Original Article

Multinational experience with next-generation sequencing: opportunity to identify transthyretin cardiac amyloidosis and Fabry disease

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Background: Sarcomeric hypertrophic cardiomyopathy (HCM) must be differentiated from phenotypically similar conditions because clinical management and prognosis may greatly differ. Patients with unexplained left ventricular hypertrophy require an early, confirmed genetic diagnosis through diagnostic or predictive genetic testing. We tested the feasibility and practicality of the application of a 17-gene next-generation sequencing (NGS) panel to detect the most common genetic causes of HCM and HCM phenocopies, including treatable phenocopies, and report detection rates. Identification of transthyretin cardiac amyloidosis (ATTR-CA) and Fabry disease (FD) is essential because of the availability of disease-specific therapy. Early initiation of these treatments may lead to better clinical outcomes.

Methods: In this international, multicenter, cross-sectional pilot study, peripheral dried blood spot samples from patients of cardiology clinics with an unexplained increased left ventricular wall thickness (LVWT) of

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≥13 mm in one or more left ventricular myocardial segments (measured by imaging methods) were analyzed at a central laboratory. NGS included the detection of known splice regions and flanking regions of 17 genes using the Illumina NextSeq 500 and NovaSeq 6000 sequencing systems.

Results: Samples for NGS screening were collected between May 2019 and October 2020 at cardiology clinics in Colombia, Brazil, Mexico, Turkey, Israel, and Saudi Arabia. Out of 535 samples, 128 (23.9%) samples tested positive for pathogenic/likely pathogenic genetic variants associated with HCM or HCM phenocopies with double pathogenic/likely pathogenic variants detected in four samples. Among the 132 (24.7%) detected variants, 115 (21.5%) variants were associated with HCM and 17 (3.2%) variants with HCM phenocopies. Variants in *MYH7* (n=60, 11.2%) and *MYBPC3* (n=41, 7.7%) were the most common HCM variants. The HCM phenocopy variants included variants in the *TTR* (n=7, 1.3%) and *GLA* (n=2, 0.4%) genes. The mean (standard deviation) ages of patients with HCM or HCM phenocopy variants, including *TTR* and *GLA* variants, were 42.8 (17.9), 54.6 (17.0), and 69.0 (1.4) years, respectively.

Conclusions: The overall diagnostic yield of 24.7% indicates that the screening strategy effectively identified the most common forms of HCM and HCM phenocopies among geographically dispersed patients. The results underscore the importance of including ATTR-CA (*TTR* variants) and FD (*GLA* variants), which are treatable disorders, in the differential diagnosis of patients with increased LVWT of unknown etiology.

Keywords: Fabry disease (FD); hypertrophic cardiomyopathy (HCM); next-generation sequencing (NGS); transthyretin cardiac amyloidosis (ATTR-CA)

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Introduction

The 2020 American College of Cardiology/American Heart Association guidelines define hypertrophic cardiomyopathy (HCM) as "a maximal end-diastolic wall thickness of ≥15 mm anywhere in the left ventricle in the absence of another cause of hypertrophy in adults" (1). The hallmark of HCM is its clinical heterogeneity, ranging from a benign asymptomatic state to arrhythmias, heart dysfunction, and sudden cardiac death (SCD) (2). In young adults, the most common etiology of HCM is SCD (3). The global prevalence of HCM among adults is 0.16–0.29% (~1:625–1:344 individuals). An even higher prevalence of 0.6% (1:167) has been reported with sensitive radiological imaging techniques, evaluation of family members, and greater use of genetic testing (4).

Variations in genes encoding cardiac sarcomere proteins account for up to 60% of HCM cases in adults (5). HCM is regarded as a monogenic cardiac disease; however, it exhibits extensive genetic heterogeneity. Due to this genetic diversity, the pathogenic genes remain unidentified in approximately 40% of HCM patients (2,5). Many genes other than sarcomeric genes have been associated with HCM; therefore, HCM is no longer considered monogenic in origin and may be due to polygenicity or oligogenicity (6,7).

Certain diseases that mimic HCM are referred to as HCM phenocopies (2). These include conditions such as protein kinase adenosine monophosphate-activated noncatalytic subunit gamma 2 (PRKAG2) cardiomyopathy, glycogen storage disorders such as Danon disease, cardiac amyloidosis, lysosomal storage disorders [e.g. Fabry disease (FD)], and other inborn errors of metabolism (4). Transthyretin cardiac amyloidosis (ATTR-CA) and FD are caused by pathogenic variants of *TTR* (18q12.1) and *GLA* (Xq22.1), respectively (8-10), and increased left ventricular wall thickness (LVWT) is a key diagnostic feature of both disorders (8-11). Amyloid cardiomyopathy results from extracellular deposits of amyloid in the myocardium, leading to symptoms of chest pain, arrhythmia, and sudden death (10). Clinically, FD may present as cardiac hypertrophy, arrhythmias, fibrosis, and heart failure due to reduced α-galactosidase A enzyme activity (8). It is crucial to differentiate sarcomeric HCM and phenotypically similar conditions because the prognosis and management differ greatly as cause-specific therapies are available (12,13).

Hence, clinicians should consider the presence of HCM phenocopies in patients with unexplained left ventricular hypertrophy (LVH) who do not meet the standard HCM diagnostic criteria, as well as predictive genetic testing in relatives of a patient with confirmed HCM or more limited hypertrophy (13–14 mm) (1). Our study explored the feasibility and practicality of the application of a 17-gene next-generation sequencing (NGS) panel to detect gene variants that cause the most common forms of HCM and HCM phenocopies in patients with unexplained increased LVWT from six countries and detection rates for pathogenic/likely pathogenic variants are reported.

