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RESEARCH LETTER
Cardiovascular disease

Retrospective variant reclassification and resequencing in hypertrophic cardiomyopathy: a reference unit centre experience

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Hypertrophic cardiomyopathy (HCM) is the most common hereditary heart disease, caused by pathogenic variants mainly in sarcomere genes.[1,2] Next generation sequencing (NGS) has significantly increased the number of variants of uncertain significance (VUS).[2–4] Thus, accurate classification is a great challenge, especially in variants classified before publication of American College of Medical Genetics (ACMG) criteria.[5] The aim of the study was to perform a retrospective reclassification and resequencing in a long-standing HCM cohort.

A total of 1454 patients referred for HCM genetic testing were recruited through the Familial Cardiomyopathies Unit of Hospital Universitario Central Asturias (HUCA; National Reference Centre of Familial Cardiomyopathies), during the period 2000–2021. This cohort was named as 'initial cohort'. An exhaustive clinical review of these patients was carried out to include only patients with definitive HCM diagnosis according to European Society of Cardiology (ESC) guidelines,[6] resulting in a 'study cohort' of 1313 patients (*Figure 1*; Supplementary material online, *Table S1*). In this cohort, 1195 cases (91%) had been previously sequenced by NGS for at least the main HCM-associated genes[7,8] (see Supplementary material online, *Tables S2 and S3*) and 118 (9%) by Sanger sequencing method, at the time of the study. Therefore, 487 (37%) patients were initially informed as carriers of a genetic variant. Among them, 41 were carriers of two variants, and one case was carrier of three variants. Thus, a total of 530 variants were identified in the 487 carriers (see Supplementary material online, *Table S4*), leading to 319 different variants (see Supplementary material online, *Table S5*). Initially, 187 of these variants (58.6%) had been classified as pathogenic/likely pathogenic (P/LP), 130 variants (40.8%) as VUS, and only two variants (0.6%) as benign/likely benign (B/LB) (*Figure 2*). In this regard, 368 patients were carriers of a P/LP variant (28%).

We reclassified all previously identified candidate variants following current ACMG criteria (see Supplementary material online, *Table S6*).[5] Varsome.com (Version: 11.3—https://varsome.com) was used to automate pathogenicity classification for all variants,[9] although all classifications were revised by at least two geneticists and two cardiologists, including new information available from literature and segregation analysis. Reanalysis resulted in significant changes in 22% of the variants (71 of 319). Fifty-three variants initially classified as P/LP and one variant as benign were reclassified as VUS (76%), seven P/LP variants, and six VUS were reclassified as B/LB (18.3%). Finally, four variants underwent a classification change from VUS to P/LP (5.6%). Thus, 131 variants (41.1%) were classified as P/LP, 174 (54.5%) as VUS, and 14 (4.4%) as B/LB, compared to 58.6%, 40.8%, and 0.6%, respectively, in the original classification. Therefore, most frequent classification after reclassification is VUS, instead of P/LP variants (*Figure 2*). The main classification change is from LP/P to VUS, mainly in cases that were classified before ACMG criteria. This is due to frequency data and in silico predictors were the main parameters to classify a variant as LP/P. However, by the ACMG criteria, these only fulfil a pathogenic moderate (PM2) and supportive (PP3) respectively, leading to a VUS classification. These variants can lead to inaccurate genetic diagnoses and inappropriate therapeutic approaches, resulting in increased morbidity and mortality. Thus, clinical translation of VUS should be performed with caution and should not be ruled out, at least until further data focused on clarifying its clinical role becomes available. On the other hand, 115 patients of the 487 (23.6%) underwent a change in classification in one of their

