myosin heavy chains, and *MYBPC3,* which codes for myosin binding protein C, and together they account for up to 50% of clinically recognized HCM cases (2). *MYH7* mutations are typically missense mutations with a dominant-negative mechanism, meaning that every codon downstream of the mutation is misgrouped, leading to an altered protein that interferes with normal sarcomere functions (2). *MYBPC3* mutations involve premature stop codons, which lead to reduced levels of protein function (2). Other, less common, mutations in *TNNT2* and *TPM1,* key sarcomere genes that regulate cardiac contraction, which code for troponin and tropomyosin, lead to impaired contraction and relaxation of the heart muscle, contributing to HCM (2). Also, while certain genes like *MYH7* and *MYBPC3* are well-established in their association with HCM, the clinical significance of variants in other genes, such as *TTN*, requires further exploration. The rarity of variants in genes like *ACTN1*, *MYH6*, *MYLK2*, *TCAP*, and *VCL* in more isolated HCM cases can also complicate diagnosis and treatment (1). HCM exhibits genetic diversity, with mutations in various sarcomeric genes contributing to the condition, as well as phenotypic variability, meaning individuals with the same mutations can experience different symptoms and disease severity (3). For example, mutations in genes like *TNNI3*, which encodes cardiac troponin 1, cause a wide spread of clinical outcomes, such as restrictive cardiomyopathy, characterized by increased mortality risk and hypertrophic cardiomyopathy with myofibrillar disarray (a key marker of HCM) even in cases without marked left ventricular hypertrophy (4). Variability in the same *TNNI3* mutation also resulted in phenotypic expression ranging from restrictive physiology to near-normal cardiac structure, further exemplifying phenotypic variability

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(4). HCM is also primarily inherited in an autosomal dominant manner, meaning that if a parent has HCM due to a genetic mutation, there is a 50% chance that the child will inherit the mutation and could develop HCM (3). Given the phenotypic variability associated with genetic mutations in HCM, genetic testing is important to identify at-risk individuals and their likelihood for severe disease progression.

Genetic testing has become a valuable diagnostic tool, detecting preclinical disease and enabling cascade testing in families (3). It plays a vital role in identifying family members who carry the inherited HCM variant, which is crucial considering that SCD can be the first symptom (3). First-line genetic testing focuses on the genes most associated with HCM, including *MYH7*, *MYBPC3*, and *TNNI3* (4). Techniques like polymerase chain reaction (PCR), in which researchers amplify specific sections of DNA to confirm mutation presence, and Whole Genome Sequencing (WGS), a whole genome analysis to identify novel mutations, are key in confirming mutations and identifying novel variants (4). Early diagnosis of key mutations through genetic testing helps prevent severe outcomes in HCM patients.

Mitochondria play a critical role in the heart, and mitochondrial dysfunction is recognized as a factor in the progression of all cardiovascular diseases (5). As the primary source of ATP for cardiomyocytes, which are cardiac cells, mitochondria are key in supporting the heart's contractile and metabolic demands (5,6). However, dysfunctional mitochondria inhibit cellular respiration and ATP production and generate reactive oxygen species (ROS), leading to oxidative stress and cellular apoptosis, severely damaging the heart (5). These observations suggest that analyzing mitochondrial function in HCM patients could clarify how much of the extent it plays in HCM progression and potentially identify mitochondrial-targeted therapies as a key intervention. Beyond sarcomeric and mitochondrial abnormalities, specific mitochondrial-regulating genes such as *PINK1* could be potential contributors to HCM progression (7). *PINK1* encodes a mitochondria-localized kinase that is believed to protect cells from stress-induced mitochondrial dysfunction (7). HCM patients, downregulation of *PINK1* and related pathways can lead to mitochondrial dysfunction and damage in cardiac cells, resulting in decreased ATP production (7). This reduction in ATP, coupled with mitochondrial dysfunction, increases oxidative stress and triggers apoptosis in cardiac cells, exacerbating HCM-related pathologies by reducing cardiac muscle mass and promoting fibrosis (7).

Although there are key genes associated with HCM, the link between specific variants and clinical outcomes remains complex. Some variants increase the risk of SCD, but phenotypic variability means that not every patient with these variants develops the disease (genotype-positive/phenotype-negative), ultimately complicating the use of these variants as reliable diagnostic tools (1). Understanding the connection between genetic predisposition, environmental factors, and epigenetic modifications is crucial for developing targeted therapies for cardiomyopathy. Multi-omic approaches, such as epigenetic and transcriptomic analyses, provide valuable insights into disease mechanisms, therapeutic targets, and potential intervention pathways. This integrated strategy shows promise for slowing disease progression and improving outcomes in cardiomyopathy patients (1).

Aortic Stenosis (AS) is a common valvular heart disease characterized by the narrowing of the aortic valve, leading to left ventricular pressure overload that can result in heart failure, chest pain, and dyspnea (8). Left ventricular hypertrophy can develop to offset the pressure load, leading to decreased contractility (8). AS is like HCM as it can lead to structural changes in the heart, but the underlying causes of both differ, as AS is caused by mechanical obstruction, while HCM is primarily genetic. Despite these differences, both conditions can result in comparable functional differences in the heart.