Methods

Sample population

The study population for this cross-sectional study included subjects from cardiology clinics located in Colombia, Brazil, Mexico, Turkey, Israel, and Saudi Arabia. Countries were selected based on interest in participation, the potential pool of undiagnosed patients in the cardiology domain, and the ability to execute the study. All participating institutions were informed and agreed to the study. From the study population, 544 samples were received by the Diagnósticos Laboratoriais Especializados (DLE) between May 2019 and October 2020. However, due to preanalytical issues and the absence of informed consent, only 535 samples were analyzed for this study.

Highlight box

Key findings

- A 17-gene next-generation sequencing (NGS) panel identified hypertrophic cardiomyopathy (HCM) variants (21.5% of 535 samples) and HCM phenocopy variants (3.2%) among patients with unexplained increased left ventricular wall thickness (LVWT), with an overall diagnostic yield of 24.7%.

What is known and what is new?

- A genetic diagnosis for patients with LVWT of unknown origin by either diagnostic genetic testing or predictive genetic testing aids in determining the prognosis and allows for timely management.
- The 17-gene NGS panel, using dried blood spot samples, effectively detected gene variants that cause the most common forms of HCM and HCM phenocopies, including the treatable disorders transthyretin cardiac amyloidosis and Fabry disease, in patients with increased LVWT from six countries.

What is the implication, and what should change now?

- This 17-gene NGS screening strategy has the potential to reduce the diagnostic challenges experienced by patients with unexplained increased LVWT and facilitate personalized care.

Inclusion criteria

Patients with an increased LVWT of ≥13 mm in one or more left ventricular myocardial segments (as measured by any imaging method—computed tomography, cardiac magnetic resonance imaging, or echocardiography), in the absence of abnormal loading disease conditions that justify increased LVWT, were included in the study.

Exclusion criteria

Patients with increased LVWT due to severe hypertension (defined as systolic blood pressure ≥180 mmHg and/or diastolic blood pressure ≥120 mmHg), severe aortic stenosis (≤1 cm^2), or genetically confirmed etiology of increased LVWT were excluded from the study.

Ethical approval and patient consent to participate

This study was performed in accordance with the ethical principles of the Declaration of Helsinki (as revised in 2013). The central laboratory study was approved by Institute of Childcare and Pediatrics Martagão Gesteira/ Federal University of Rio de Janeiro under approval No. CAAE 53630421.3.0000.5264. Signed informed consent forms for blood sampling and analysis were obtained at the participating sites from all patients included in the study.

Procedures

Peripheral dried blood spot (DBS) samples were collected from patients onto a filter paper and stored at room temperature. Demographic details, including age, gender, and country of origin, were simultaneously recorded. Clinical and cardiac imaging data of patients were not collected in this pilot study. Deoxyribonucleic acid (DNA) from blood spots was extracted automatically using a QIAsymphony Investigator Kit® (Qiagen®). Both DBS and extracted DNA were kept refrigerated (4 ℃). Anonymized samples were analyzed at the DLE.

Sequencing analysis

In selecting genes to be included in the NGS panel, the global prevalence, national and regional epidemiology,

Table 1 A 17-gene panel to detect hypertrophic cardiomyopathy and its phenocopies

Gene	Locus	Exons sequenced	OMIM	HCM or HCM phenocopy
ACTC1	15q14	7	102540	HCM
TNNI3	19q13.42	8	191044	HCM
MYBPC3	11p11.2	35	600958	HCM
MYH7	14q11.2	40	160760	HCM
MYL2	12q24.11	7	160781	HCM
MYL3	3p21.31	7	160790	HCM
TPM1	15q22.2	14	191010	HCM
TNNT2	1q32.1	17	191045	HCM
TNNC1	3p21.1	6	191040	HCM
PRKAG2	7q36.1	22	602743	HCM phenocopy
PTPN11	12q24.13	16	176876	HCM phenocopy
TTR	18q12.1	4	176300	HCM phenocopy
LAMP2	Xq24	10	309060	HCM phenocopy
GLA	Xq22.1	7	300644	HCM phenocopy
PLN	6q22.31	2	172405	HCM phenocopy
FLNC	7q32.1	48	102565	HCM phenocopy
DES	2q35	9	125660	HCM phenocopy

OMIM, Online Mendelian Inheritance in Man; HCM, hypertrophic cardiomyopathy.

and local technical capabilities were considered. The pathogenicity of variants was classified according to the consensus recommendations published by the American College of Medical Genetics and Genomics (ACMG) and the Association for Molecular Pathology (AMP) (14,15) into categories such as pathogenic, likely pathogenic (probability greater than 90% of being pathogenic), uncertain significance, likely benign (more than 90% likely to be benign), and benign. ACMG uses a series of criteria to establish a scoring system based on variant information (e.g., protein effect, information in literature, position in the transcript, functional assays, prediction software, and database). The variant classification is determined based on the presence or absence of certain traits. A 17-gene panel was developed to detect known splice regions and flanking regions (20 base pairs adjacent to each exon) of the targeted genes using DBS samples. Information on the 17 genes included in the panel is presented in *Table 1*.

Library preparation and NGS

Initially, the dilution of genomic DNA (10–500 ng) was performed with ultrapure water. Genomic libraries were prepared according to instructions provided by Agilent (Agilent Sure Select XT HS and XT Low Input Custom 1-499 kb 96 reactions Design ID: 3223981). Qualitative and quantitative validation of all DNA libraries was performed using an automatic gel electrophoresis method (TapeStation D1000 Screen Tape) and a fluorescence-based measurement method (Qubit™ dsDNA HS Assay Kit), respectively.

SureSelect XT HS and XT Low Input were used for pooling and hybridizing successfully validated DNA libraries to synthesize probes that were in accordance with the human genome reference sequence version GRCh37 (hg19). In-house-designed, biotin-labeled oligonucleotide probes were generated for binding with targeted gene sequences.

