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Investigating the dual role of mitochondrial and nuclear genome variants in pediatric cardiomyopathies

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Mitochondrial defects can lead to cardiomyopathies, which can be particularly severe in children. However, many cases of pediatric cardiomyopathy have no known etiology. To address this, we sought to explore if mitochondrial genome defects might be a contributor, as this could offer insights into disease mechanisms and guide targeted interventions. We first sequenced cardiomyopathy-related genes in twenty-seven pediatric patients diagnosed with primary non-syndromic cardiomyopathy and performed whole mtDNA sequencing in both patients and thirty-one healthy controls. The initial sequencing identified pathogenic variants in seven patients but subsequent mtDNA sequencing revealed additional insights. Specifically, a variant in *FOXRED1*, encoding FAD-dependent oxidoreductase domain-containing protein-1 which functions in mitochondrial complex I stability, and another variant in cytochrome c oxidase-I, *MT-CO1*, crucial for aerobic metabolism, were identified in two siblings with hypertrophic cardiomyopathy. In another case with hypertrophic cardiomyopathy, a variant in cytochrome b, *MT-CYB*, is likely a key factor in the abnormal contraction of cardiac muscle contraction. Furthermore, a novel 12 S rRNA variant was found in a patient with left ventricular non-compaction, and this offers a promising explanation for the pathogenesis, given the gene's high expression in the left ventricle. Taken together, mtDNA variants act synergistically with others, potentially disrupting myocardial bioenergetics.

Cardiomyopathies comprise a genetically heterogeneous group of myocardial disorders. Gerull et al. emphasize that key subtypes, including hypertrophic cardiomyopathy-HCM, dilated cardiomyopathy-DCM, arrhythmogenic cardiomyopathy-ACM/ARVC, restrictive cardiomyopathy-RCM, and left ventricular noncompaction-LVNC, are most often driven by mutations in sarcomeric, desmosomal, cytoskeletal, or nuclear envelope genes[1]. HCM is commonly caused by pathogenic variants in thick filament genes, particularly *MYH7* (myosin heavy chain-7) and *MYBPC3* (myosin binding protein-C, cardiac type), which together account for many familial cases[2,3]. DCM is associated with variants in *TTN* (titin), *LMNA* (lamin A/C), *MYH7*, and other genes affecting force transmission, calcium handling, and transcriptional regulation[4,5]. ACM is primarily linked to desmosomal genes such as *PKP2* (plakophilin-2), *DSP* (desmoplakin), *DSG2* (desmoglein-2), and *DSC2* (desmocollin-2)[6], while RCM involves sarcomeric genes; mostly *TNNI3* (troponin-I3, cardiac type), *TNNT2* (troponin-T2, cardiac type), *ACTC1* (actin, alpha cardiac muscle-1), and *MYH7*[7]. LVNC shows overlap with sarcomeric and ion channel gene variants, including *MYH7*, *MYBPC3*, *ACTC1*, and *RYR2* (ryanodine receptor-2)[8]. Modifier variants and other factors, including sex, mitochondrial haplogroups, and the presence of multiple rare variants, influence phenotypic expression and disease severity and noncoding enhancer variants also contribute to cardiomyopathy presentation[9,10].

In the pediatric population, heart failure may result from a variety of underlying causes, including inflammatory or infectious processes such as myocarditis and metabolic disorders that lead to cardiomyopathy. However, it can also be a consequence of hemodynamic imbalances associated with syndromic or non-syndromic congenital structural defects[11]. Although cardiomyopathies are one of the leading causes of heart failure and sudden cardiac death among the young all over the world, present molecular studies are still ongoing,

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and therapeutic approaches are used mostly only to relieve the symptoms of the disease[12,13]. Moreover, the prevalence of pediatric cardiomyopathy subtypes differs based on the accuracy of diagnostic evaluations[14–16], and the rarity of the disease complicates data collection. Given the complexity of genetic factors involved, there is limited but growing understanding of the portion of heritability that remains unexplained interactions between genes, and factors that modify the progression of each type of cardiomyopathy[17]. However, pediatric cardiomyopathies require an elaborative and multidisciplinary evaluation approach starting from diagnosis to prevent rapid progressive case of the disease[18].

The genetic basis for isolated pediatric cardiomyopathies may overlap with those found in adult cases, but the mode of inheritance can be variable and may also occur due to solely *de novo* mutations[17,19]. Among those, although much smaller than the nuclear genome (nDNA), studies on the mitochondrial genome (mtDNA) also have become quite important for understanding cardiomyopathies[20] but also for investigating other complex diseases[21]. It is also evident that mitochondria serve a pivotal function in the generation of this energy and in the regulation of cardiac bioenergetics[22]. Therefore, we reasoned that analyzing the genetic profiles of pediatric cardiomyopathy patients, sequencing the associated nuclear genes along with mtDNA sequencing would provide valuable insights for the unexplained etiologies.

