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ORIGINAL RESEARCH

Epigenetic Study of Cohort of Monozygotic Twins With Hypertrophic Cardiomyopathy Due to MYBPC3 (Cardiac Myosin-Binding Protein C)

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BACKGROUND: Hypertrophic cardiomyopathy is an autosomal dominant cardiac disease. The mechanisms that determine its variable expressivity are poorly understood. Epigenetics could play a crucial role in bridging the gap between genotype and phenotype by orchestrating the interplay between the environment and the genome regulation. In this study we aimed to establish a possible correlation between the peripheral blood DNA methylation patterns and left ventricular hypertrophy severity in patients with hypertrophic cardiomyopathy, evaluating the potential impact of lifestyle variables and providing a biological context to the observed changes.

METHODS AND RESULTS: Methylation data were obtained from peripheral blood samples (Infinium MethylationEPIC BeadChip arrays). We employed multiple pair-matched models to extract genomic positions whose methylation correlates with the degree of left ventricular hypertrophy in 3 monozygotic twin pairs carrying the same founder pathogenic variant (*MYBPC3* p.Gly263Ter). This model enables the isolation of the environmental influence, beyond age, on DNA methylation changes by removing the genetic background. Our results revealed a more anxious personality among more severely affected individuals. We identified 56 differentially methylated positions that exhibited moderate, proportional changes in methylation associated with left ventricular hypertrophy. These differentially methylated positions were enriched in regions regulated by repressor histone marks and tended to cluster at genes involved in left ventricular hypertrophy development, such as *HOXA5*, *TRPC3*, *UCN3*, or *PLSCR2*, suggesting that changes in peripheral blood may reflect myocardial alterations.

CONCLUSIONS: We present a unique pair-matched model, based on 3 monozygotic twin pairs carrying the same founder pathogenic variant and different phenotypes. This study provides further evidence of the pivotal role of epigenetics in hypertrophic cardiomyopathy variable expressivity.

Key Words: DNA methylation ■ epigenetics ■ HCM ■ monozygotic twins ■ *MYBPC3* pathogenic variant ■ phenotypic expressivity

Hypertrophic cardiomyopathy (HCM) is an inherited heart condition characterized by a left ventricular hypertrophy (LVH) not ascribable to other overloading conditions.[1–3] HCM is associated with myocardial fibrosis, diastolic dysfunction, and potential obstruction of the left ventricular outflow tract. Recent research has revealed abnormalities in calcium handling, fibroblast activation, fetal genes dysregulation, and impaired protein and energy homeostasis.[2,4–6] HCM follows an autosomal dominant inheritance

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CLINICAL PERSPECTIVE

What Is New?

- We evaluated a cohort of monozygotic twins, carriers of the same founder pathogenic *MYBPC3* variant but with different hypertrophic cardiomyopathy phenotype expression, and provided a unique model to isolate the environmental influence articulated by epigenetics from the genetic background.
- The epigenetic imprint of hypertrophic cardiomyopathy could be recapitulated in blood samples.
- We found that different environmental factors, such as lifestyle or a more anxious personality, could be related with more severe left ventricular hypertrophy via epigenic changes found in highly relevant genes for left ventricular hypertrophy, heart function, and stress.

What Are the Clinical Implications?

- Further studies to evaluate the epigenic-influence on hypertrophic cardiomyopathy expression are encouraged.
- Lifestyle changes or stress-targeted treatments may help to avoid the epigenetic negative adaptative alterations found in this cohort.

Nonstandard Abbreviations and Acronyms

CpGI	CpG islands
DMP	differentially methylated positions
HCM	hypertrophic cardiomyopathy
HOXA5	homeobox A5
MYBPC3	myosin-binding protein C, cardiac type
TRPC3	transient receptor potential cation channel, subfamily C, member 3

pattern, with genetic screening recommended for first-degree relatives of identified pathogenic variant carriers due to its penetrance and variable expressivity.[1] Accumulated abnormal protein is thought to increase energy expenditure, hampering cardiomyocyte function and prompting compensatory responses to maintain cardiac output, such as myocardial hypertrophy.[5,7] Furthermore, high levels of inflammatory cytokines and myocardium lymphoid infiltration promote disease progression.[8] Approximately half of patients with HCM present pathogenic variants at genes encoding heart sarcomere proteins, notably *MYBPC3* and *MYH7*, the former accounting for most cases with more than 500 reported pathogenic variants resulting in cMyBP-C (cardiac myosin-binding protein C) dysfunction.[2,9–11] In other cases, the disorder can be attributed to alterations in proteins involved in calcium handling or part of the cytoskeleton.[2]

However, in some patients, genetic testing fails to identify pathogenic variants. Genome-wide association studies have shown a strong polygenic influence in a significant portion of patients with sarcomere-negative HCM.[12] Modifiable risk factors like hypertension, obesity, or intense physical activity were associated with HCM development, suggesting a 2-hit model combining a genetic predisposition with environmental factors that trigger or modify its phenotypic expression.[12,13]

The lack of a consistent correlation between specific pathogenic variants and the resulting phenotype suggests the involvement of the epigenetic machinery.[12,14,15] Epigenetics refers to inheritable changes in gene expression that occur without altering the underlying DNA sequence. It plays a central role in dynamic biological processes such as differentiation or aging and integrates environmental stimuli with genomic information.[16–21] It mainly relies on changes in the methylation state of DNA cytosine nucleotides and various histone covalent modifications that together determine transcription machinery accessibility and ultimately regulate gene expression. DNA cytosine methylation tends to occur at symmetrical CpG dinucleotides.[22] Although CpG are typically methylated throughout the mammalian genome, clusters of unmethylated CpG called CpG islands (CpGI) often congregate at regulatory regions of actively transcribed genes and can be subject of global or site-specific changes during development and disease.[22] Functionally, high methylation rates at both transcription start sites and first exon or intron have been strongly linked to gene repression, whereas gene body methylation correlates with active transcription.[23,24] Although the role of DNA methylation in HCM has not been fully explored yet, recent studies demonstrated its implication in LVH. For instance, myocardial-specific *Dnmt1* knockout rat models showed an upregulation of pathways involved in myocardial protection, whereas samples of patients with HCM showed significantly high transcriptional levels of this gene.[25]

Understanding epigenetic interindividual variability plays a key role in unraveling how environmental factors regulate or trigger the phenotypic expression of a given disorder and how they induce the divergence in methylation patterns over time, or, alternatively, how a disorder may lead to the systemic dysregulation of DNA methylation landscape.[26,27] In this regard, the use of monozygotic twin models, which isolate the epigenetic regulation from genetic influences and age differences, can provide a valuable insight into the interplay between DNA methylation, environmental factors and the variable phenotypic course of HCM.[28,29] In this study,

we integrated clinical variables with blood methylation profiles of a cohort of 3 pairs of monozygotic twins carrying the nonsense pathogenic variant p.Gly263Ter at *MYBPC3* to study their distinct LVH (Figure 1).