The Agilent SureSelect XT HS and XT Low Input

Table 2 Demographics of patients with hypertrophic cardiomyopathy and its phenocopies

	Overall[a]	HCM + HCM phenocopies	HCM[b]	HCM phenocopies[c]	ATTR-CA	FD
N	535	128	113	17	7	2
Mean age (SD), years	49.9 (17.7)	42.8 (17.9)	42.0 (17.4)	46.5 (20.1)	54.6 (17.0)	69.0 (1.4)
Median age [range], years	51 [4–92]	43 [4–85]	43 [4–85]	46 [11–73]	44 [31–73]	69 [68–70]

[a], excluded were samples with missing (n=5) or incorrect age (n=5); [b], HCM variants: *MYBPC3*, *MYH7*, *TNNI3*, *TNNT2*, *TNNC1*, *TPM1*, *MYL3*, *MYL2*, and *ACTC1*; [c], HCM phenocopy variants (this includes two patients with double mutations, each with an HCM variant and an HCM phenocopy): *DES*, *GLA*, *FLNC*, *LAMP2*, *PLN*, *PRKAG2*, *PTPN11*, and *TTR*. HCM, hypertrophic cardiomyopathy; ATTR-CA, transthyretin cardiac amyloidosis; FD, Fabry disease; SD, standard deviation.

preparation automation guide (Agilent) was utilized to prepare the target-captured library as per the manufacturer's protocol, with slight modifications for samples on DBS. In addition, the automatic gel electrophoresis method (TapeStation D1000 Screen Tape) was employed to identify the final, size-adjusted concentration of the target-captured DNA fragments.

The sequencing of target-enriched DNA fragments was performed by Illumina NextSeq 500 and NovaSeq 6000. These sequencers carried out the sequencing via pair-end and two 150-base pair reads having an average coverage of a minimum of 20× to ensure the quality of the data. A fully characterized positive control was included in each sequencing run.

NGS quality and data analysis

High-end quality control (QC) procedures were conducted along the sequencing process to identify the samples. Apart from sample identification, these procedures were also performed to ensure good quality of isolated DNA, library preparation, target capture, and sequencing. Furthermore, mapping and variant QC metrics were computed and used on the sequencing output to exclude and rerun failed samples. A bioinformatics analysis pipeline was utilized to align the sequencing data against the human genome reference sequence version GRCh37 (hg19) and obtain relevant information. An in-house software ('DLE-Tool') that uses Burrows-Wheeler for alignment (0.7.15), variant calling (single-nucleotide variants and indels) based on mpileup (1.3.1), and Ensembl Variant Effect Predictor (v105) annotation was employed to assess the pathogenicity [or variant of uncertain significance (VUS)] of all identified variants. Franklin variant interpretation (Genoox) was used especially in cases with novel variants.

Statistical analysis

Demographic and gene variant data were summarized using descriptive statistics.

Continuous variables were described using mean with SD and median with range, and N was calculated for categorical variables.

Results

A total of 535 samples from patients across Colombia, Brazil, Mexico, Turkey, Israel, and Saudi Arabia were analyzed [311 (58.1%) males, 222 (41.5%) females, and gender not recorded for 2 (0.4%) individuals]. *Table 2* provides the demographic characteristics of the included patients.

HCM and HCM phenocopy variants considered to be pathogenic/likely pathogenic were identified in a total of 128 patient samples (23.9%). In four of the 128 samples (0.7%), a second pathogenic/likely pathogenic variant (associated with HCM in two samples and with an HCM phenocopy in two samples) was detected in addition to the HCM variant. Among the 132 detected variants, 115 (21.5%) variants were associated with HCM and 17 (3.2%) variants with HCM phenocopies. The mean [standard deviation (SD)] age of the 128 patients was 42.8 (17.9) years. The distribution of variants by country is shown in *Figure 1*, and classifications of pathogenicity and nucleotide and amino acid changes are provided in Table S1.

Of the patients with pathogenic/likely pathogenic HCM variants (n=113), 56 patients (49.6%) were male, and 57 patients were female (50.4%). Overall, the most prevalent HCM variants were *MYH7* (n=60, 11.2%) and *MYBPC3* (n=41, 7.7%) (*Figure 2*).

HCM phenocopy variants considered to be pathogenic/likely pathogenic were identified in 17 patients [9 males