Here, we sought to better understand the molecular mechanism regarding HCM and identify changes in gene expression unique to HCM compared to other cardiac diseases. First, to determine whether the gene expression changes found through the multi-omic analysis are specific toward HCM rather than to broader cardiac pathology, we included aortic stenosis (AS) patients as a disease comparison group. We hypothesized that HCM would exhibit disease-specific gene expression and methylation patterns, primarily in non-sarcomeric pathways, compared to healthy controls and AS patients. To identify the genetic signatures of HCM and their functional enrichments, we conducted a multimodal integrative analysis of transcriptomic and epigenomic datasets from HCM patients, reporting alterations in genes related to energy and metabolism. These findings enhance our understanding of HCM genetics and could lead to more effective treatment strategies.

RESULTS

To assess whether the HCM transcriptome is distinguishable from that of healthy donors, we conducted differential expression analysis on RNA-seq data from the heart tissue of HCM patients (n = 13) and healthy controls (n = 7), identifying 5,006 differentially expressed genes (DEGs) between HCM and healthy controls, with 3,392 genes upregulated in healthy controls relative to HCM and 1,614 genes upregulated in HCM relative to healthy controls (**Figure 1A**)

To further understand the magnitude of these gene expression differences between HCM and healthy controls, we created heatmaps showcasing the normalized z-score distribution of the most upregulated and most downregulated genes. The most differentially expressed genes were identified in these comparisons. Differential expression analysis revealed significant expression changes between HCM and healthy controls, with the Z-score distribution suggesting that downregulation of genes may play a more prominent role in HCM progression than upregulation, as HCM exhibited relatively higher expression levels compared to healthy controls with the downregulated genes (Figure 1B). The top ten genes upregulated in healthy controls compared to HCM patients were *SAA1*, *SAA2*, *SAA2-SAA4*, *FAM83B*, *LINC02822*, *RNASE2*, *C4orf54*, *LOC105372926*, *PTPRN*, and *IL1RL1*(*p*-values < 0.05, |log2 fold change |($\log_2$fc)| > 1, **Figure 1B**). Genes upregulated in HCM relative to controls, including *RFPL4A* and *EDN3*, were also identified (*p*-values < 0.05, |$\log_2$fc | > 1, **Figure 1B**).

To identify the biological processes associated with these

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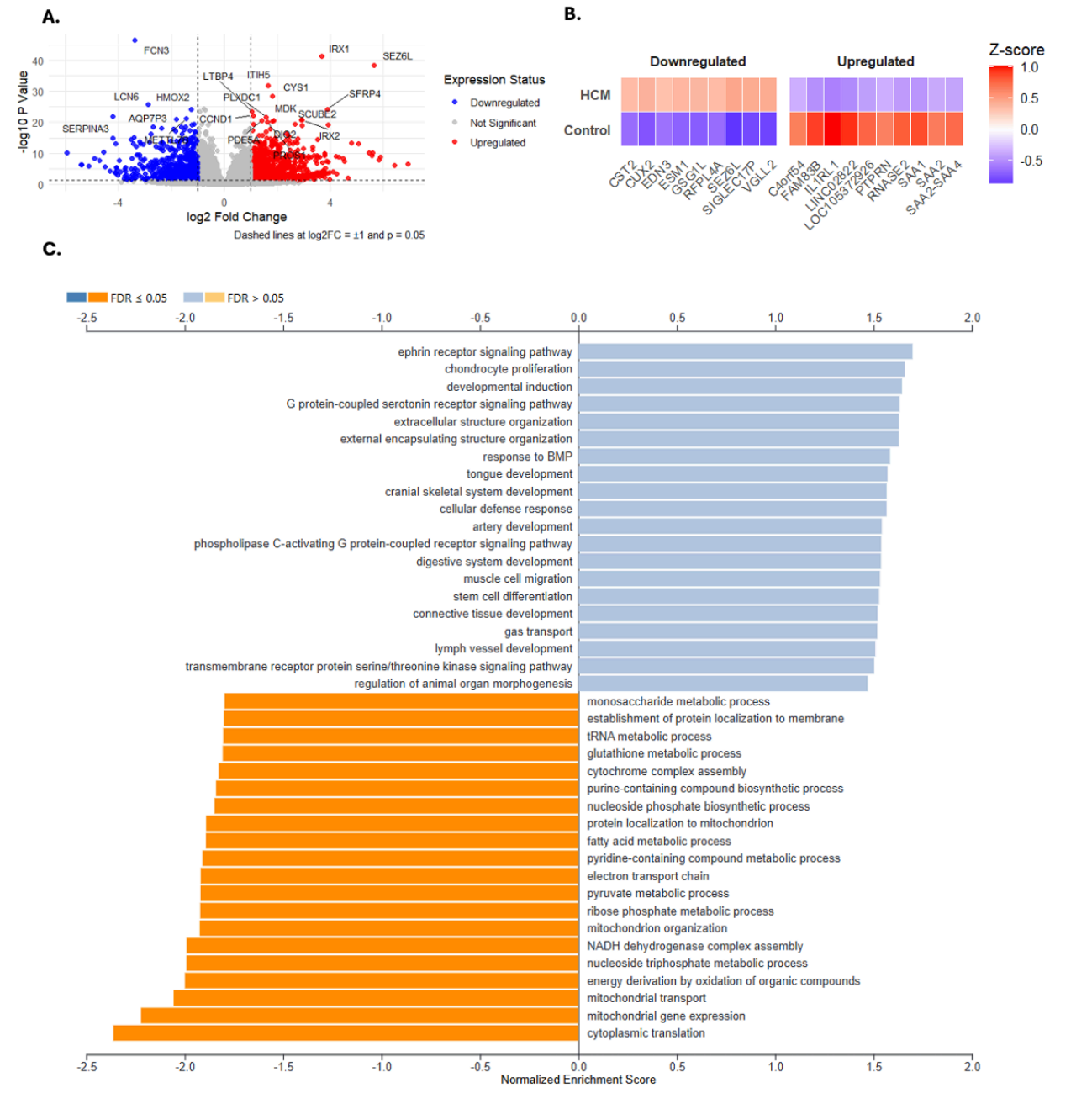