METHODS

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Study Population

The study included 3 pairs[1–3] of monozygotic twins, consisting of a severely affected individual with HCM (P) and a mildly affected one (C) (Table). They were all carriers of NM_000256.3 (*MYBPC3*): c.787G>T (p.Gly263Ter) variant, a founder pathogenic variant in our region.[30] PowerPlex 16 HS System kit was used to confirm the genetic concordance of each twin pair, as reported elsewhere.[15] Moreover, pairs 1 and 2 were related by an aunt–nephew relationship, with demonstrated identical mitochondrial DNA.[15]

Data Acquisition

The study protocol was approved by the Local Ethical Committee and all participants signed the informed consent (2022.350). Clinical, demographic, and general lifestyle data were collected and anonymized by the Cardiology Department of the Hospital Universitario Central de Asturias.[15] Additional retrospective lifestyle variables were collected following the validated FANTASTIC questionnaire.[31,32] Cell type composition was predicted from DNA methylation data using the Houseman algorithm implemented in the *EpiDISH* package (v.2.14.1).[33] Statistical comparisons were performed with the nonparametric tests for paired samples using the statistical software R (v.4.2.2).

DNA Methylation Data Acquisition and Preprocessing

Genomic DNA methylation profiling of white blood fractions was performed with *Illumina Infinium Human MethyationEPIC v2.0 BeadChip* platform after bisulfite conversion following the EZ-96 DNA Methylation Kit conversion protocol (Zymo Research). All *MethylationEPIC BeadChip* data analyses were performed using the statistical software R (v.4.2.2). First, IDAT files were processed with the *minfi* package (v.1.44.0).[34] Self-reported sex and twin pair-belonging were validated from sex chromosome and single-nucleotide variant probes using the *getSex* and *getSnpBeta* functions from *minfi*. Probes were filtered out if (1) detection *P* value was >0.01 in any sample; (2) they were located in sex chromosomes; (3) they were cross-reactive or multimapping[35,36]; and (4) they included single-nucleotide variants with minor allele frequency ≥0.01 at their CpG or single base extension sites (dbSNP v.147). The intensity values from the 774 772 remaining probes were then subjected to background correction with *minfi* ssNOOB algorithm (offset=15, dyeCorr=TRUE and dyeMethod="single") and resulting β-values were normalized using the BMIQ method from R/Bioconductor package *ChAMP* (v2.28.0).[37,38] *M*-values were obtained by the logit transformation of the normalized β-values with the *beta2m* function from R/Bioconductor package *lumi* (v2.30.0) and were used for statistical purposes assuming homoscedasticity.[39] A surrogate variable analysis was performed to account for possible batch effects or confounding variables using the *sva* package.[40]

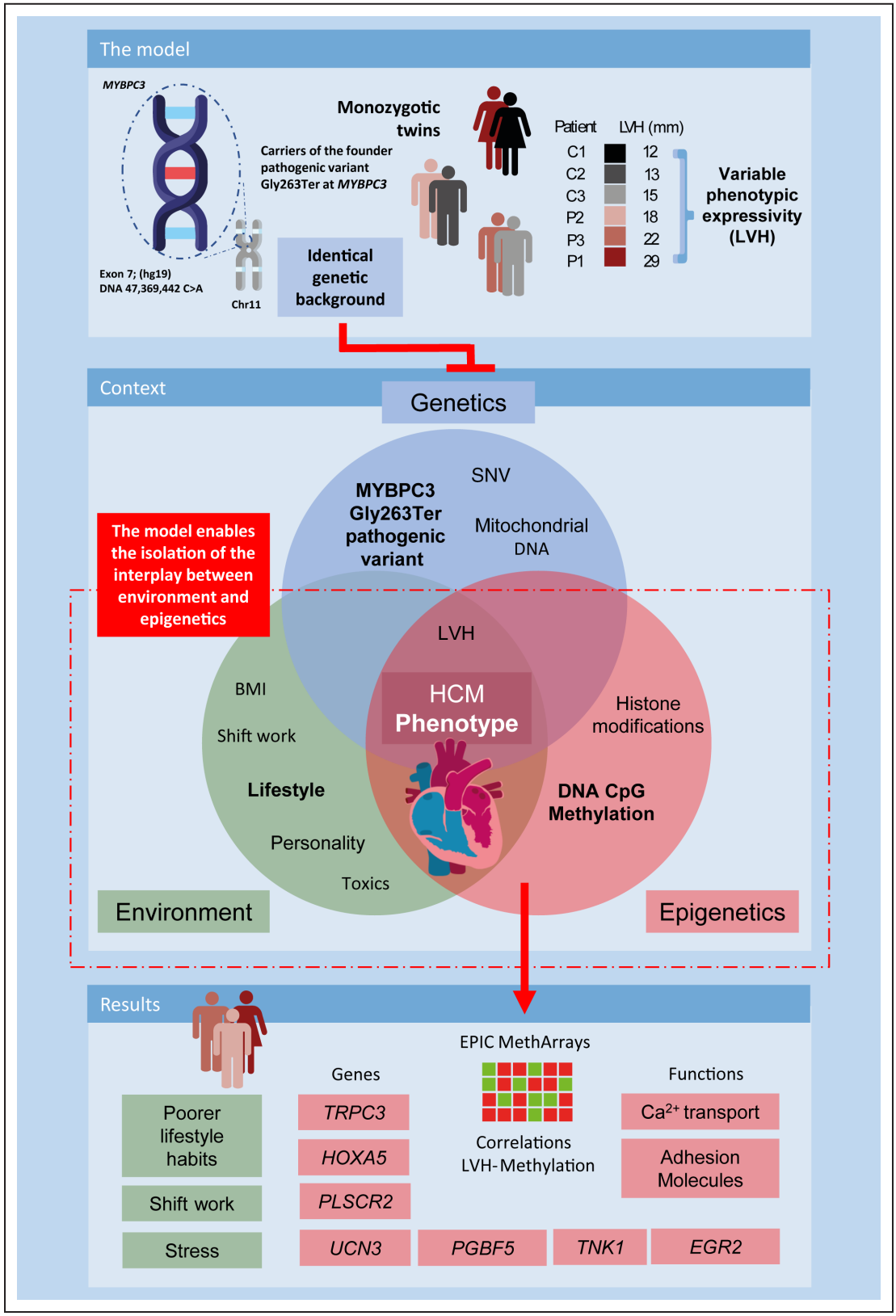
Differential Methylation Analysis

Linear mixed models were built using the *limma* package (v3.54.2) to detect differentially methylated positions (DMP) fitting *M*-values (dependent variable) and LVH in mm (independent variable). All models were pair matched and included neutrophil proportion as confounder to avoid cell type composition bias. DMP were defined by contrasting coefficients using an empirical Bayes-moderated *t* test and keeping those with |logFC|>0.20 (biological filtering) and an adjusted $P<0.05$ (statistical filtering). *P* values were corrected for multiple testing using the Benjamini–-Hochberg method for controlling the false discovery rate. Additionally, we defined biological DMP (bDMP) as the subset of positions resulting from applying only the biological filtering (|logFC|>0.20). The methylation profiles of 18 known HCM-causal genes (*MYBPC3, MYL2, MYL3, ALPK3, TNNT2, TNNI3, TNNC1, TPM1, ACTC1, PLN, FLNC, MYH7, JPH2, ACTN2, CSRP3, FHOD3, TRIM63, KLHL24)* were examined.[2,6] Corrected β-values were used for graphical purposes and expressed in terms of adjusted methylation (arbitrary units) after removing the effect of model confounders.