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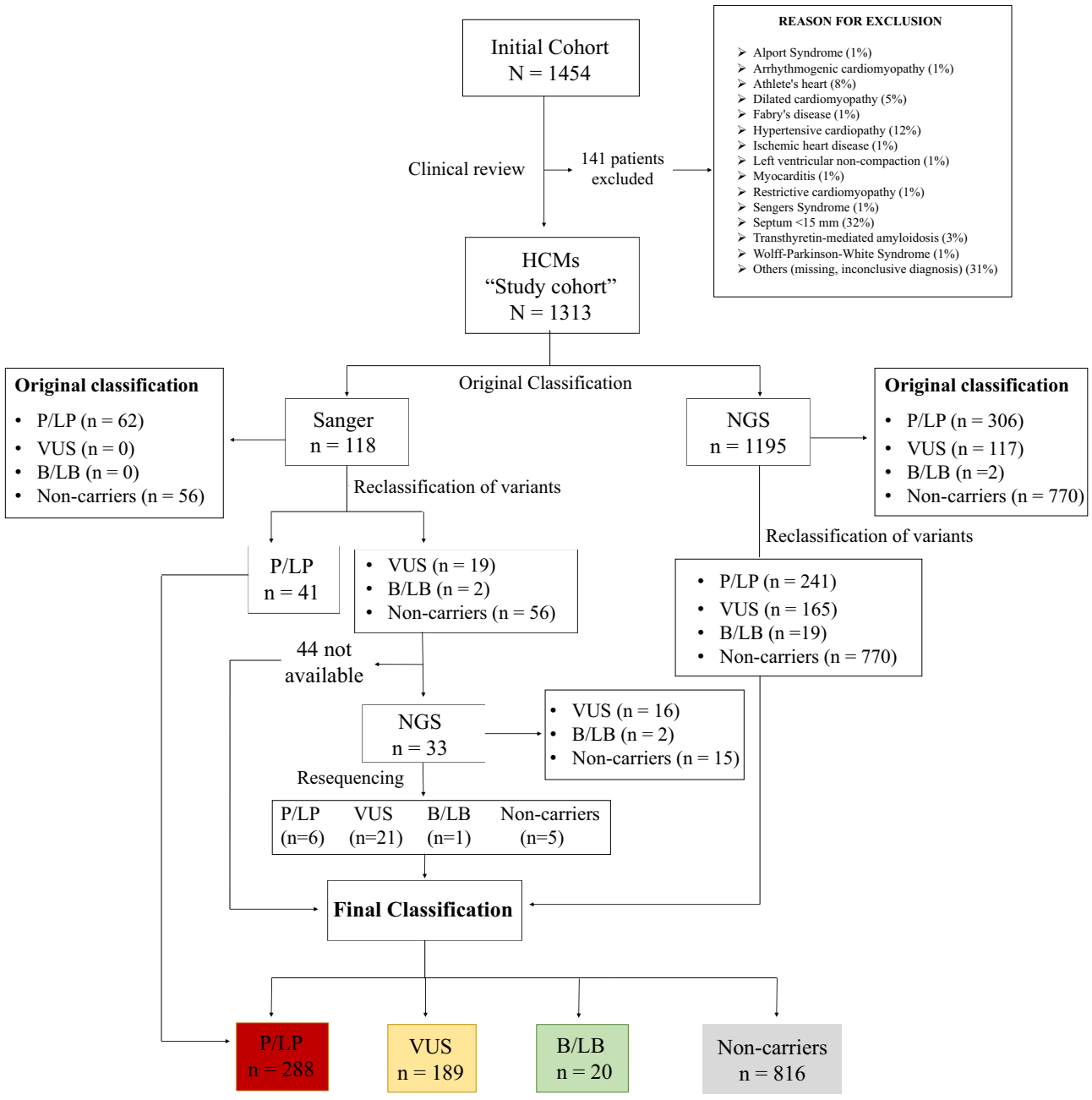

Figure 1 Workflow implemented for the reclassification and resequencing in the cohort of patients with hypertrophic cardiomyopathy. NGS, Next Generation sequencing; VUS, variant of uncertain significance; P, pathogenic; LP, likely pathogenic; B, benign; LB, likely benign.

variants when the current classification criteria of the ACMG were applied. Therefore, 466 patients (35.5%) carried at least one P/LP or VUS variant, compared to 485 (37%) in the original classification. Specifically, 184 cases (14%) carried only VUS, in contrast to the original 117 (9%) cases ($P = 0.0001$; odd ratio (OR):1.6660; 95% confidence interval (CI):1.3032–2.1298), and 282 patients (21.5%) had at least one P/LP variant, compared to the 368 cases (28%) originally identified ($P = 0.0004$, OR:0.7024; 95%CI:0.5876–0.8396). All patients were classified into each of these categories based on the most severe variant they carried (*Figure 2*). These classification changes have an impact on the patient's and relative's clinical management and genetic counselling. For instance, when a P/LP variant is identified, all negative carriers of the variant are ruled out of cardiological follow-up. On the other hand, if it is a VUS, both negative and positive carriers (at least first-degree relatives), are clinically follow-up. In addition, if a P/LP variant is misclassified, a real causal variant could not be identified, leading to a false negative. Furthermore, the emotional and psychological impacts due to changes in a variant's classification can have long-lasting effects. Moreover, genotype-phenotype studies have implicated several variants as highly malignant defects in HCM. For example, *MYH7* p.R403Q and *TNNT2* p.R92W have been associated with a high incidence of sudden cardiac death.[10,11] Thus, these results support an urgent periodic reassessment of variants through family segregations and functional studies.