Figure 1: Differential gene expression and functional pathway alteration between Hypertrophic Cardiomyopathy (HCM) patients and Healthy Controls. RNA-seq analysis was performed on heart tissue samples from HCM patients (n = 13) and Healthy Controls (n=7) to identify transcriptional differences and associated biological pathways. (A) Volcano Plot depicting differentially expressed genes in heart tissue samples from HCM patients and healthy controls. Each point represents an individual gene plotted by $\log_2$ fold change($\log_2$fc) of HCM patients relative to control based on DESeq2 normalized counts and -log10 p-value. Positive x-axis values(red) indicate higher gene expression in HCM patients, while negative values(blue) indicate genes with higher expression in healthy controls. Significant genes are classified as those with p-values < 0.05 and $|\log_2 fc| > 1$. Labeled genes represent the top 20 genes with both large differential expression and high statistical significance. (B) Heatmap depicting the top 10 upregulated and downregulated genes in HCM compared to Control Patients. Color illustrates normalized z-scores for the genes with the highest and lowest $\log_2$ fold changes between HCM patients and healthy Controls. Dark red indicates higher expression levels, while white to light blue indicates no variability in expression. (C) Gene Ontology enrichment analysis of differentially expressed genes between HCM patients and healthy controls. Bar chart depicting functional processes related to differential gene expression, with the x-axis representing normalized enrichment scores, indicating the magnitude of gene set enrichment. Positive normalized enrichment score (NES) values (blue bars) indicate processes upregulated in HCM patients relative to healthy controls (FDR > 0.05) and negative NES values (orange bars) indicate processes significantly downregulated in HCM patients relative to healthy controls (FDR < 0.05).

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gene expression patterns in HCM patients, we performed a functional enrichment analysis using WebGestalt (Web-based Gene Set Analysis Toolkit) on all differentially expressed genes between HCM and healthy controls. We aimed to uncover the functional patterns between these patient groups and gain insights into the underlying mechanisms of HCM. Specific genes upregulated in HCM patients compared to healthy control patients are involved in processes such as ephrin receptor signaling pathways, chondrocyte proliferation, and developmental induction, but these pathways are not significant (FDR > 0.05, **Figure 1C**). Conversely, processes such as cytoplasmic translation and mitochondrial gene expression are significantly downregulated in HCM compared to healthy controls (FDR < 0.05, **Figure 1C).** The pronounced downregulation of mitochondrial and translation processes highlights key functional pathways that are altered in HCM.

To determine whether the gene expression differences were specific to HCM or reflective of broader cardiac pathology, we conducted differential expression analysis on RNA-seq data from the heart tissue of HCM patients (n = 8) and AS patients (n = 5), identifying 5,382 DEGs, with 2,531 upregulated in HCM relative to AS and 2,851 downregulated in HCM relative to AS (**Figure 2A**). By using the AS condition as the control, we identified genes that specifically impact HCM in contrast to other heart diseases. Our analysis revealed that multiple genes exhibited consistent downregulation in AS relative to HCM, with the directionality of expression, indicating that these genes are expressed at lower levels in AS compared to HCM, which may suggest that genes involved in AS and potentially other heart diseases are more heavily downregulated than those involved in HCM. To further understand these differences and similarities in gene expression between patients with HCM and AS, we created heatmaps showcasing the normalized count z-score distribution of the most upregulated and most downregulated genes of each dataset **(Figure 2B).** The AS heatmap reveals that genes upregulated in HCM relative to AS show consistently lower expression in the AS group, while downregulated genes exhibit z-scores closer to zero, reflecting less variability between the two conditions (**Figure 2B**)**.** The ten most upregulated genes in HCM relative to AS were *LOC102723846, ACSL6-AS1, LOC105370849, LOC105372096, CPNE6, GARIN1B, LOC105374296, LOC149684, LOC107987013,* and *EVX1* **(**p-values < 0.05, $|\log_2 fc)| > 1$, **Figure 2B**). Genes downregulated in HCM relative to AS included *MTRNR2L10, CTAG2, CLVS2, LOC105371292, LINC00701, PANCR, PWRN1, CLEC3A, MTRNR2L3,* and *UGT3A2* (p-values < 0.05, $|\log_2 fc)| > 1$, **Figure 2B**). Overall, these heatmaps suggest that HCM and AS share broadly similar expression profiles, with downregulation as a common feature in both conditions; however, the z-score distributions indicate this downregulation is more pronounced in AS than in HCM, while the largest magnitude of expression differences was observed between HCM and healthy controls (**Figure 1B**, **Figure 2B**).

Next, we identified pathways with genes differentially expressed between HCM and AS patients. Processes like cell-cell adhesion mediated by plasma membrane adhesion molecules and extracellular structure organization were significantly enriched in AS patients relative to HCM (FDR < 0.05, **Figure 2C**). In contrast, HCM patients show an upregulation of processes like cytoplasmic translation, mitochondrial gene expression, and NADH dehydrogenase complex assembly compared to AS patients **(**FDR < 0.05, **Figure 2C).** Notably, these processes, which are upregulated in HCM relative to AS, are the same ones that are downregulated in HCM when compared to healthy controls but exhibit even further downregulation in AS. A distinctive observation is that the processes involved in both the genetic downregulation in HCM and the genetic upregulation relative to AS are primarily related to mitochondrial function and energy metabolism.