Enrichment Analysis

The *IlluminaHumanMethylationEPICanno.ilm10b4.hg19* package (v0.6.0) was used to assign each probe to its CpGI and gene location status. A single annotation was assigned to each region according to the following criteria (1) for CpGI status, Island>Shore>Shelf>OpenSea; and (2) for gene locations, 1stExon>Transcription start site (TSS)200>TSS1500>ExonBoundary>5′ untranslated region (UTR)>3'UTR>Body>Intergenic. TSS200 and TSS1500 were then grouped together as promoter and exon boundaries included into gene bodies.

A biological contextualization of bDMP was performed using R/Bioconductor *MissMethyl* package, as well as the Gene Ontology database.[41,42] Chromatin



enrichment analyses were performed with the R/Bioconductor package LOLA (v1.8.0).[43] bDMP enrichments in 6 histone marks (H3K4me1, H3K4me3, H3K27me3, H3K36me3, H3K9me3, and H3K27ac) were calculated using chromatin immunoprecipitation followed by sequencing tracks from different stem cell

Figure 1. Graphical description of the cohort, design, and results.
The upper section shows the model used, consisting of 3 pairs of monozygotic twins carrying the same pathogenic variant in *MYBPC3* but with differential expression of the LVH phenotype quantified in mm. The middle part shows the aim of comparing homozygotic, allowing a nongenetically biased study of the environmental influence on phenotypic expression through epigenetic changes. In the lower part, the results of clinical analysis and methylation profiling, showing differentially methylated genes, affected functions, and relevant health determinants in the development of pathology. BMI indicates body mass index; HCM, hypertrophic cardiomyopathy; LVH, left ventricular hypertrophy; *MYBPC3*, myosin-binding protein C, cardiac type; and SNV, single-nucleotide variant.

and tissue epigenomes obtained from the Encyclopedia of DNA Elements and the National Institutes of Health Roadmap Epigenome Consortia.[44,45] Chromatin state data from these same samples were obtained from the National Institutes of Health Roadmap's ChromHMM expanded 18-state model (obtained from http://egg2.wustl.edu/roadmap/). For the different comparisons, appropriate background including all filtered CpG probes interrogated by the MethylationEPIC BeadChip platform was used to calculate statistical significance. Odds ratio (OR) enrichment and statistical significance were calculated by means of 2-sided Fisher's tests in all analysis.

RESULTS

Exploratory Analysis

Initially, an exploratory analysis was conducted on clinical and lifestyle variables (Figure 2A). We observed a discrete drop in the estimated neutrophil proportions at the expense of the other leukocyte populations in severely affected twins (P=0.02). Overall, no statistical correlation between LVH and variables such as body mass index, tobacco and alcohol consumption, or years of intense physical activity was observed. However, severely affected twins showed a higher number of years working shifts (P=0.10) and a lower total FANTASTIC lifestyle assessment score (P=0.05) compared with their siblings. Accordingly, examining FANTASTIC questionnaire results across the explored dimensions, a general trend for severely affected twins to score lower was shown, representing a poorer lifestyle (Figure 2B), which could be statistically supported at stress (P=0.04) or toxic substance consumption (P=0.01) dimensions. Furthermore, these patients consistently reported in an open-ended question a worse tolerance to stressful situations and more anxious personalities compared with their siblings.

Regarding global methylation, we observed the expected bimodal distribution, with most probes showing a similar methylation fraction across cell types (gathering around 1 or 0 values) (Figure 3A). To get an overview of the degree of similarity regarding the DNA methylation profiles among individuals, a principal component analysis was performed using the total number of screened CpG. This nonsupervised analysis simplifies the complexity of multidimensional sample spaces into distinct principal components (PC) capable of explaining decreasing percentages of intersample variability. Most of the interindividual variability was contained in PC1 and PC2, together explaining approximately 85% of data divergence (Figure 3B). These PC clearly identified the 3 twin pairs, showing that the genetic background is the most relevant factor determining epigenetic differences between them. In addition, we found a striking proximity of pair 2 siblings when compared with pairs 1 and 3, which also reflects their less divergent phenotype. PC3 and PC5 were able to distinguish mildly from severely affected individuals but explained a much lower degree of intersample variability, indicating that the epigenetic differences underlying distinct LVH are of a much smaller magnitude (Figure 3C). This PC space preserves the relationship between cotwins, either in one or the other principal component.

Because methylation measurements (1) translate the mean value of the studied complex sample and methylation profiles differ between cell types, and (2) are mainly influenced by the genetic background as shown in Figure 3B, they were corrected considering a pair-matched model (removes genetic background) that includes neutrophil proportions (as estimated by Houseman deconvolution) as covariate to reduce bulk tissue heterogenicity. The relevance of these variables in the methylation profiles could be further supported through their correlation with the calculated surrogate variables that explain the variability in the methylation profiles not related to LVH (Figure 3D).

Analysis of Differentially Methylated Positions

Our patient set exhibited a continuous phenotype (LVH) spectrum (Figure 4A), prompting us to stratify methylation profiles based on their ventricular thicknesses and not to their affection (severely versus mildly); in other words, if methylation changes would reflect HCM expressivity, we would expect their intensity to proportionally vary with the increasing LVH. Therefore, DMP were extracted using pair-matched models that included LVH as independent variable and the estimated neutrophil proportion as confounder (Figure 4B). A total of 2486 bDMP were obtained, 1718 hypo- and 768 hypermethylated. Of them, 38 hypo- and 18 hypermethylated corresponded to DMP that showed an adjusted P value <0.05 (Figure 4C). Extracted DMP were sufficient to stratify our patients in an unsupervised analysis

Table. Clinical and Lifestyle Data

	Age, y	Phenotype severity	Twin pair	Sex	LVH, mm	Shift work years	Pack years	FANTASTIC test total	BMI
C1	89	Mild	1	F	12-13	0	0	118	26.7
P1	89	Severe	1	F	29	0	0	92	23.1
C2	47	Mild	2	M	13	1	31.25	91	27.7
P2	47	Severe	2	M	18	4	26	64	28.5
C3	49	Mild	3	M	15	0	0	99	21.7
P3	49	Severe	3	M	22	17	0	93	22.12

BMI indicates body mass index; and LVH, left ventricular hypertrophy.

according to their corresponding phenotypic expression, showing 2 main probe populations consisting of either hypo- or hypermethylated CpG (Figure 4D). Hypomethylated DMP were enriched at CpGI shores ($P<0.001$) and first exons ($P<0.05$), hypermethylated ones at intergenic regions ($P<0.05$) (Figure 4E).

To validate the consistency of the relation between the observed changes and the HCM phenotype, we included a cohort of 10 external peripheral blood samples from healthy donors not affected by HCM (GSE42861). The set of 56 DMP remained effective in accurately stratifying individuals after their phenotype on a principal component analysis (Figure 4F). Validation cohort DMP corrected methylation profiles were similar to those of mildly affected twins, thereby supporting the association of the observed changes with LVH development (Figure 4G).

Then, the adjusted methylation of CpG located within or in the vicinity (±1000 bp) of genes of known involvement in HCM was explored. *MYBPC3* presented no statistically significant changes (Figure 5A). Among all 18 known causal genes, only *JPH2* presented a single bDMP (Figure 5B), and no DMP were found.