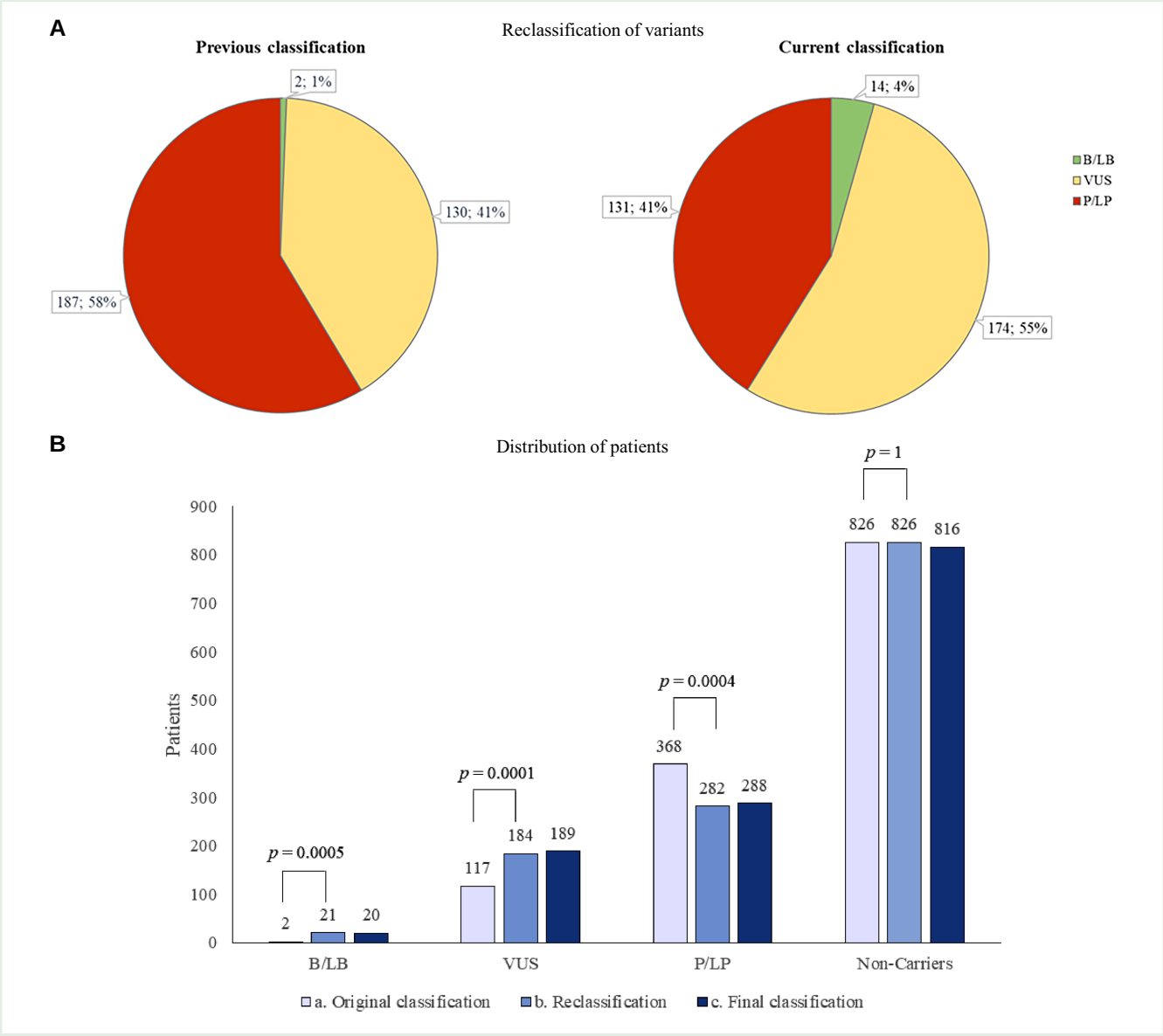

Figure 2 (*A*) Reclassification of variants. The classification of the 319 identified variants is represented, before and after the reanalysis. The number and percentage of variants is included inside each section. In this regard, originally most variants were pathogenic/likely pathogenic (187 of 319; 58.6%), but after reanalysis, the predominant classification of variants was variant of uncertain significance (174 of 319; 54.5%), in contrast to 40.8% (130 of 319) before the reanalysis. (*B*) Distribution of patients ($n = 1313$) carriers of a pathogenic/likely pathogenic, variant of uncertain significance, or benign/likely benign variant; and non-carriers. The number of patients in each class before and after reclassification and resequencing are represented. (*A*) Originally, a total of 485 carriers of a pathogenic/likely pathogenic or variant of uncertain significance variant (37%) were identified. Specifically, 368 patients were carriers of a pathogenic/likely pathogenic variant (28%; 368 of 1313 patients). (*B*) After variant reclassification, a total of 466 patients (35.5%) carried at least one pathogenic/likely pathogenic, or variant of uncertain significance compared to 485 (37%) in the original classification. Specifically, 282 patients had at least one pathogenic/likely pathogenic variant (21.5%), in contrast to the 368 cases (28%) originally identified ($p = 0.0004$). (*C*) After resequencing, six new carriers of a pathogenic variant and five new carriers of at least one variant of uncertain significance were identified. Therefore, a total of 477 patients (36.3%) finally had a pathogenic/likely pathogenic or variant of uncertain significance. In this regard, 288 patients (21.9%) carried at least one pathogenic/likely pathogenic variant, in contrast to the 368 cases (28%) originally identified.

After reclassification, available patients ($n = 33$) partially sequenced by Sanger sequencing who did not carry any P/LP variant were resequenced by NGS of a 205 cardiovascular gene panel (see Supplementary material online, *Table S7*). Sequencing was performed in an Ion GeneStudio S5 Plus Sequencer (ThermoFisher Scientific, Waltham, MA, USA). The detailed procedure was previously reported.[2] Ion Reporter (ThermoFisher Scientific) and Genome One (DREAMgenics S.L., Oviedo, Asturias, Spain) software were used for variant annotation. We selected candidate variants, and familial studies were performed in all available relatives by Sanger sequencing (see Supplementary material online, *Table S8*).

Thus, we identified six new carriers of a pathogenic variant (*MYH7* p.Phe488Leu (c.1462T > C), *TNNI3* p.Arg136Gln (c.407G >