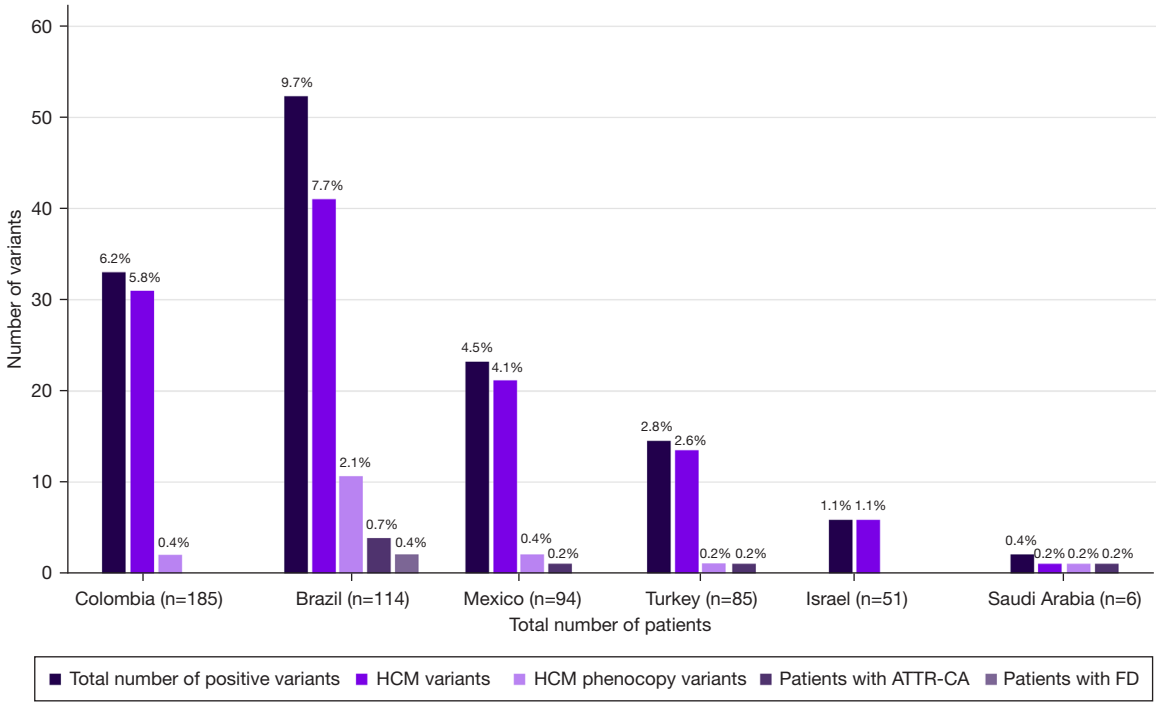

Figure 1 Distribution of pathogenic/likely pathogenic variants associated with hypertrophic cardiomyopathy or its phenocopies within the total population. HCM, hypertrophic cardiomyopathy; ATTR-CA, transthyretin cardiac amyloidosis; FD, Fabry disease.

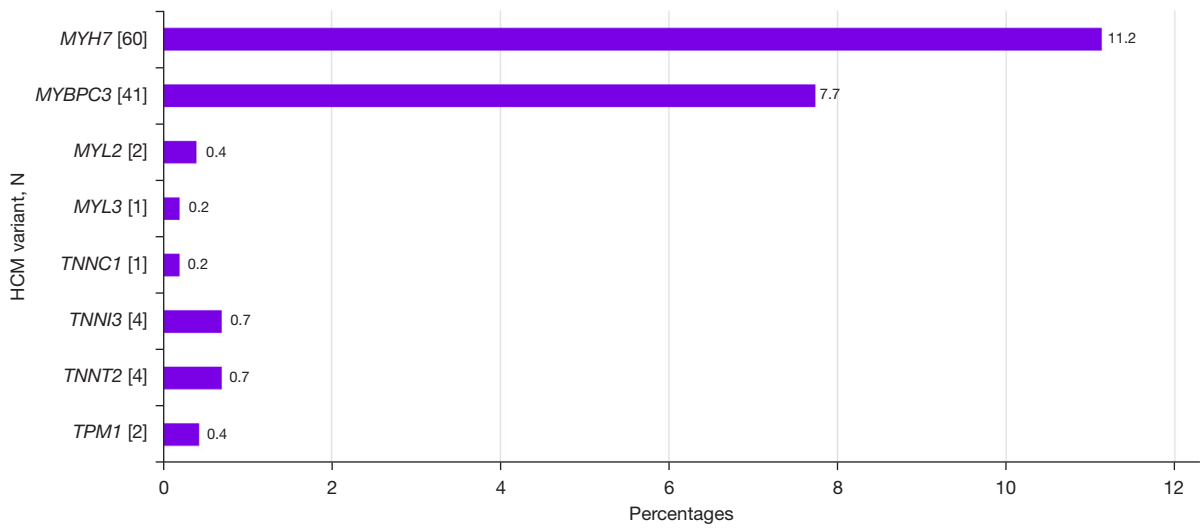

Figure 2 Frequencies of patients with confirmed hypertrophic cardiomyopathy gene variants. No samples tested positive for *ACTC1*.

(52.9%) and 8 females (47.1%)]. *TTR* variants were the most prevalent HCM phenocopy variants (n=7; 1.3%) and were found in 5 males and 2 females [mean (SD) age 54.6 (17.0) years]. Other pathogenic/likely pathogenic HCM phenocopy variants detected in more than one sample included *LAMP2* (n=3, 0.6%), *DES* (n=2, 0.4%), and *GLA* variants (n=2, 0.4%). The *GLA* variants were found in female patients [mean (SD) age 69.0 (1.4) years] and were predicted to be associated with the 'classic' phenotype (c.413delG) and a 'later-onset' phenotype of FD (c.644A>G)

according to the International Fabry Disease Genotype–Phenotype (dbFGP) database (http://dbfgp.org/dbFgp/fabry/Mutation.html), which was available online during the assessment of data.

Discussion

HCM is the most common inherited form of cardiomyopathy and is associated with considerable genotypic variations. Advances in gene sequencing provide opportunities to improve diagnostic certainty and differentiate genetic sarcomeric HCM from phenocopies (16). However, as a result of an incomplete understanding of the genetic variants and their pathogenicity, patients may remain with a diagnosis unconfirmed by molecular diagnostics (5). Establishing an early diagnosis is essential as it allows the determination of the prognosis and appropriate therapeutic intervention. In this pilot study of 535 patients with unexplained increased LVWT, the 17-gene NGS panel showed a diagnostic yield of 24.7% for pathogenic/likely pathogenic variants associated with HCM or HCM phenocopies.

The diagnostic yield of the panel for confirmed HCM was found to be 21.5%. Evidence from published studies presented in Table S2 indicates that the diagnostic yield of NGS screening methods for HCM ranged from 21% to 79% (17-26). The most prevalent HCM gene variants in the present study were *MYH7* (11.2%) and *MYBPC3* (7.7%). HCM phenocopies were confirmed in 3.2% of patients, which is less frequent compared with the published range of 5–10% for testing projects for HCM phenocopies (27). The present study also reported ATTR-CA as the most frequent phenocopy (1.3%). Six of the published studies investigated the diagnostic yield for ATTR-CA among patients with HCM (Table S2). Five studies did not detect variants in the *TTR* gene (17,18,20-22). A study conducted in Italy included 343 patients who had been referred with a tentative HCM diagnosis and reported a diagnostic yield of 3.5% and 2% for ATTR-CA and FD, respectively (11-gene panel) (26). The diagnostic yield for FD (0.4% in the current study) ranged from 0% to 5.6% in NGS-based studies among HCM patients (Table S2) (17-20,22,24-26). Less stringent risk stratification and greater geographic dispersion of patients in the current study may have contributed to the differences in the diagnostic yield rates compared with the other studies discussed above.