The extracted DMP often clustered in regions with other CpG exhibiting similar changes, affecting genes encoding (1) the cell growth regulator TNK1 (tyrosine kinase nonreceptor 1; Figure 5C); (2) Ca^{2+} voltage-gated channel subunits, such as *CACNA1* and *CACNG2*, or

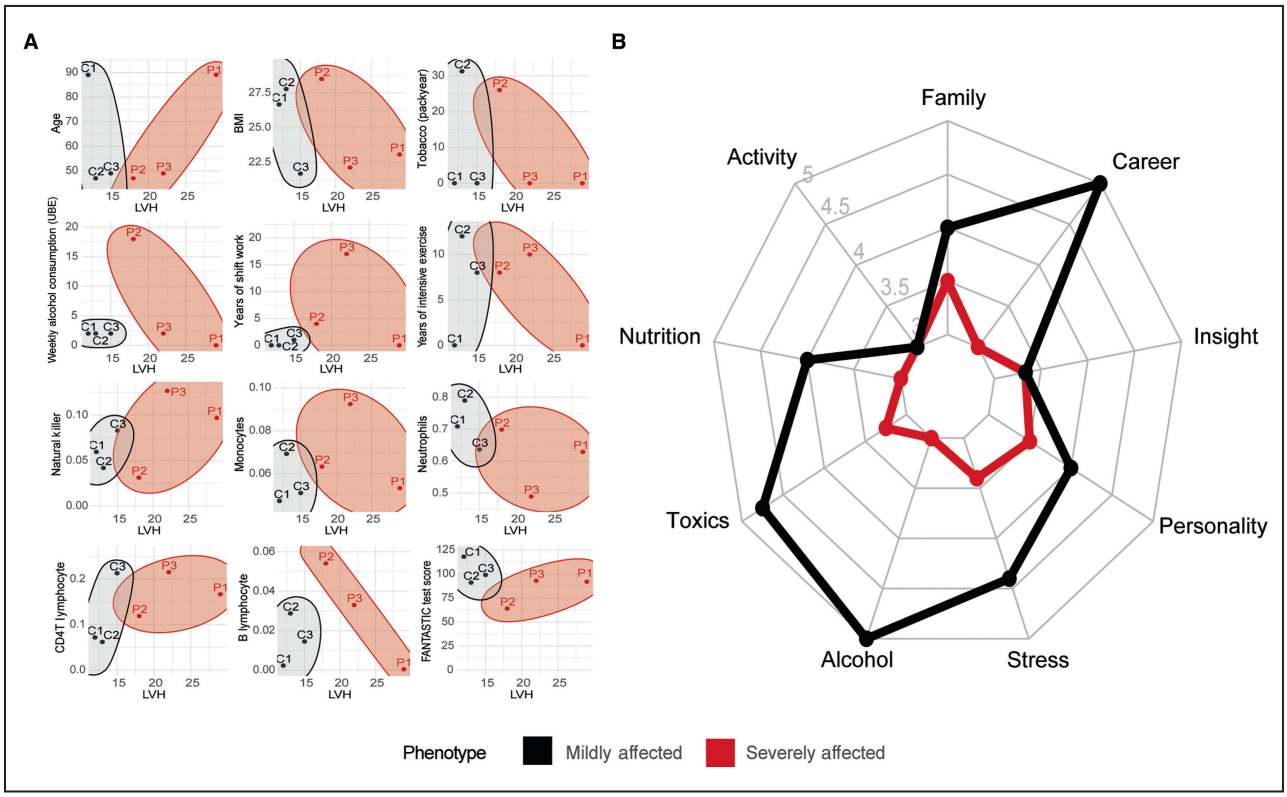


Figure 2. Exploratory analysis of clinical and lifestyle data.
A, Exploratory correlations between all sampled clinical and lifestyle variables against their corresponding LVH (mm). **B**, Radar plot with the FANTASTIC lifestyle test median scores for mildly and severely affected twins across assessed dimensions. BMI indicates body mass index; LVH, left ventricular hypertrophy; and UBE, standard drink of alcohol consumption.

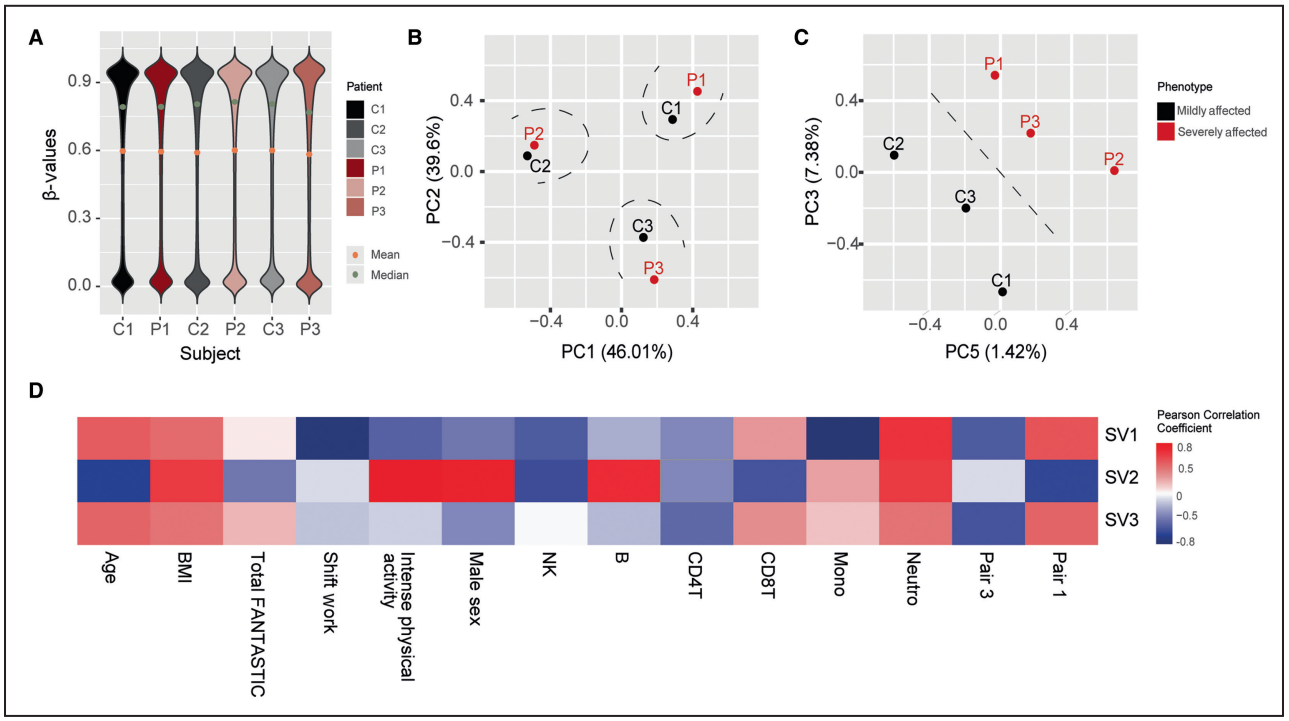


Figure 3. Exploratory analysis of DNA methylation data.
A, Violin plots depicting 5-methylcytosine distribution at screened probes. **B**, PCA for the 774 772 CpG sites across all samples included in the DNA methylation study. PC1-PC2 combination segregate epigenetic data after their genetic background; **C**, Same, but segregating data after their HCM phenotype at PC3-PC5 space. **D**, Pearson correlations between surrogate variables explaining epigenetic variability not ascribable to LVH differences and other collected variables. BMI indicates body mass index; HCM, hypertrophic cardiomyopathy; LVH, left ventricular hypertrophy; NK, natural killer; PC, principal component; PCA, principal component analysis; and SV, surrogate variable.