To deepen the analysis of gene expression in HCM, we utilized a publicly available parallel DNA methylation dataset, consisting of HCM and AS patients. Methylation analysis was specifically chosen to narrow the list of DEGs from the RNA-sequencing analysis by identifying genes that also exhibit differential methylation (DMGs). Differential methylation highlights DNA chemical modifications that can alter gene expression without changing the coding sequence (9). This is critical for pinpointing potential gene therapy targets, as genes that are both DEGs and DMGs are more likely to be biologically significant and relevant for gene therapy intervention and treatment. When the methylation data corroborates the RNA sequencing results, it enhances the reliability of the findings by providing an additional lens through which these group-specific patterns are present (9). We identified 121 overlapping genes, reinforcing the likelihood that these genes significantly contribute to HCM **(Figure 3).** In our dataset, these 121 genes were hypomethylated, meaning they had decreased methylation levels, which is typically associated with increased gene expression. However, in this case, both HCM and AS showed downregulation of these differentially expressed genes, contradicting the expected effect of hypomethylation. This unexpected pattern suggests that other epigenetic mechanisms may be suppressing the usual effects of methylation to influence gene expression. Restoring proper methylation patterns could therefore be a potential therapeutic strategy. This level of specificity enhances the confidence to identify optimal gene targets for HCM gene therapy.

Among the 121 genes that are both differentially methylated and differentially expressed, we identified *PINK1* as a key target, as it was consistently found in the downregulated mitochondrial pathways from the functional analysis. *PINK1* had high functional analysis scores across multiple energy-related pathways, specifically the electron transport chain, protein localization to mitochondria, mitochondrial organization, and mitochondrial transport. To investigate the importance of energy-related pathways in HCM, we compared *PINK1* to *MYH7*, a gene well-known for its clinical relevance to the disease. Based on differential expression analysis, *PINK1* expression was significantly reduced in the HCM group compared to healthy controls (log2fc = -0.2119, $p = 0.0026$) and significantly elevated in HCM compared to AS patients ($p = 0.0408$, log2fc = 0.2833), findings supported by one-way ANOVA with Tukey HSD post-hoc analysis, confirming significant differences in PINK1 expression across all three patient groups (Figure 4. In contrast, although *MYH7* expression was elevated in the HCM and AS samples, the

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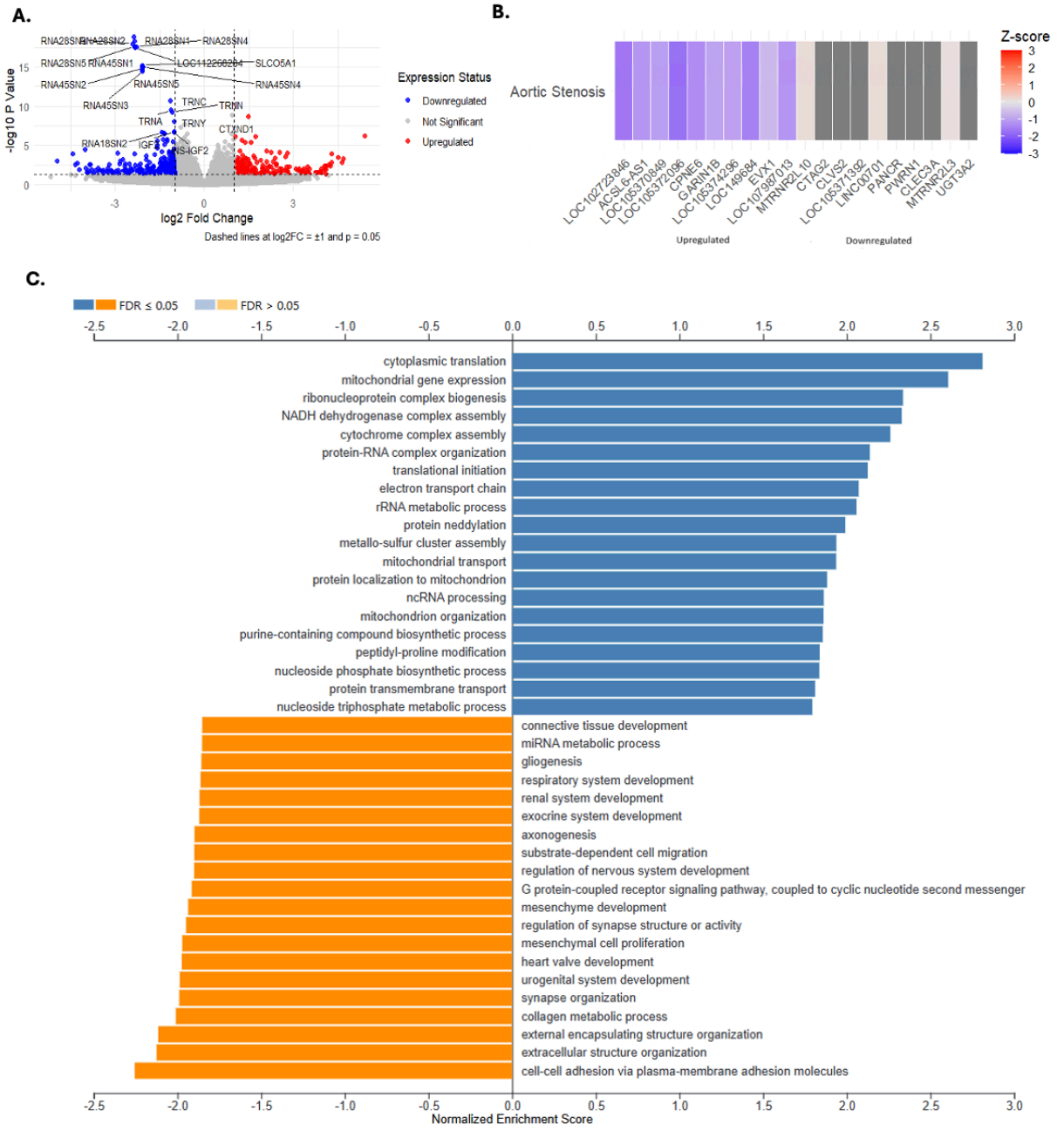