It is noteworthy that the present study enrolled the highest number of patients compared to other published NGS-based studies that analyzed HCM patients as summarized in Table S2. Additionally, it is also the only study that enrolled patients from different countries. Interestingly, the gene panels used in three of the ten studies did not include the *TTR* gene. The results of the present study signify the importance of the inclusion of *TTR* in the NGS screening panels.

Hereditary ATTR-CA is an autosomal-dominant disease with considerable phenotypic heterogeneity with certain *TTR* variants causing exclusively infiltrative cardiomyopathy (10). FD is an X-linked disorder with phenotypes varying from the classic phenotype, with pediatric-onset and multi-organ involvement, to later-onset, a predominantly cardiac phenotype. Manifestations are diverse in female FD patients in part due to variations in residual enzyme activity and X chromosome inactivation patterns (13). Lack of recognition of these clinical entities, non-specific symptoms, and co-morbidities often lead to delayed diagnosis, resulting in disease progression (10,13).

A confirmed diagnosis of ATTR-CA or FD allows appropriate and timely initiation of cause-specific therapy (i.e. stabilizing molecules or genetic silencers for ATTR-CA (12), and enzyme replacement therapy or chaperone therapy for FD (13,28) to relieve symptoms and to improve the prognosis.

The diagnostic yield for pathogenic variants and VUS is enhanced by using the NGS-based technique. The clinical interpretation of NGS testing results was based on the pathogenicity of the sequencing variants, which was ascertained as per the ACMG/AMP guidelines (14,15). Additionally, a VUS temperature scale may be used to assess the variant classification (14). Other web-based databases of genetic variants that may contain additional disease-relevant information on VUS are presented in Table S3. These resources serve to support clinicians in the interpretation of genetic testing results, aiding in the accurate assessment and understanding of genetic variants associated with HCM and HCM phenocopies.

This pilot study provided the first evidence of the feasibility of NGS testing using the 17-gene panel and its effectiveness in increasing the diagnostic yield for HCM and its phenocopies, including ATTR-CA and FD. Therefore, it has the potential to reduce the diagnostic odyssey experienced by patients with an unexplained increased LVWT and their families and facilitate appropriate genetic counseling (including identification of relatives at risk of having an inherited heart condition) and a better outcome of current therapies (29).

An important limitation of this pilot study is that the clinical data of patients were not collected. Furthermore, cardiac imaging data were not collected because of a lack of access to cardiac magnetic resonance imaging in the participating countries. Thus, we were not able to perform critical analyses of genotype–phenotype associations. Data on the ethnicity of patients were not collected. Such data are important as variations in ethnic background affect the penetrance and expressivity of genetic variants. The gene panel used in our study was limited to 17 genes associated with the most common forms of HCM and HCM phenocopies. This approach challenged the detection of other genes, including novel genes and deep intronic variants. Lastly, the number of patients included in the current pilot study was relatively small. A larger confirmatory study involving 2,068 patients from 21 countries is currently ongoing.

Conclusions

A diagnostic pilot study was conducted to investigate the feasibility of using a 17-gene NGS panel to detect gene variants associated with the most common forms of HCM and HCM phenocopies in patients with an unexplained increased LVWT. DBS samples derived from 535 patients from six countries were analyzed. This strategy was effective in the identification of patients with pathogenic/likely pathogenic variants associated with HCM (21.5%) or HCM phenocopies (3.2%) with an overall diagnostic yield of 24.7%. Early confirmation of the underlying etiology of HCM and HCM phenocopies is essential as patient outcomes largely depend on the early initiation of cause-specific therapy, if available.

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Footnote

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for current research and manuscript. The authors have no other conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. This study was performed in accordance with the ethical principles of the Declaration of Helsinki (as revised in 2013). The central laboratory study was approved by Institute of Childcare and Pediatrics Martagão Gesteira/Federal University of Rio de Janeiro under approval No. CAAE 53630421.3.0000.5264. Signed informed consent forms for blood sampling and analysis were obtained at the participating sites from all patients included in the study.

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Supplementary

Table S1 Classifications of pathogenicity and nucleotide and amino acid changes for all hypertrophic cardiomyopathy or hypertrophic cardiomyopathy phenocopy-positive variants