A), *MYBPC3* p.Ala627Val (c.1880C > T) in homozygosis, *MYBPC3* p.Ser478Ter (c.1433_1434delinsGA), *FLNC* p.Ala1539Thr (c.4615G > A), and *TNNC1* p.Ala8Val (c.23C > T) (see Supplementary material online, *Figure S1*). Therefore, a total genetic yield of 18% was obtained (18% vs. 21% of our general population). Previous sequencing had missed these variants because years ago only a few sarcomeric genes were studied, and sometimes even only the most mutated exons were analysed. By NGS technologies, not only new genes were identified, but also we were able to study the full coding sequence plus at least 5 flanking intronic base pairs.[2–4]

Finally, a total of 477 patients had a P/LP or VUS variant (36.3%), compared to 485 (37%) in the original classification. Among them, 288 patients (21.9%) carried at least one P/LP variant (134 different P/LP variants including 33 not previously reported in the ClinVar Database, which increase the genetic knowledge of HCM), in contrast to the 368 cases (28%) originally identified (*Figure 2*).

In conclusion, the change of classification occurred in 22% of these variants when the current ACMG criteria were applied. These results support an urgent further reanalysis of each HCM variant if they were not initially classified following ACMG recommendations. Furthermore, we identified a P/LP variant in 18% of negative partially sequenced cases, close to our general HCM population (21.9%). Thus, variant reclassification and resequencing are crucial to perform an accurate molecular diagnosis and genetic counselling, especially in long-standing Reference Unit Centres.

Ethics committee approval

This study was approved by the Ethics Committee of Principado de Asturias (CEImPA 2022.254), and all the patients signed the informed consent for the genetic study.

Supplementary material

Supplementary material is available at *European Journal of Preventive Cardiology*.

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Data availability

Further data is available by mailing to the corresponding author (juan.gomezde@sespa.es).

Author contributions

Conceptualization, R.L., J.G., and E.C.; methodology, J.G. and E.C.; formal analysis, E.C.-L. and J.G.; investigation, E.C.-L., R.L., M.S., C.G.-L., J.R.-R., R.R.-L., V.E.-H., A.P.-C., D.V.-C., and I.P.; resources, J.G.; data curation, E.C.-L.; writing—original draft preparation, E.C.-L.; writing—review and editing, E.C.-L, R.L., and J.G.; supervision, E.C. and J.G.; project administration, E.C. and J.G.; funding acquisition, E.C. and J.G. All authors have read and agreed to the published version of the manuscript.

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