Figure 2: Differential gene expression and functional pathway alteration between Hypertrophic Cardiomyopathy (HCM) patients and Aortic Stenosis (AS) patients. RNA-seq analysis was performed on heart tissue samples from HCM patients (n = 8) and AS (n = 5) to identify transcriptional differences and associated biological pathways. (A) Volcano Plot depicting differentially expressed genes on heart tissue samples between HCM patients and AS patients (n = 5). Each point represents an individual gene plotted by $\log_2$ fold change of HCM patients relative to AS patients based on DESeq2 normalized counts and -log10 *p*-value. Positive x-axis values(red) indicate higher gene expression in HCM patients, while negative values(blue) indicate higher gene expression in AS patients. Significant genes are classified as those with p-values < 0.05 and $|\log_2$ Fold Change| > 1. Labeled genes represent the top 20 genes with both large differential expression and high statistical significance. (B) Heatmap showing the top 10 upregulated and downregulated genes in HCM and their expression in AS. Colors indicate normalized z-scores for genes with the highest and lowest $\log_2$ fold changes between HCM and AS patients, calculated for the AS group using the HCM mean and standard deviation as reference, allowing visualization of how AS gene expression deviates from HCM. Negative z-scores (blue/purple) represent lower expression in AS relative to HCM, while positive Z-scores(beige/blue) represent higher expression in AS relative to HCM. (C) Gene ontology enrichment analysis of differentially expressed genes between HCM and AS patients. Bar chart depicting functional processes related to differential gene expression, with the x-axis representing normalized enrichment scores, indicating the magnitude of gene set enrichment. Positive NES values (blue bars) indicate processes upregulated in HCM patients relative to AS patients (FDR < 0.05) and negative NES values (orange bars) indicate processes downregulated in HCM patients relative to AS patients (FDR < 0.05).

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Figure 3: Venn Diagram depicting the overlap between differentially expressed genes (DEGs) and differentially methylated genes (DMGs). Diagram highlights the number of genes from the methylation analysis uniquely identified as DEGs, DMGs, and both DEGs and DMGs

difference was not statistically significant (p = 0.5903), and ANOVA similarly reveled no significant difference between HCM and healthy controls (Figure 5). These gene expression patterns are clearly visualized as *PINK1* demonstrates a clearer separation between groups, whereas *MYH7* shows substantial overlap in expression between HCM patients and healthy controls **(Figures 4 and 5).** This suggests that *PINK1* downregulation may be a more specific marker of HCM pathology, as its expression differs significantly from both AS and healthy control samples. In contrast, *MYH7* did not show statistically significant differences and may therefore be a less reliable marker for distinguishing HCM.

DISCUSSION

This study aimed to identify the molecular mechanisms that are specific to HCM from other forms of cardiovascular disease, such as AS, and from healthy cardiac function. We integrated transcriptomic and epigenetic analyses to better understand how gene expression and epigenetic modification contribute to HCM progression. Our initial hypothesis was that HCM patients would exhibit disease-specific gene expression and methylation patterns, primarily in non-sarcomeric pathways, in comparison to healthy controls and AS patients. The results support this hypothesis as we identified distinct gene expression profiles for HCM patients, with the most notable pattern being downregulation in HCM relative to healthy controls and upregulation relative to AS. Functional analysis identified significant downregulation of genes associated with mitochondrial and metabolic pathways, suggesting that HCM has a unique transcriptional profile, characterized by pronounced energy-related deficiencies. While mitochondrial dysfunction is a feature shared with other cardiovascular diseases, its downregulated gene expression patterns appear more specific to HCM. In addition, DNA methylation analysis revealed that several of these same genes were both differentially methylated and differentially expressed, such as *PINK1*, suggesting that epigenetic regulation could be contributing to these transcriptional changes in HCM. *MYH7* and *MYBPC3*, two known sarcomere genes with mutations common in HCM pathogenesis, were differentially expressed across all groups, while mitochondrial pathway gene expression patterns appeared more specific and pronounced in HCM. Collectively, these findings suggest that the downregulation of mitochondrial-related pathways is a defining feature of HCM and must be targeted, rather than focusing solely on sarcomere mutations, to more effectively slow down HCM progression.

Our analysis revealed that several mitochondrial pathways are consistently downregulated in HCM, with the highest downregulation magnitude being observed in mitochondrial gene expression, mitochondrial transport, NADH Dehydrogenase Complex Assembly, mitochondrial organization, and the electron transport chain. These processes are essential for ATP synthesis, indicating a widespread energetic deficiency in HCM (10). This highlights the urgency to address energy-related functions within HCM patients, which have been previously neglected in favor of addressing sarcomere-related pathological hypotheses. The differential expression analysis revealed a distinctive pattern regarding the downregulation in HCM relative to healthy controls but upregulated relative to AS. This indicates that the downregulation of mitochondrial genes can create mitochondrial changes that may reflect a phenotype of HCM, contributing to our understanding of the disease's pathophysiology based on the available data. Given the significance of the these results and the overlap of processes identified in both datasets, targeting the following processes may be crucial for developing new treatments: cytoplasmic translation, mitochondrial gene expression, NADH dehydrogenase complex assembly, cytochrome complex assembly, electron transport chain, mitochondrial transport, protein localization to mitochondrion, mitochondrion organization, nucleoside phosphate biosynthetic process, and nucleoside triphosphate metabolic process.