Variant	ACMG classification	Nucleotide change	Amino acid change	Novel HGMD®/ ClinVar	Number of cases	Countries
HCM variants (n=115)						
MYH7	Pathogenic	c.428G>A	Arg143Gln	–	1	Brazil[a]
MYH7	Likely pathogenic	c.431G>A	Gly144Asp	+/–	1	Mexico
MYH7	Pathogenic	c.655C>G	Gln219Glu	–	1	Mexico
MYH7	Pathogenic	c.715G>A	Asp239Asn	–	1	Colombia
MYH7	Pathogenic	c.727C>T	Arg243Cys	–	2	Colombia, 2
MYH7	Pathogenic	c.746G>A	Arg249Gln	–	4	Brazil, 4
MYH7	Pathogenic	c.751C>A	His251Asn	–	1	Colombia
MYH7	Pathogenic	c.788T>C	Ile263Thr	–	1	Brazil
MYH7	Pathogenic	c.1063G>A	Ala355Thr	–	1	Brazil
MYH7	Pathogenic	c.1207C>T	Arg403Trp	–	1	Colombia
MYH7	Pathogenic	c.1208G>A	Arg403Gln	–	2	Mexico, 2
MYH7	Pathogenic	c.1223A>G	Asn408Ser	+/+	2	Colombia, 2
MYH7	Pathogenic	c.1357C>T	Arg453Cys	–	6	Brazil, 6
MYH7	Likely pathogenic	c.1511A>G	Glu504Gly	+/+	1	Mexico
MYH7	Pathogenic	c.1750G>C	Gly584Arg	–	1	Brazil
MYH7	Pathogenic	c.1988G>A	Arg663His	–	3	Mexico, 1; Brazil, 1; Turkey, 1
MYH7	Pathogenic	c.2011C>T	Arg671Cys	–	2	Colombia, 1; Columbia: 1[e]
MYH7	Pathogenic	c.2012G>A	Arg671His	–	1	Mexico
MYH7	Pathogenic	c.2146G>A	Gly716Arg	–	1	Colombia
MYH7	Pathogenic	c.2155C>T	Arg719Trp	–	1	Brazil
MYH7	Pathogenic	c.2156G>A	Arg719Gln	–	1	Colombia
MYH7	Pathogenic	c.2167C>T	Arg723Cys	–	1	Turkey
MYH7	Pathogenic	c.2207T>C	Ile736Thr	–	1	Israel
MYH7	Pathogenic	c.2221G>C	Gly741Arg	–	1	Israel
MYH7	Pathogenic	c.2302G>A	Gly768Arg	–	1	Brazil[e]
MYH7	Pathogenic	c.2347C>T	Arg783Cys	–	1	Colombia
MYH7	Pathogenic	c.2389G>A	Ala797Thr	–	5	Brazil, 2; Colombia, 1; Mexico, 2
MYH7	Pathogenic	c.2466G>A	Met822Ile	+/–	2	Mexico, 2
MYH7	Likely pathogenic	c.2555T>G	Met852Arg	+/–	2	Turkey, 2
MYH7	Pathogenic	c.2602G>C	Ala868Pro	–	1	Mexico

Table S1 (*continued*)

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Table S1 (*continued*)

Variant	ACMG classification	Nucleotide change	Amino acid change	Novel HGMD®/ ClinVar	Number of cases	Countries
MYH7	Pathogenic	c.2633T>C	Val878Ala	–	3	Colombia, 3
MYH7	Pathogenic	c.2770G>A	Glu924Lys	–	1	Mexico
MYH7	Pathogenic	c.4499G>A	Arg1500Gln	–	1	Brazil
MYH7	Pathogenic	c.4954G>T	Asp1652Tyr	–	2	Brazil, 1; Colombia, 1
MYH7	Pathogenic	c.5342G>A	Arg1781His	–	1	Brazil[b]
MYH7	Likely pathogenic	c.2242_2243delTCinsAT	Ser748Ile	+/–	1	Colombia
MYH7	Pathogenic	c.2623_2625del	Glu875del	–	1	Brazil
MYBPC3	Pathogenic	c.622C>T	Gln208*	+/–	1	Turkey
MYBPC3	Pathogenic	c.772G>A	Glu258Lys	–	2	Brazil, 1; Colombia, 1
MYBPC3	Pathogenic	c.1483C>T	Arg495Trp	–	2	Turkey, 1[c]; Colombia, 1
MYBPC3	Pathogenic	c.1484G>A	Arg495Gln	–	2	Brazil, 2
MYBPC3	Likely pathogenic/VUS	c.1828G>A	Asp610Asn	–	1	Brazil
MYBPC3	Pathogenic	c.2429G>A	Arg810His	–	1	Brazil[a]
MYBPC3	Pathogenic	c.2670G>A	Trp890*	–	2	Brazil, 1; Colombia, 1
MYBPC3	Pathogenic	c.3641G>A	Trp1214*	+/–	1	Brazil
MYBPC3	Pathogenic	c.3694A>T	Lys1232*	–	1	Israel
MYBPC3	Pathogenic	c.3773T>G	Leu1258*	–	1	Turkey
MYBPC3	Pathogenic	c.1928-2A>G	Splicing	–	2	Mexico, 2
MYBPC3	Pathogenic	c.3491-2A>T	Splicing	–	1	Colombia
MYBPC3	Pathogenic	c.1800delA	Lys600Asnfs*2	–	3	Brazil, 2; Mexico, 1
MYBPC3	Pathogenic	c.2511delC	Ile837Metfs*42	+/–	1	Mexico
MYBPC3	Pathogenic	c.1351+2T>C	Splicing	–	1	Israel
MYBPC3	Pathogenic	c.2994+1G>A	Splicing		1	Turkey
MYBPC3	Pathogenic	c.3190+5G>A	Splicing	–	3	Mexico, 1; Turkey, 1; Colombia, 1
MYBPC3	Likely pathogenic	c.613_614insTGACC	Gln205Leufs*97	+/+	1	Turkey
MYBPC3	Pathogenic	c.560delC	Pro187Leufs*13	+/–	1	Turkey
MYBPC3	Pathogenic	c.913_914delTT	Phe305Profs*27	–	1	Brazil
MYBPC3	Pathogenic	c.1358delC	Pro453Leufs*13	–	2	Brazil, 2
MYBPC3	Pathogenic	c.1526_1527delGA	Arg509Thrfs*21	+/–	1	Brazil
MYBPC3	Pathogenic	c.2230_2233delGAAG	Glu744Metfs*9	+/–	2	Colombia, 2
MYBPC3	Pathogenic	c.2864_2865delCT	Pro955Argfs*95	–	1	Mexico
MYBPC3	Pathogenic	c.1838dupA	Asp613Glufs*25	–	2	Israel, 2
MYBPC3	Likely pathogenic	c.2418_2419dupCA	Ile807Thrfs*16	+/+	1	Colombia

Table S1 (*continued*)

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Table S1 (*continued*)

