Peer Review File

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REVIEWER A:

Comment A1: As mentioned in the discussion, no clinical or imaging data are available. Also understandable by the given reasons it nevertheless will make it difficult to compare this study with various others in the field thus providing not much more than the results of a genetic screening.

Reply A1: We acknowledge this important limitation of our pilot study which was mentioned in the original version of our manuscript (lines 434-437): "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 MRI in the participating countries").

Comment A2: The numbers and ratios of positive *GLA* cases are confusing. You report consistently 3 cases with pathogenic *GLA* variants, but the relative number is given with either 0.6% (line 265) or 2.2% (line 271 and figure 2). I understand 0.6% (3/535) but I don't understand, how 2.2% is calculated.

Reply A2: We now present the percentage of positive *GLA* cases calculated based on the total number of samples (n=535). To address comment A3 from this reviewer, we have corrected the percentage to 0.4% (instead of 0.6%; lines 103, 301, 363) to only include HCM phenocopy-positive *GLA* cases. The original Figure 2 has been replaced by a new Figure 2.

Comment A3: The *GLA* variant D313Y is not only "benign" from the phenotypic point of view (dbFGP) but also from the genetic. From 25 entries in ClinVar, 21 are classifying the variant as "benign" or "other". Applying ACMG/AMP criteria together with the regular publications of the ClinGen Sequence Variant Interpretation (SVI) Work Group this variant cannot be longer classified as VUS and should therefore not be reported.

Reply A3: We now only report detection rates of (likely) pathogenic variants associated with hypertrophic cardiomyopathy or hypertrophic cardiomyopathy-phenocopies in our revised manuscript (all listed in the new Supplementary Table 1).

Comment A4: There is no parameter in the ACMG/AMP guidelines (at least to my knowledge) that would qualify "response to a specific treatment" as evidence for reclassification to likely pathogenic as you suggest (lines 378-381).

Reply A4: The sentence "Assessing the response to a specific treatment may also be useful for obtaining evidence that allows for the reclassification of the VUS as "likely pathogenic." (lines 425-426) has been deleted. In fact, the whole paragraph ("The following steps could be helpful…..of the VUS as "likely pathogenic.", lines 418-426) has been deleted as recommended by reviewer C (comment C9).

Comment A5: Your diagnostic pathway of Fabry disease (figure 4) in females is not in accordance with international recommendations[1]. In females, the identification of pathogenic genetic variants in the *GLA* gene shall always be the first step. Relying on enzyme activity and/or lyso-Gb3 measurement as a primary test in females will lead to a considerable amount of false-negative results. Especially in cases of late-onset (predominant) cardiac variants like N215S you will find substantial numbers of females with normal values for both parameters.

Reply A5: The data are now described without particular focus on transthyretin cardiac amyloidosis and/or Fabry disease to address reviewers' comments. The original text describing the approach to diagnosis of Fabry disease (lines 393-401) and the original Figure 4 ("Diagnostic approach of Fabry disease") have been removed.

Comment A6: One minor remark belongs to line 64 in the abstract: As far as I understood your method, you investigated 17 genes NOT 17 single-nucleotide variants.

Reply A6: We have changed the sentence (lines 90-93) accordingly: "NGS included the detection of known splice regions and flanking regions of 17 genes using the Illumina NextSeq 500 and NovaSeq 6000 sequencing systems."

REVIEWER B

Comment B1. The authors performed an interesting study on patients from different countries and compared their results to those previously published. The results of the study contribute to current knowledge of the genetics of HCM and the correct approach to genetic testing.
The length of the article could be shortened, particularly the Discussion section where too much information is provided (as in a text on medical genetics).

Reply B1: We have carefully evaluated the content of our manuscript and have considerably reduced the word count to 2518 words, also to address comments from other reviewers. The "Highlight Box" (line 119) has been amended accordingly.

REVIEWER C

Comment C1. The patient number is adequate for a pilot study, even if the unavailability of clinical data (not also no MRI that is not so frequently performed, but also no medical history, Echocardiodoppler, and ECG) represents a strong for supporting the strength of results.

Reply C1: We acknowledge this important limitation of our pilot study which was mentioned in the original version of our manuscript (lines 434-437): "An important limitation of this pilot study was that the clinical

data of patients were not collected. Furthermore, cardiac imaging data were not collected because of a lack of access to cardiac MRI in the participating countries").

Comment C2. Line 150-159 ("We have investigated the…… differentiate sarcomeric HCM from HCM phenocopies.") and lines 299-305. ("This pilot study…. mean [SD] age, 42.6 [17.8] years) please summarize.

Reply C2: We have summarized the text, as indicated by the reviewer, also to address the recommendations from other reviewers to shorten the manuscript. Please see lines 160-168 and lines 341-349.

Comment C3. Listed Ref 1 is not that of the EU Guidelines but of the AHA: please verify the references vs. citations in the text.

Reply C3: All references and citations have been verified and updated where necessary (also to address the editorial comment; i.e. original references 3, 6, 7, 11, 27, and 29, with associated textual changes). Lines 122-125 have been corrected to reflect that reference no. 1 are guidelines from the American College of Cardiology/American Heart Association.

Comment C4. Line 191 please describe how blood samples are stored from collection to use and how they are processed for sequencing.

Reply C4: Lines 207-208 have been changed to include the information that DBS samples were stored at room temperature. Moreover, lines 210-212 now include information on how samples were processed for sequencing.

Comment C5. Please list the VAR and VUS in Table 5 (AA change, and/or nucleotides).