The observed downregulation of mitochondrial pathways in HCM supports the idea that energy deficits play a central role in pathogenesis (10). Deficiencies in oxidative phosphorylation (OXPHOS) and mitochondrial gene expression – two of the most significantly downregulated pathways from our functional analysis – disrupt ATP production, exacerbating oxidative stress, leading to compensatory pathological remodeling in HCM cardiomyocytes, such as downregulation of protective genes and activation of stress-response pathways, which accelerate disease progression and lead to hypertrophy (10). In healthy cardiomyocytes, mitochondria generate ATP via oxidative phosphorylation of fatty acids and glucose (6). However, dysfunctional mitochondria generate excess reactive oxygen species (ROS), increase oxidative stress, and induce apoptosis (5). Reduced expression of mitochondrial genes thus contributes to diminished ATP and ADP levels, impaired complex II and IV activity, and worsened oxidative phosphorylation capacity (7). These energetic deficiencies are also linked to structural changes in HCM such as septal hypertrophy in genotype-negative HCM and contractile dysfunction, by keeping the heart energetically

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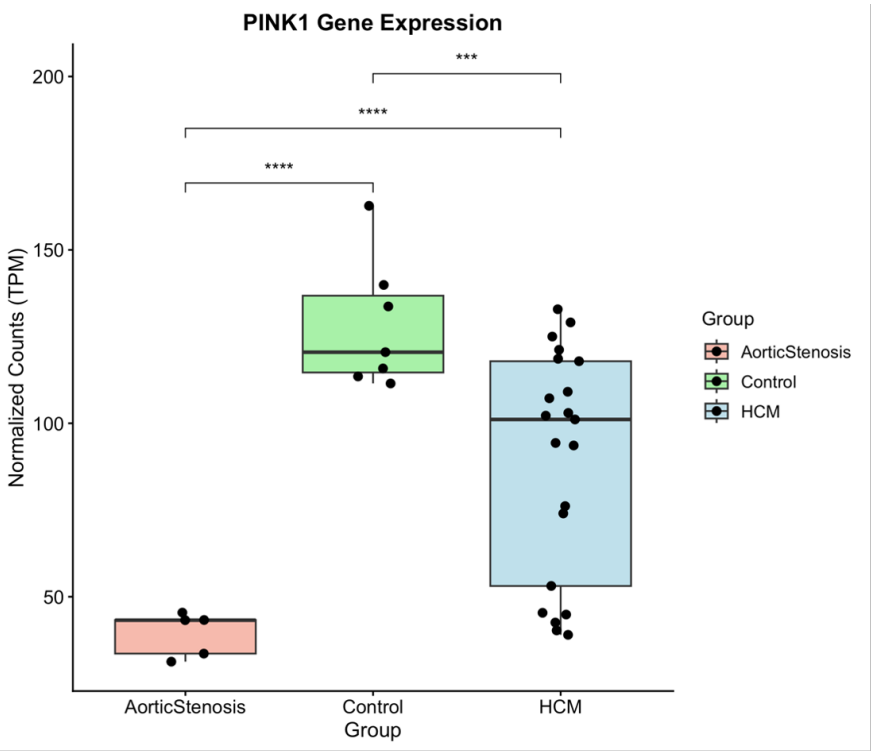

Figure 4: Boxplot of PINK1 gene expression in Healthy Controls, Hypertrophic Cardiomyopathy (HCM), and Aortic Stenosis (AS) Patients. Distribution of normalized Transcripts Per Million (TPM) counts for the *PINK1* gene across Healthy Controls (n = 7), HCM patients (n = 21), and AS patients (n = 5). TPM values were calculated using RNA-seq data, and individual sample points and interquartile range are displayed for each group. Group differences were calculated using a one-way ANOVA followed by Tukey's Honest Significant Difference (HSD) post-hoc test. Significance levels: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, ns = not significant.