TRPC3 (Figure 5D), a Ca^{2+} sensing channel; (3) transcriptional regulators such as EGR2 (early growth response protein 2; Figure 5E), or HOX (homeobox) factors A3, A5 (Figure 5F) and A6; (4) surface adhesion molecules such as the PLSCR2 (phospholipid scramblase 2; Figure 5G) or multiple members coded by the protocadherin gene cluster, such as PCDHGB1 (protocadherin gamma), PCDHB1/5/14, or PCDHGA1/2/3; and (5) the PiggyBac transposase coded by *PGBD5* (Figure 5H), drug allergy-related proline rich protein PRR23B, or UCN3 (urocortin 3; Figure 5I), which belongs to the corticotropin-releasing family. These changes, although of moderate intensity, tended to collocate with elements of known regulatory function, such as CpGI or gene promoters.

DMP Enrichment Analysis

To assess cell functions affected by epigenetic changes that reflect the LVH phenotype and due to the low number of statistically significant DMP, the enrichment analysis was performed on the set of 2486 bDMP. Gene ontologies were used to estimate affected biological functions (Figure 6A). With high statistical significance and large gene ratios (percentage of genes affected in relation to the total number of genes related to a given biological function), gene ontologies showed enrichment (especially regarding hypomethylated bDMP) in genes involved in cell-to-cell contact and communication, either through homophilic surface adhesion molecules like protocadherins or through receptors and membrane ion transport channels.

Using chromatin immunoprecipitation followed by sequencing data of 6 histone marks across different cell cultures and tissues obtained from the Encyclopedia of DNA Elements and the National Institutes of Health Roadmap Epigenome Consortia, we looked for bDMP enrichments in genomic regions associated with specific histone modifications (Figure 6B). Regarding both hyper- and hypomethylation, the explored positions showed an up to 6-fold enrichment in DNA areas marked by H3K9me3, related to pericentromeric heterochromatin and necessary to the maintenance of genomic stability, and by H3K27me3, associated with inactive gene promoters. Based in the histone code theory, these findings could be ascribed to various chromatin states with different biological implications (Figure 6C). Both hypo- and hypermethylated bDMP showed a clear enrichment in states associated with ZNF (zinc finger proteins) repeats, gene clusters highly enriched in sequences encoding structurally similar ZNF family proteins frequently containing DNA-binding

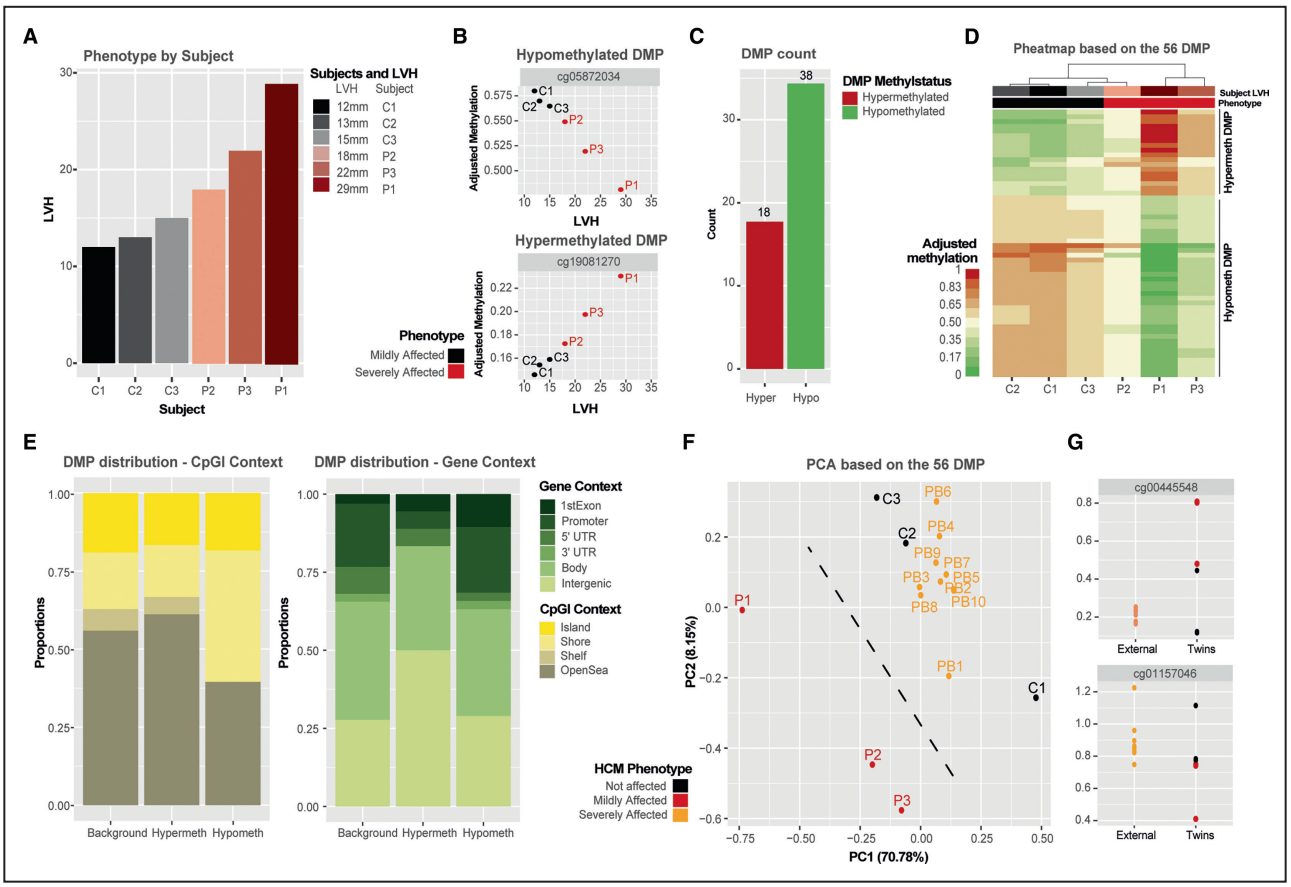


Figure 4. DMP analysis, contextualization, and validation.
A, LVH (mm) of all 6 individuals. **B**, Example of hypo- and hypermethylated DMP. DNA methylation was corrected by twin pair belonging and neutrophil proportion. **C**, Total number of hyper- and hypomethylated DMP (P<0.05). **D**, Heatmap generated using the 56 DMP. The methylation values have been normalized to the range 0 to 1 for each probe. The top bars show the phenotypic annotations, with a correct stratification of patients after their phenotype. **E**, Stacked barplots displaying the relative frequency of hyper- or hypomethylated DMP in relation to their CpGI (left) or gene (right) context. The background distribution of the array is included for interpretation purposes. **F**, PCA based on the methylation profiles of the 56 DMP from all 6 twins and an external cohort of 10 peripheral blood samples from healthy donors. **G**, Examples of 2 DMP in both twins and external healthy validation cohort. CpGI indicates CpG islands; DMP, differentially methylated positions; HCM, hypertrophic cardiomyopathy; LVH, left ventricular hypertrophy; PC, principal component; and PCA, principal component analysis.

domains. Hypermethylated bDMP were enriched in chromatin states controlled by polycomb repressors.