Reply C5: We now only report (likely) pathogenic variants associated with hypertrophic cardiomyopathy or hypertrophic cardiomyopathy-phenocopies in our revised manuscript (i.e., variants of uncertain significance have been deleted). A new table has been included as Supplementary Table 1 ("Classifications of pathogenicity and nucleotide and amino acid changes for all hypertrophic cardiomyopathy or hypertrophic cardiomyopathy phenocopy-positive variants") and includes the nucleotide and amino acid changes.

Comment C6. Line 318 -334. The web-based dataset should be presented in a dedicated table, adding the website, ref, and type of information provided. A brief statement in the manuscript may explain that they may support clinicians in the interpretation of genetic testing results (I respectfully remember to the authors that this is not a review).

Reply C6: We have adjusted the section (lines 410-417) by deleting the information on web-based databases. That information has now been incorporated in a new Supplementary Table 3 ("Web-based databases"). The sentence (lines 408-410) "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." has been added.

Comment C7. Lines 332 -334. The present study does not have patient' follow-up, this statement is speculative.

Reply C7: The sentence "Double sarcomere gene variants, identified in 2% of patients in the current pilot study, may confer a gene dosage effect in HCM, thereby predisposing them to adverse disease progression (31)." has been removed (lines 378-381).

Comment C8. Lines 332 -334. algorithms from patients as in Figure 3 -4 are not results from this paper but a modification of already published figures (as correctly stated in the legends). I understand that the general idea is that the results from this genetic study could be propaedeutics to follow a pathway or another in these flowcharts, to improve the diagnosis, but this concept may be simply, briefly stated and the figure deleted or put in Supplementary data adding a box to identify where the results of this work may putatively play a decisional role. Moreover, no information is given about the fact that these diagnostic pathways are followed in the countries participating in this study or not.

Reply C8: The data are now described without particular focus on transthyretin cardiac amyloidosis and/or Fabry disease, also to shorten the discussion section. The algorithms (original Figures 3 and 4) and associated text (lines 388-401) have been removed.

Comment C9. Lines 371-379: generic, not essential statements, please delete.

Reply C9: The paragraph has been removed (lines 418-426).

REVIEWER D

Comment D1. Please check for the title, if this is about Transthyretin Cardiac Amyloidosis and Fabry Disease, the result and conclusion should be emphasized for these 2 genes and give more detail about pathogenic variants in these 2 genes. It's unrelated to the results and conclusion of 24% of positive testing cases.

Reply D1: Our manuscript presents results of screening of the most common forms of HCM and HCM phenocopies. The data are now described without particular focus on transthyretin cardiac amyloidosis and/or Fabry disease. However, we propose not to change the title given the importance of early identification of patients with treatable HCM phenocopies (lines 78-79 and 461-463).

Comment D2. The authors should add the results of pathogenic variants for all positive cases/ the pathogenic variants of ATTR-CA and FD, whether novel or known previous variants, and the detail of classification from ACMG (as your ref 17).

Reply D2: A new table has been included as Supplementary Table 1 ("Classifications of pathogenicity and nucleotide and amino acid changes for all hypertrophic cardiomyopathy or hypertrophic cardiomyopathy phenocopy-positive variants"). This table includes nucleotide and amino acid changes for all (likely) pathogenic variants. A column has been added to indicate whether novel variants are listed in the Human Gene Mutation Database or ClinVar. Moreover, the sentence "Franklin variant interpretation (Genoox) was used especially in cases with novel variants." has been added (lines 271-272).

Comment D3. Please give more detail on whether all positive cases have pathogenic variants.

Reply D3. We refer the reviewer to our response to comment D2.

Comment D4. In the abstract, all gene names should be in italics.

Reply D4: All gene names are now presented using italics font type.

Comment D5. Figure 1: Please add the meaning of 'dark blue bar'. - What does 'other variants (not positive HCM)*' mean?

Reply D5: Figure 1 ("Distribution of (likely) pathogenic–variants associated with hypertrophic cardiomyopathy or its phenocopies within the total population") has been recreated to show numbers and percentages only for (likely) pathogenic variants in a clear manner.

Comment D6. Figure 1 and Table 3 are the same; the authors can delete Table 3.

Reply D6: The previous Table 3 has been deleted and Figure 1 has been retained.

Comment D7. Table 5 does not significantly demonstrate, and it'd be more interesting if the authors add all pathogenic/ likely pathogenic of this study instead.

Reply D7: We refer to our response to comment #2 from this reviewer.

Comment D8. Table 6 is quite confusing. The detection rate from NGS depends on the test whether the gene panels, whole exome, or whole genome. The number and genes that are included in the panel.

Reply D8: The content of the table (now Supplementary Table 2) has been simplified to increase comprehensibility. We confirm that gene panels were used in these studies and show the numbers of genes

included. It is not feasible to present all genes included as, for example, the panel used in the study by Bonaventura, et al. tested 229 genes.

Comment D9. Figures 3 and 4 seem not related to this study.

Reply D9: The data are now described without particular focus on transthyretin cardiac amyloidosis and/or Fabry disease, also to shorten the discussion section. The algorithms (original Figures 3 and 4) and associated text (lines 388-401) have been removed.

Re-review comments

Comment Reviewer A: I'm satisfied with the most part of the revised version. Only one issue is if the authors insist to maintain with the title. You should mention more about TTR and FD more in both abstract and discussion part.

Authors Response: Thank you for your feedback. We have added lines 82-84 to the abstract ("Identification of 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"). In addition, we have added lines 323-330 to briefly discuss the phenotypic variability of ATTR-CA and FD and related diagnostic challenges ("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)").

In our opinion, this should appropriately inform the reader, in combination with lines 146-152 which were already included ("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)").

Please note that diagnostic algorithms for ATTR-CA and FD (Figures and associated text) were removed to address comments made during the initial review of our manuscript.