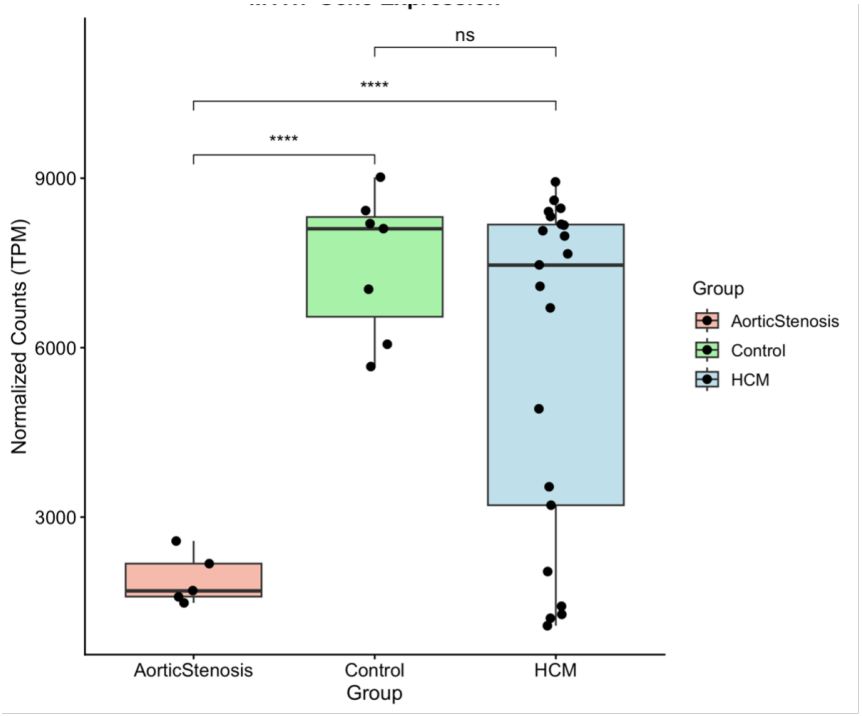

Figure 5: Boxplot of *MYH7* gene expression in Healthy Controls, Hypertrophic Cardiomyopathy (HCM), and Aortic Stenosis (AS) Patients. Displays the distribution of normalized Transcripts Per Million (TPM) counts for the *MYH7* gene across Healthy Controls (n = 7), HCM patients (n = 21), and AS patients (n = 5). TPM values were calculated using RNA-seq data, and individual sample points and interquartile range are displayed for each group. Group differences were calculated using a one-way ANOVA followed by Tukey's Honest Significant Difference (HSD) post-hoc test. Significance levels: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, ns = not significant.

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decompensated (7,11).

Beyond hypertrophic changes, our data also revealed that NADH dehydrogenase complex assembly and the electron transport chain (ETC) were among the most significantly downregulated mitochondrial pathways in HCM. This downregulation is likely to impair NADH-linked respiration, reducing ATP production in cardiomyocytes (10). Disorganized interfibrillar mitochondria are also associated with dysfunction in these pathways and can contribute to hypertrophy (10). Furthermore, this downregulation is linked to a 30% overall reduction in ETC activity, which can elevate oxidate stress and increase the risk of atrial fibrillation (11).

When comparing these findings to AS, both HCM and AS exhibited notable downregulation compared to healthy controls, especially in the mitochondrial and metabolic pathways of the functional analysis; however, AS consistently exhibited a more prominent downregulation compared to HCM, reflecting its distinct mechanisms compared to HCM and how those differences can be used to help guide treatment. In HCM, the mitochondrial gene downregulation observed involves pathways such as oxidative phosphorylation and the electron transport chain, leading to impaired energy and metabolic function (11). This downregulation is intrinsic to HCM pathogenesis as it is genetically linked to deficiencies in OXPHOS, for example, rather than due to secondary mitochondrial and cellular structural abnormalities and degradation (11). Conversely, the more intense mitochondrial dysfunction observed in AS reflects a secondary response to the pressure overload due to valvular narrowing and the hypertrophy that results from it, as mitochondrial downregulation occurs after that (12). Furthermore, inflammation and degeneration of the aortic valve drive mitochondria-induced apoptosis, contributing to valve calcification. Thus, mitochondrial function in AS is extrinsic, as metabolic changes, such as increased fatty acid oxidation and reduced angiogenesis, occurred due to pressure overload, not as a cause of pressure overload (13). This distinction between HCM and AS highlights that genetic changes in HCM are not a result of hypertrophy but involve significant downregulation of mitochondrial and metabolic pathways.

Mitochondrial dysfunction, however, is not only seen in HCM but also in a wide range of cardiac diseases, such as heart failure, ischemic heart disease, and coronary artery disease (13). In coronary artery disease, mutations in mitochondrial tRNA led to deficiencies in mitochondrial protein synthesis, elevating ROS and reducing ATP production (13). In ischemia, however, the upregulation of mitochondrial-related genes and enzymes leads to mitochondrial dysfunction, which in turn causes myocardial damage (13). Furthermore, mitochondrial dysfunction in ischemic heart disease occurs as a secondary effect of the initial ischemia, which induces ROS, inhibiting mitophagy - the degradation of damaged mitochondria - leading to cardiomyocyte apoptosis (13). In heart failure, protein deficiencies lead to inhibited ETC function, leading to dysfunction and cardiomyocyte apoptosis, while increased expression of other proteins suppresses mitochondrial function and energy production by inhibiting key regulators (13). These patterns show that mitochondrial dysfunction is seen in various cardiac diseases, but its early and intrinsic nature in HCM makes it a unique and key aspect in driving disease progression, meaning early targeting and prevention are key.

Targeting the downregulation of these mitochondrial pathways offers potential therapeutic strategies for HCM. Many commonly used therapies target mitochondrial health to treat heart conditions. For example, Elamipretide, a tetrapeptide drug, stabilizes complex 1 within the electron transport chain, restoring ATP production, stabilizing cardiolipin, and minimizing electron leakage in the electron transport chain, improving energy transfer in cardiac cells (6,11). NAD+ supplementation also significantly improves NADH-linked respiration in HCM tissue, increasing energy production and slowing disease progression (11). SGLT2 inhibitors primarily aid in restoring the previously disrupted balance between glycolysis and oxidative phosphorylation in heart failure patients, thus supporting cardiac health (10). Antioxidants like Coenzyme Q10 (CoQ10) and MitoQ reduce oxidative damage, improve mitochondrial function, and can enhance survival rates (10).