Variant	ACMG classification	Nucleotide change	Amino acid change	Novel HGMD®/ ClinVar	Number of cases	Countries
MYBPC3	Likely pathogenic	c.2556dupC	Gly853Argfs*31	+/+	1	Turkey
MYBPC3	Likely pathogenic	c.3758_3773dup16	Glu1261Hisfs*10	+/+	1	Mexico
MYBPC3	Likely pathogenic	c.3774dupA	Gln1259Thrfs*7	+/+	1	Colombia
TNNT2	Pathogenic	c.274C>T	Arg92Trp	–	1	Turkey
TNNT2	Pathogenic	c.305G>A	Arg102Gln	+/–	1	Brazil
TNNT2	Likely pathogenic	c.838A>C	Lys280Gln	+/–	1	Saudi Arabia
TNNT2	Pathogenic	c.881G>A	Trp294*	+/–	1	Brazil
TNNI3	Pathogenic	c.434G>A	Arg145Gln	–	2	Brazil, 1; Turkey, 1[c]
TNNI3	Pathogenic	c.470C>T	Ala157Val	–	1	Colombia
TNNI3	Pathogenic	c.485G>T	Arg162Leu	+/–	1	Colombia
TPM1	Pathogenic	c.523G>A	Asp175Asn	+/–	2	Brazil, 2[d]
MYL2	Pathogenic	c.401A>C	Glu134Ala	–	1	Mexico
MYL2	Likely pathogenic	c.53T>C	Phe18Ser	+/–	1	Mexico
MYL3	Pathogenic	c.517A>G	Met173Val	–	1	Colombia
TNNC1	Pathogenic	c.23C>T	Ala8Val	–	1	Colombia
HCM phenocopies (n=17)						
TTR	Pathogenic	c.148G>A	Val50Met	–	1	Brazil
TTR	Pathogenic	c.250T>C	Phe84Leu	–	1	Brazil
TTR	Pathogenic	c.325G>C	Glu109Gln	–	1	Turkey
TTR	Pathogenic	c.424G>A	Val142Ile	–	4	Brazil, 2[d]; Mexico, 1; Saudi Arabia, 1
LAMP2	Pathogenic	c.864+1G>A	Splicing	–	1	Brazil
LAMP2	Likely pathogenic	c.972_973insA	Leu325Thrfs*25	+	1	Brazil[b]
LAMP2	Pathogenic	c.973dupC	Leu325Profs*25	–	1	Brazil
GLA	Pathogenic	c.644A>G	Asn215Ser	–	1	Brazil
GLA	Likely pathogenic	c.413delG	Gly138Glufs*27	+/+	1	Brazil
PRKAG2	Pathogenic	c.905G>A	Arg302Gln	–	2	Brazil, 1; Mexico, 1
PTPN11	Pathogenic	c.1528C>G	Gln510Glu	–	1	Brazil
DES	Pathogenic	c.893C>T	Ser298Leu	–	1	Colombia
DES	Likely pathogenic	c.1219A>T	Lys407*	+/+	1	Colombia

Double variants were detected in 4 cases: [a], *MYH7* and MYBPC3; [b], *MYH7* and *LAMP2*; [c], *MYBPC3* and *TNNI3*; [d], *TPM1* and *TTR*. [e], age is unknown. ACMG, American College of Medical Genetics; ClinVar, public archive of interpretations of clinically relevant variants; HGMD®, Human Gene Mutation Database; HCM, hypertrophic cardiomyopathy; VUS, variant of uncertain significance; –, published variant listed in HGMD®; +/+, variant not reported either in HGMD® or ClinVar; +/–, variant not listed in HGMD® but listed in ClinVar; (*) marks stop codon.

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Table S2 Studies reporting the diagnostic yield of hypertrophic cardiomyopathy, transthyretin cardiac amyloidosis, and Fabry disease using an NGS screening method

Study no.	Author; country (year): study design	No. of genes in the panel	No. of patients included, M/F ratio	Inclusion criteria	Diagnostic yield of HCM, M/F ratio	Diagnostic yield of ATTR-CA, M/F ratio	Diagnostic yield of FD, M/F ratio
1	Current Cardio NGS study; Colombia, Brazil, Mexico, Turkey, Israel, and Saudi Arabia: Prospective study	17	N=535, M/F =1.4:1	LVWT ≥13 mm	21.5% (n=115) M/F =1:1	1.3% (n=7) M/F = 2.5:1	0.4% (n=2) M/F = 0:2
2	Jääskeläinen P, *et al.* (17); Finland (2019): Prospective study	59	N=382, M/F =1.6:1	LVH ≥15 mm in probands and ≥13 mm in relatives	38.2% All were pathogenic or likely pathogenic	0%	0.5% (n=2)
3	Tran Vu MT, *et al.* (18); Vietnam (2019): Prospective study	23	N=104 M/F =1.7:1	LV wall thickness ≥15 mm	42.3% (n=44) M/F =1.4:1	0%	0.9% (n=1)
4	Zhao Y, *et al.* (19); China (2017): Prospective study	19	N=18 M/F =1.5:1	LV septum and/or interventricular septal thickness ≥15 mm	66.7% (12/18) M/F =1:1	TTR gene was not included in the panel	5.6% (n=1)
5	Hayashi T, *et al.* (20); Japan (2018): Retrospective study	67	HCM patients (N=46) M/F =1.4:1 RCM patients (N=7) M/F ratio=6:1	LVH ≥15 mm in probands and ≥13 mm in relatives	In HCM patients, 78% (n=36/46) M: 88% In RCM patients, 71% (n=5/7)	0%	0%
6	Bonaventura J, *et al.* (21); Czech Republic (2020): Prospective study	229	HCM patients (N=336) M/F =1.4:1	LVH ≥15 mm	21% (n=70)	0%	0.6% (n=2)
7	Nagyova E, *et al.* (22); Bratislava, Slovakia (2019): Prospective study	46	Cardiomyopathy patients (N=16); dilated (DCM) (n=6); hypertrophic (HCM) (n=8); and noncompaction (NNCM) (n=2); cardiomyopathy M/F: 3:1	Patients with HCM, DCM, and NNCM	In HCM patients: 62.5% (n=5) M: 80% F: 20%	0%	0%
8	Norrish G, *et al.* (23); London, England (1994–2017): Retrospective study	NGS panels were available since 2011: small (≤21 genes) or expanded (>21 genes) panels	First-degree child relatives with HCM (N=57)	≤18 years of age; maximal LVWT, 13 mm	69% (n=27/39) Median age 6 years (IQR, 3.75–10 years)	No information on whether TTR was included in the panel	No information whether GLA was included in the panel
9	Rubattu S, *et al.* (24); Rome, Italy (2016): Prospective cohort study	17	HCM patients (N=70) with both early-onset (EO) (n=35) and later-onset (LO) (n=35) HCM; EO: M/F =3.3:1 LO: M/F =0.3:1	Age: EO HCM: ≤25 years and LO HCM: ≥65 years LVWT >15 mm in adults	Overall; 40% (n=28/70)	TTR was not included in the panel	0%
10	Cecconi M, *et al.* (25); Italy (2016): Retrospective study	19	19 DNA samples of HCM patients in the discovery set	LVWT ≥15 mm	79% (n=15/19)	TTR was not included in the panel	5.3% (n=1)
11	Maurizi N, *et al.* (26); Italy (2019): Prospective study	11	N=343, M: 58% F: 42% M/F =1.3:1	HCM patients at age ≥40 years	73% (n=251)	3.5% (n=12)	2% (n=6)