DISCUSSION

Environmental Exposition Triggers Methylation Pattern Divergence

The unstable phenotypic expression of HCM-related variants has been proposed to be governed by the environment–epigenetic interplay.[15] The strength of this work relies on the possibility of isolating the environmental influence on epigenetic patterns by studying a cohort of monozygotic twins, carriers of the same founder pathogenic variant, representing a unique model of controlled genetic background, as already shown by other authors.[21,46–49] More severely affected twins showed evidence of an overall worse lifestyle and a predisposition to modify their DNA methylation patterns at genes related to intercellular interactions and calcium handling, processes heavily involved in the mechanism of cardiac contraction.[2,4–6,12] DMP clustered within genomic elements involved in gene expression regulation, such as CpGI or first exon hypomethylation, a functional pattern closely associated with active gene transcription.[23]

Our data suggest that twin pairs with a greater clinical disparity, like twin pair 1, exhibit a more pronounced divergence in their methylation patterns. This could be explained by normal aging, this pair being 89 years old and, consequently, implying a longer exposure time to the environmental influences driving epigenetic drift. The divergences found in cotwins are subtle but must be considered in the context of an identical

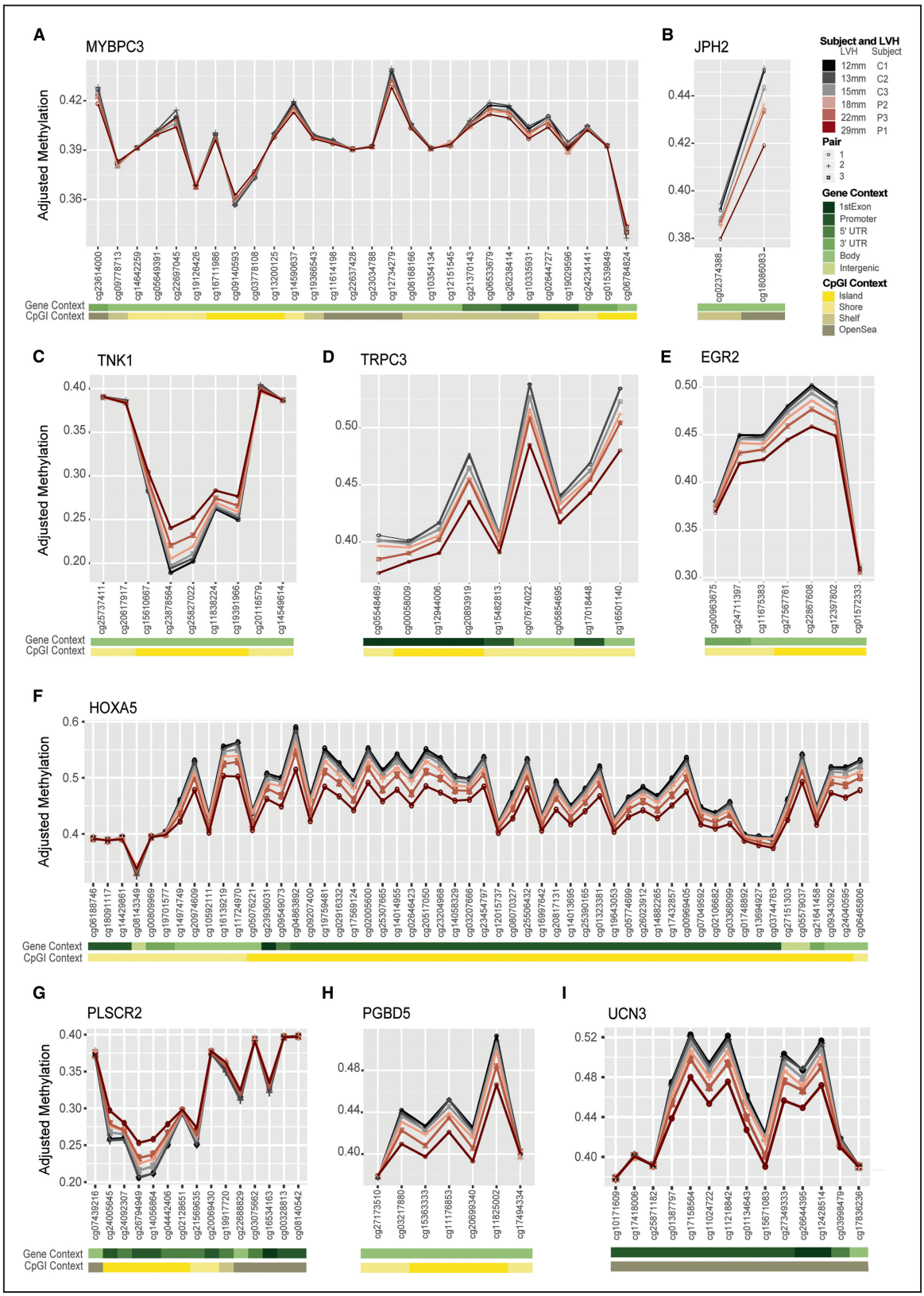


Figure 5. Gene candidates.

A, Adjusted methylation profiles at *MYBPC3*. Color code represents individual's phenotype. The genomic context of each CpG is expressed in the bars below according to their relationship with gene structure and local CpG density. **B**, bDMP at *JPH2*. **C** through **I**, Candidate gene adjusted methylation profiles. bDMP indicates biological differentially methylated positions; CpGI, CpG islands; and LVH, left ventricular hypertrophy.