Other epigenetic factors, like DNA methylation, also play a crucial role in HCM progression. Our functional enrichment and DNA methylation analysis identified 121 genes that were both DEGs and DMGs, with *PINK1* as the key gene being downregulated in the mitochondrial pathways found. While hypomethylation is associated with increased gene expression, the dataset revealed that *PINK1* was hypomethylated yet downregulated in HCM in AS patients. This suggests that epigenetic modifications like DNA methylation do not solely account for the observed suppression of gene expression and may reflect the involvement of additional regulatory mechanisms such as histone modifications or chromatin remodeling. *PINK1* functional deficiencies impair mitophagy, reducing ATP function and compromising metabolism and mitochondrial quality, both of which are crucial for healthy cardiac function (14). Experimental models show that downregulation of *PINK1* increased cardiac hypertrophy by inhibiting mitophagy and promoting mitochondrial DNA release, which activated inflammatory signaling that exacerbated hypertrophy (15). This finding shows that boosting *PINK1* activation is a potential strategy for limiting HCM progression (15). These findings have implications for epigenetic regulation of non-sarcomere genes in HCM pathology, further supporting our hypothesis.

While our analysis reveals critical findings on mitochondrial dysfunction and energy deficits in HCM, several limitations should be noted. The small sample size (n=13 HCM patients, n=7 healthy controls) may limit the generalizability of our results. TPM values for boxplot visualization were derived from both datasets together; however, as both studies utilized left ventricle RNA-seq data from HCM patients, the samples are comparable for the purposes of directional expression visualization, though slight dataset differences may introduce variability in the HCM group distribution. Additionally, patient heterogeneity introduces variability that affects associations between mitochondrial dysfunction and clinical outcomes. The phenotypic diversity of HCM complicates the understanding of underlying mechanisms, leading to inconsistencies in result interpretations. Although we established correlations between

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HCM and mitochondrial dysfunction, causation cannot be definitively inferred, and the cross-sectional nature of this study limits insights into the progression of mitochondrial dysfunction over time, potentially missing critical insights into how these functional processes impact HCM patients

To address these limitations, future research should focus on larger, more diverse patient populations to enhance the generalizability of the findings. Longitudinal studies assessing treatments on HCM outcomes would be valuable in understanding the progression of mitochondrial dysfunction and the impact of other energy-related processes downregulation over time.

Targeted mitochondrial therapies present promising avenues for managing HCM. Additionally, incorporating energy-related therapies, including elamipretide, SGLT2 inhibitors, and empagliflozin, could significantly restore energy balance within hypertrophic cardiac cells. These targeted mitochondrial therapies could provide a novel approach to managing HCM and other related cardiomyopathies. Ultimately, these findings support our hypothesis that gene expression differences beyond the sarcomere play a critical role in HCM progression, highlighting new targets for novel treatment.

MATERIALS AND METHODS

Data availability

The datasets used in this study are all publicly available from the NCBI Gene Expression Omnibus (GEO). GSE180313 contains gene expression data of left ventricle samples from patients with HCM (n=13), and healthy controls (n=7), from a comprehensive multi-omics study (transcripts, metabolites, and complex lipids) (16). GSE207095 is a transcriptome data analysis coupled with DNA methylation profile analysis that includes samples from HCM patients (n=8) and AS patients (n=5) (17).

RNA sequencing data analysis

FastQC was utilized for quality control of the datasets, and all files were subsequently compiled into MultiQC (18,19). Low-quality reads – those with poor base quality scores and adapter contamination – were discarded. Trimmomatic was employed to trim adapter sequences (20). Following this, clean reads were then aligned to the human reference genome GRCh38.p14 (hg38) to create a raw count matrix. The raw counts were normalized to transcripts per million (TPM) to account for gene length and transcriptome size. Differential expression analysis was performed on the raw gene expression counts using DESeq2 (21). Comparative gene analysis was subsequently conducted using the DESeq2 results, along with Z-score transformation and column-wise scaling, followed by gene set enrichment analysis of gene ontologies using Web-based Gene SeT AnaLysis Toolkit (WebGestalt) (22). Genes were ranked by log2fold change from DESeq2 results and enrichment was assessed using the Gene Ontology (GO) Biological Process database, with significance determined by FDR correction < 0.05. To visualize and compare individual gene expression across groups, normalized TPM counts for *PINK1* and *MYH7* were plotted as boxplots across HCM samples from both datasets, with group differences assessed using a one-way Analysis of Variance (ANOVA) followed by Tukey's Honest Significant Difference (HSD) post-hoc test to determine pairwise significance between Healthy Controls, HCM, and AS patients.

Bisulfite sequencing data analysis

For the epigenetic analysis of HCM patients, pre-analyzed parallel bisulfite sequencing data were used alongside the transcriptomic datasets above. The dataset was originally derived from raw bisulfite sequencing data that was pre-processed and formatted from each patient to include HCM-related symptoms and biological markers such as maximum left ventricular wall thickness (17). Differentially methylated CpG sites in the myocardium of HCM patients were identified, recording chromosome location, coordinates, strand, probe type, CpG landmark, and associated gene (17). The dataset encompassed methylation data on 2,000,000 CpG sites, based on the Illumina Methylation EPIC platform, determining which CpG sites are included in the microarray (17).

Statistical analysis and data visualization

All statistical analyses and data visualizations were conducted using R version 4.3.3 (2024-02-29 ucrt). The following R packages were used: ggplot2 (v3.5.1), reshape2 (v1.4.4), ggpubr (v0.4.0), tidyverse (v2.0.0), broom (v1.0.9), viridis (v0.6.5), ggrepel (v0.9.5), grid (v4.3.3), and VennDiagram (v1.7.3) (23 - 31). WebGestalt 2024 was also used for their automatic figure production regarding functional gene analysis.

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