ATTR-CA, transthyretin cardiac amyloidosis; DCM, dilated cardiomyopathy; EO, early onset; F, females; FD, Fabry disease; *GLA*, α-galactosidase A gene; HCM, hypertrophic cardiomyopathy; LO, later onset; LV, left ventricle; LVH, left ventricular hypertrophy; LVWT, left ventricular wall thickness; M, males; NGS, next-generation sequencing; NNCM, noncompaction cardiomyopathy; N, number/sample size; RCM, restrictive cardiomyopathy; *TTR*, transthyretin gene.

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Table S3 Web-based databases

Name of the webpage	How to access	Information given
Population frequency		
Varsome	https://varsome.com/	A global genomics community of 500,000+ healthcare professionals and researchers who share their findings and expertise and look to establish collaborations. Varsome features as a knowledge database consisting of +140 data resources and a powerful variant search engine
Alamut® Visual	https://extranet.interactive-biosoftware.com/AlamutVisual-2.15_Documentation/SNPs.html	Provides convenient access to several databases of known variants, including NCBI (dbSNP), gnomAD, Swiss-Prot variants, NHLBI GO ESP, HGVD, and GoNL
dbSNP	http://www.ncbi.nlm.nih.gov/SNP/	dbSNP is a public-domain archive for a broad collection of simple genetic polymorphisms designed to support submissions and research into a broad range of biological problems, including physical mapping, functional analysis, pharmacogenomics, association studies, and evolutionary studies
gnomAD	https://gnomad.broadinstitute.org/	gnomAD is a collaborative effort by international researchers to gather and standardize exome and genome sequencing data from diverse large-scale projects, with the aim of providing accessible summary data to the broader scientific community
Mutation/Polymorphism and Novel/Published		
HGMD®	https://www.hgmd.cf.ac.uk/ac/index.php	HGMD® is a comprehensive compilation of (published) gene lesions associated with inherited human diseases
ClinVar	https://www.ncbi.nlm.nih.gov/clinvar/	ClinVar is a public archive of reports of the relationships among human variations and phenotypes, with supporting evidence, serving as a valuable resource for understanding the impact of genetic variations on human well-being
Pathogenic/Benign		
Franklin	https://franklin.genoox.com/clinical-db/home	Franklin is a connectivity hub across the medical genetics domain. The network effect generated by the Franklin community extends the actionable genomic information that impacts patients' care
PolyPhen-2	http://genetics.bwh.harvard.edu/pph2/	PolyPhen-2is a computational tool that employs physical and comparative assessments to predict the potential effects of an amino acid substitution on the structure and function of a human protein
SIFT	https://sift.bii.a-star.edu.sg/	SIFT utilizes sequence homology and the physical characteristics of amino acids to forecast the impact of an amino acid substitution on protein function, applicable to both naturally occurring nonsynonymous polymorphisms and experimentally induced missense mutations
MutationTaster	https://www.mutationtaster.org/	A web-based application that assesses the disease-causing potential of DNA sequence variants, utilizing a series of computational tests to estimate the impact of the variant on the gene product or protein
CADD	https://cadd.gs.washington.edu/	CADD is a tool utilized for evaluating the deleterious nature of single nucleotide variants and insertion/deletion variants in the human genome by assigning them scores
Related to a specific disorder/incidental		
OMIM®	https://www.omim.org/	OMIM®is a compendium providing comprehensive and authoritative information on human genes, genetic phenotypes, and Mendelian disorders, with over 16,000 genes covered

CADD, combined annotation-dependent depletion; ClinVAR, public archive of interpretations of clinically relevant variants; DbSNP, the single nucleotide polymorphism database; DNA, deoxyribonucleic acid; gnomAD, genome aggregation database; GoNL, the genome of the Netherlands consortium; HGMD®, the human gene mutation database; HGVD, the Japan human genetic variation database; NCBI, national center for biotechnology information; NHLBI GO ESP, national heart, lung, and blood Institute grand opportunity exome sequencing project; OMIM®, online mendelian inheritance in man; PolyPhen-2, polymorphism phenotyping v2; SIFT, sorting intolerant from tolerant; Swiss-prot, curated protein sequence database.

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