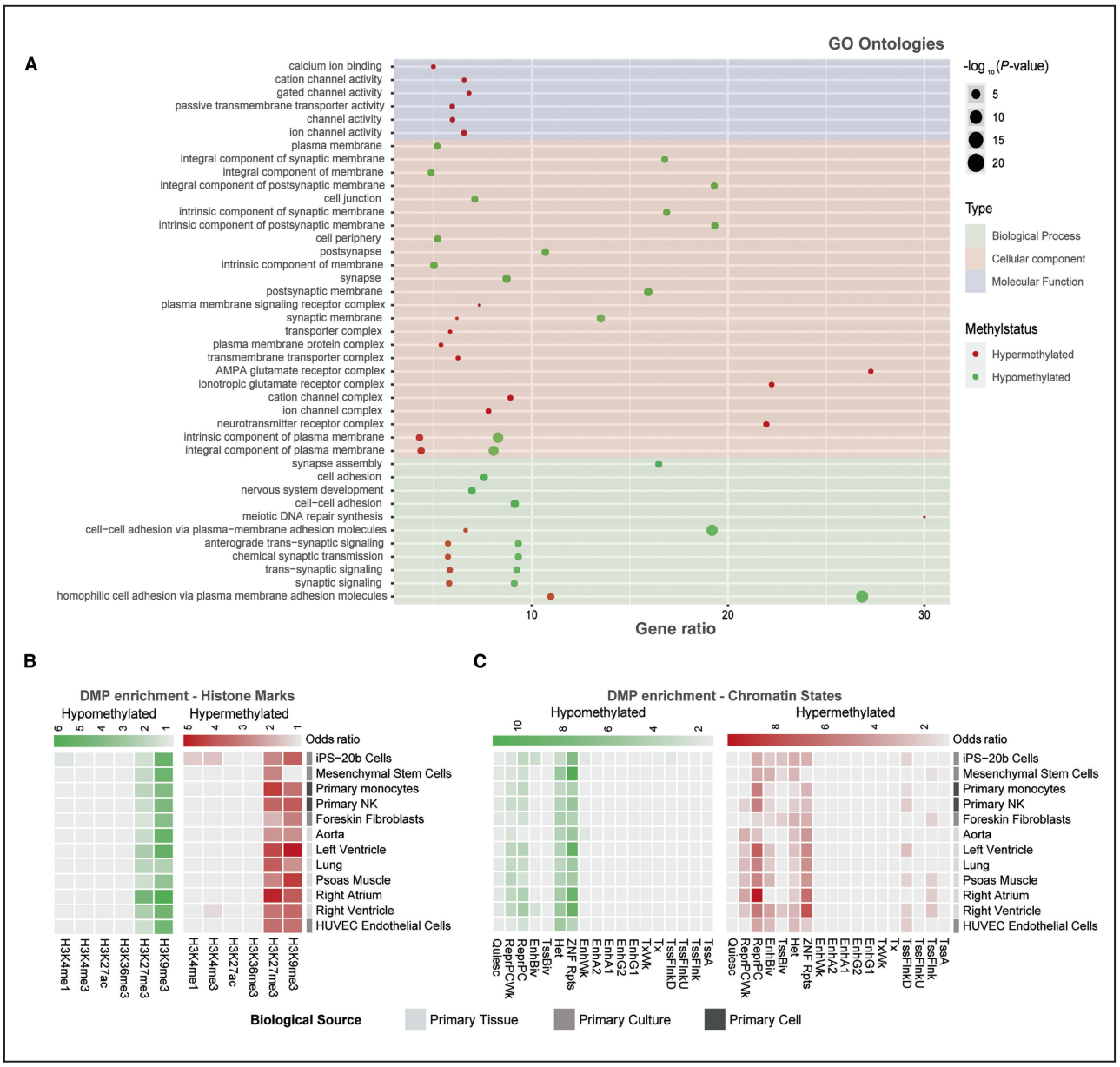


Figure 6. Enrichment analysis.
A, GO enrichment analysis of extracted bDMP at different annotated biological processes, cellular components, and molecular functions. Statistical signification is represented using different dot sizes, and the percentage of affected genes per category is represented by the gene ratio. **B**, Heatmap illustrating histone mark enrichment analyses of hyper- and hypomethylated bDMP. Color scales represent the odds ratio obtained across 6 common histone modifications from the NIH Roadmap Epigenome consortium as compared with the background distribution of the used platform. The legend indicates the biological origin of the used references for these comparisons. **C**, Same as (**B**) but displaying chromatin state enrichment analysis across 18 chromatin states obtained from the NIH Roadmap Epigenome consortium. bDMP indicates biological differentially methylated positions; DMP, differentially methylated positions; GO, Gene Ontology; HUVEC, human umbilical vein endothelial cell; NIH, National Institutes of Health; and NK, natural killer.

genetic background and a very similar environment and lifestyle.[15,21,28,46,47]

Candidate Genes and Their Potential Role in LVH

One key finding is that the extracted DMP were located at genes with known or feasible biological implication LVH development. This indicates, on the one hand, that the epigenetic machinery is indeed behind the connection between genotype and HCM phenotype, and on the other, that we might be able to recapitulate epigenetic changes associated with cardiac pathology at a systemic level, as functional epigenetic biomarkers.

To infer the influence of our findings, we have to resort to the typical behavior of DNA methylation.[24,50]

The binding of transcription factors onto regulatory elements, especially promoters containing CpGI, usually prevents maintenance DNA methyltransferases from methylating their CpG, whereas active gene bodies, already unfolded and accessible to the transcription machinery, are good targets for methyltransferases and tend to show higher methylcytosine levels.[24] Under these premises, we could estimate that, in more severely affected patients with HCM, genes that may prevent LVH could be downregulated, whereas pro-LVH genes could result upregulated.

For instance, we observed a marked CpGI hypermethylation at 2 genes widely related to LVH: *PLSCR2* and *TNK1*, translating a potential transcriptional repression. *PLSCR2* encodes a member of the phospholipid scramblase family, proteins that mediate calcium-dependent, nonspecific movement of membrane phospholipids and phosphatidylserine exposure.[51] It interacts with VCP (valosin-containing protein), a protein with cardioprotective properties against overload-related cardiac hypertrophy, so its epigenetic silencing could contribute to LVH.[51] *TNK1* is a negative regulator of the Ras-MAPK cascade,[52] a pathway that has proven responsible of LVH development in HCM mouse models.[53] Thus, its repression could explain the development of the phenotype in most affected twins.

On the contrary, other genes such as *HOXA5, TRPC3*, or *UCN3*, presented hypomethylated DMP at their regulatory elements, potentially resulting in their upregulation. A CpGI at *HOXA5* promoter region known to be bound by this transcription factor itself[54] showed extensive hypomethylation. Gene upregulation could lead to its own hypomethylation due to the steric hindrance of the maintenance methyltransferases at its binding site. *HOXA5* involvement in the development of HCM has been largely explored.[54–56] It controls *NEXN* expression, a gene coding a Z-disc protein involved in LVH.[57] Zhang et al. demonstrated the prohypertrophic role of *HOXA5* in murine models: cardiac-specific accumulation of HINT1, a suppressor of *HOXA5*, showed a cardioprotective effect that alleviates LVH.[58] Furthermore, *HOXA5* knockdown models impaired the cardioprotective effect of *HINT1* overexpression.[58] Under normal conditions, *HOXA5* should not be transcribed in myocardial tissue or peripheral blood. Its expression has been shown to be repressed by promoter hypermethylation or through binding of miRNA-196a to the 3'UTR of its transcript.[54] As stated, our data showed the hypomethylation of the CpGI that governs its expression in severely affected patients with HCM, which suggests a transcriptional activation of this prohypertrophic factor. Besides, *HOXA5* is part of the fibroblast differentiation cluster based on single-cell gene expression databases, so its upregulation could be related to the increased interstitial fibrosis observed in HCM as well.[59]

Similarly, *TRPC3* promoter CpGI hypomethylation could represent its upregulation. *TRPC3* encodes a short transient receptor potential channel that regulates reactive oxygen species production and intracellular Ca^{2+} homeostasis and that has been shown to be involved in cell growth, proliferation, and pathological hypertrophy.[60,61] Its prohypertrophic action has been demonstrated experimentally in different scenarios.[60–62] Combined blockade of *TRPC3* and *TRPC6* by selective small-molecules or genetic deletion inhibited pathological cardiac hypertrophy pathways in cardiomyocytes.[62] It has been shown to be responsible for basal Ca^{2+} levels and its activity leads to cell depolarization, affecting both the cardiac rhythm and neurohumoral regulation.[60,63,64] Not only that, this protein also promotes interstitial fibrosis by amplifying mechanical stress-induced reactive oxygen species signaling, eventually affecting all hallmarks of heart failure.[60,65] Additionally, *MYBPC3* is essential to constrain the myosin-actine cross bridging to sustain normal ejection in a Ca^{2+}-dependent fashion, so its deficiency results in an increased contractility, which sustains the pathophysiology of LVH in patients with HCM.[66,67] This is further promoted by the additional repression of *TRPC3*, because it promotes cardiomyocyte depolarization.[63]

Another very interesting finding was the hypomethylation of both the promoter and the first exon of *UCN3*, a less-known paralog of *UCN2* and *UCN1*.[68,69] UCN proteins are peptides associated with stress response that belong to the corticotropin releasing factor family.[69] UCN isoforms present an inotropic effect on myocardium and have been shown to improve cardioprotection after ischemia by preventing cardiac remodeling and maintaining Ca^{2+} homeostasis.[68,70] The mechanism of action of these promising candidates is still poorly understood, although it appears to be mediated by miRNA.[68,71] Together with their role in the development of the pathology, the more anxious temperament of the most affected twin of each pair, suggests a possible involvement of stress-related UCN proteins in LVH.

Central therapies with proven benefit in heart failure have largely focused on preventing the maladaptive neurohormonal systemic response.[51,65] Main therapies address the increased circulating levels of substances, like adrenaline or noradrenaline, and try to inhibit the maladaptive response, including sinus tachycardia, which further increases the myocardial oxygen demand and impairs myocardial perfusion.[65] The only lifestyle significant differences identified among cotwins were, in fact, stress related, the twin with the most severe LVH being the one who not only presented a poorer lifestyle but also a more anxious personality. In this regard, the identification of differential methylation at stress-related genes is extremely interesting provided the context of the clinical findings. This makes

us wonder whether this would be a possible preventive strategy to be addressed in future investigations.

Heart failure therapies address various cardiomyocyte impaired mechanisms, including contractility defects related to Ca^{2+} handling, disbalanced metabolism from β-oxidation to glycolysis, and reactive oxygen species overproduction.[65] Our study identified significant DNA methylation changes at genes related to all these functions, especially Ca2+ handling, so targeting these epigenetic alterations could potentially prevent or delay LVH progression.

As for the *EGR2* and *PGBD5* genes, it is difficult to make possible functional associations through hypomethylation of a CpGI in their gene body. However, we do know that EGR2 binds to several points of the HOXA cluster, altering the expression of the profibrotic factor HOXA4.[72] Its repression is mediated by miR-150 and attenuates maladaptive myocardial remodeling.[72]

As mentioned, we also found strong changes at the protocadherin gene cluster, as also revealed by gene ontologies. Protocadherins are homophilic surface adhesion molecules involved in cell-to-cell junctions and cytoplasmic signal transduction.[73] They are well described to be expressed in a combinatorial fashion to specify neuronal identity for coding synaptic connectivity and to gather stochastic methylation in developing neurons.[29] Their role in the development of congenital heart defects has recently been reported and they appear to be neatly regulated throughout the cardiovascular system.[73] We found up to 28 bDMP in the cluster associated with *PCDHGA3*, mainly within its first exon and promoter by hypermethylation. This protein has been shown to be part of the intercalated disks, essential for the contractile and coordinated function of the myocardium.[74] Changes in *PCDHGA3* were strongly associated with a fall in stroke volume and ventricular dysfunction: the higher its expression, the greater left ventricular end-systolic diameter.[74] Planterose-Jiménez et al. also found protocadherin loci to accumulate DNA methylation variability between monozygotic cotwins in an universal epigenetic interindividual dissimilarity.[29] They also found that almost half of the affected CpG in peripheral blood were also affected in adipose tissue, consistent with the idea of capturing tissue-specific shifts at the systemic level.[29] The nature of the many protocadherins as individual epigenetic fingerprints and the fact that they accumulate—as demonstrated both in our work and in previous literature—methylation alterations suggest their role in the interplay between environment and phenotype.

In summary, our results show, on the one hand, that the gradual LVH corresponds to proportional modifications on DNA methylation levels affecting regions involved in the development of the pathology, and, on the other hand, that the imprint of HCM could be recapitulated in blood samples.

The Potential Role of Other Actors

Environmental stimuli are also able to articulate phenotypic variation through changes in other epigenomic layers, for instance affecting regions controlled by particular histone marks.[26,75,76] In our case, the tendency for selective hypo- and hypermethylation of areas regulated by the repressor marks H3K27me3 and H3K9me3 may indicate the upregulation of otherwise silenced genes in relation to the development of LVH. The involvement of H3K9me3 in LVH has already been described: its suppression contributes to ventricular mass growth in murine models through activation of *FHL1*, a key molecule in the development of HCM.[26] Furthermore, another study supported its role in LVH by demonstrating that fluid overload led in ventricular tissue to H3K9me3 depletion *NPPA* and *NPPB* promoters, 2 hallmark genes for LV maladaptive remodeling.[26]

Study Limitations

There are also some technical limitations that prevented us from investigating causal relationships for the varying expressivity of the studied mutation, located at *MYBPC3*. These include sample size, which likely limited the power to detect more subtle alterations; cellular heterogeneity; the bulk view provided by the array that yields average methylation of the 3 alleles of multiple cell types; and, most important, the fact of using peripheral blood samples to target a myocardial-related pathology.

Although the external validation supports the relation between obtained DMP and the studied phenotype, we cannot know if DMP are present in their myocardium counterparts and underpin the cause of HCM expressivity, or whether they are a systemic consequence of an increased LVH. However, phenotypic differences are proportionally reflected in the peripheral blood methylation patterns. The fact that *MYBPC3* and other HCM causal genes are not expressed in peripheral blood may explain why no changes could be observed in their methylation patterns, as this epigenetic mark has a known functionality and is not usually subject to regulation at inactive regions.[24] We cannot discard the presence of differential methylation or imprinting phenomena at these genes within cardiomyocytes and further studies are needed to shed light on this matter.

These facts do not undermine the value of our findings, which are able to demonstrate systemic changes in DNA methylation at various positions along with the increased severity of HCM. Project follow-ups should focus on the obtention of samples of paired cardiac tissue, the validation of our findings in an external cohort of patients carrying the studied mutation, the performance of allele-specific analysis of *MYBPC3*, and even the integration of methylation data with expression

profiles to look for further functionality in the target tissue.

CONCLUSIONS

We present a unique pair-matched model, based on 3 monozygotic twin pairs carrying the same founder pathogenic variant (*MYBPC3* p.Gly263Ter) and different phenotypes. Thanks to the possibility to remove the genetic background we were to isolate the environmental influence, beyond age, on DNA methylation changes. The epigenetic imprint of HCM could be recapitulated in blood samples.

We found that different environmental factors, such as lifestyle or a more anxious personality, could promote the development of a more severe LVH. Moreover, we found a moderate number of epigenetic changes correlating with phenotype severity that were located in highly relevant genes for LVH, heart function, and stress.

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Disclosures

None.

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