Results

As shown in Fig. 1, we used a two-stage sequencing approach to identify genetic variants in pediatric cardiomyopathies. First, a panel of 137 nuclear genes was sequenced in all 27 patients, leading to seven diagnoses. We then performed whole mitochondrial DNA sequencing in all patients and 31 healthy controls. The results highlight the potential contribution of mitochondrial variants to disease pathogenesis. Further details are provided in the following sections.

Clinical evaluation

We initially summarized the demographic information of the patients based on data collected after their examination in the genetics clinic. Age distribution of patients according to subtypes is presented in Fig. 2a. The mean age of the patient cohort was 6,6 years, while the mean age of the control group was 10,3 years. No statistically significant difference was identified between the two groups following the Mann-Whitney comparison test regarding age. Following a medical genetic examination, 70,3% (19/27) of 27 patients had no family history of cardiomyopathy, while 29,6% (8/27) had a positive family history with only respect to cardiomyopathy. The most common symptom among patients is chest pain with 40,7% (11/27). Eight of the patients, 29,6%, had parents who were related or at least married from the same region, see Fig. 2b (see Supplementary Table 1 A and Supplementary Fig. 1a-c for detailed clinical information).

Cardiomyopathy-associated variants

We started by examining nuclear gene variants derived from the sequencing of 137 genes linked to cardiomyopathy. The most frequently occurring variants were identified in *TTN* (OMIM: 188840), with 91 (33.8%) variants, followed by *PLEC* (OMIM: 601282), with 24 (8.9%) variants. A scatter plot, which provides a summary of the variants grouped according to genes and corresponding genotypes, is presented in Supplementary Fig. 2. Following evaluation in accordance with the ACMG criteria, 61 variants of uncertain significance (VUS) or pathogenic variants were identified in 29 genes. Next, the expression levels of these genes in the heart were analyzed using the The Genotype-Tissue Expression project (GTEx) database, and the resulting graph is presented in Fig. 2c. The analysis identified four clusters, with *MYH7* (OMIM: 160760), myosin light chain 3 (*MYL3*, OMIM: 160790), *TNNT2* (OMIM: 191045), and troponin C1 encoding (*TNNC1*, OMIM: 191040) exhibiting higher expression levels in the left ventricle than other genes. According to the ACMG criteria, the variants are categorized as either *variant of uncertain significance* (VUS) or *pathogenic* and presented in a heatmap in Fig. 2d presenting the strengths of the individual criteria (e.g., PVS1-Strong). Upon further analysis of the variants, it was determined that those in the *MYH7*, triadin encoding (*TRDN*, OMIM: 603283) gene and mitochondrial elongation factor Ts encoding (*TSFM*, OMIM: 603283) genes were classified as pathogenic according to the criteria PVS1-Very strong (null variant in a gene where LOF is a known mechanism of disease), the variant in *MYH7* in one patient was classified as pathogenic with PP5-Very strong, the homozygous variant in *TNNC1* was detected in two cousins and was classified as pathogenic regarding PS3 & PP3-Very strong (PS3:well established functional studies for damaging effect, PP3: multiple computational evidence supporting deleterious effect), and these variants lacked any benign supporting criteria. Consequently, seven patients, including two cousins, were diagnosed with pathogenic variants in four genes. Moreover, majority of the variants in the VUS category based on both PM2 and BP1 criteria (33/61; 54.1%). A list of all the variants, classified according to disease subtype, can be found in Fig. 2e. Regarding pathogenic variants, patient-8, −13 and −14, who were diagnosed with HCM, were found to have a pathogenic variant in *MYH7*. Patient-21, who exhibited LVNC, was found to have a pathogenic frameshift deletion in *TRDN*. Patient-19 with LVNC had pathogenic variants in both *TSFM* and *MYH7*. In the remaining 20 patients, 54 variants (88.5%) detected in various genes were concluded to be VUS.

Mitochondrial variants

As a next step, we sequenced mitochondrial variants in both the patient and control groups. We filtered the patient variants against those identified in the control group. The most prevalent variant type was single-nucleotide polymorphisms (SNPs), with a frequency of 677 (97,1%). This was followed by insertions (INS), with a frequency of 10 (1,4%), deletions (DEL), with a frequency of 5, and multi-nucleotide polymorphisms (MNP), with a frequency of 3. Figure 3a illustrates the distribution of all variants across the disease subtypes in a solar plot. Results showed that homoplasmic variants (≥ 95%) were the most common, with 688, while heteroplasmic variants were found in 7 occurrences in only five patients. Four of heteroplasmic variants were in the coding

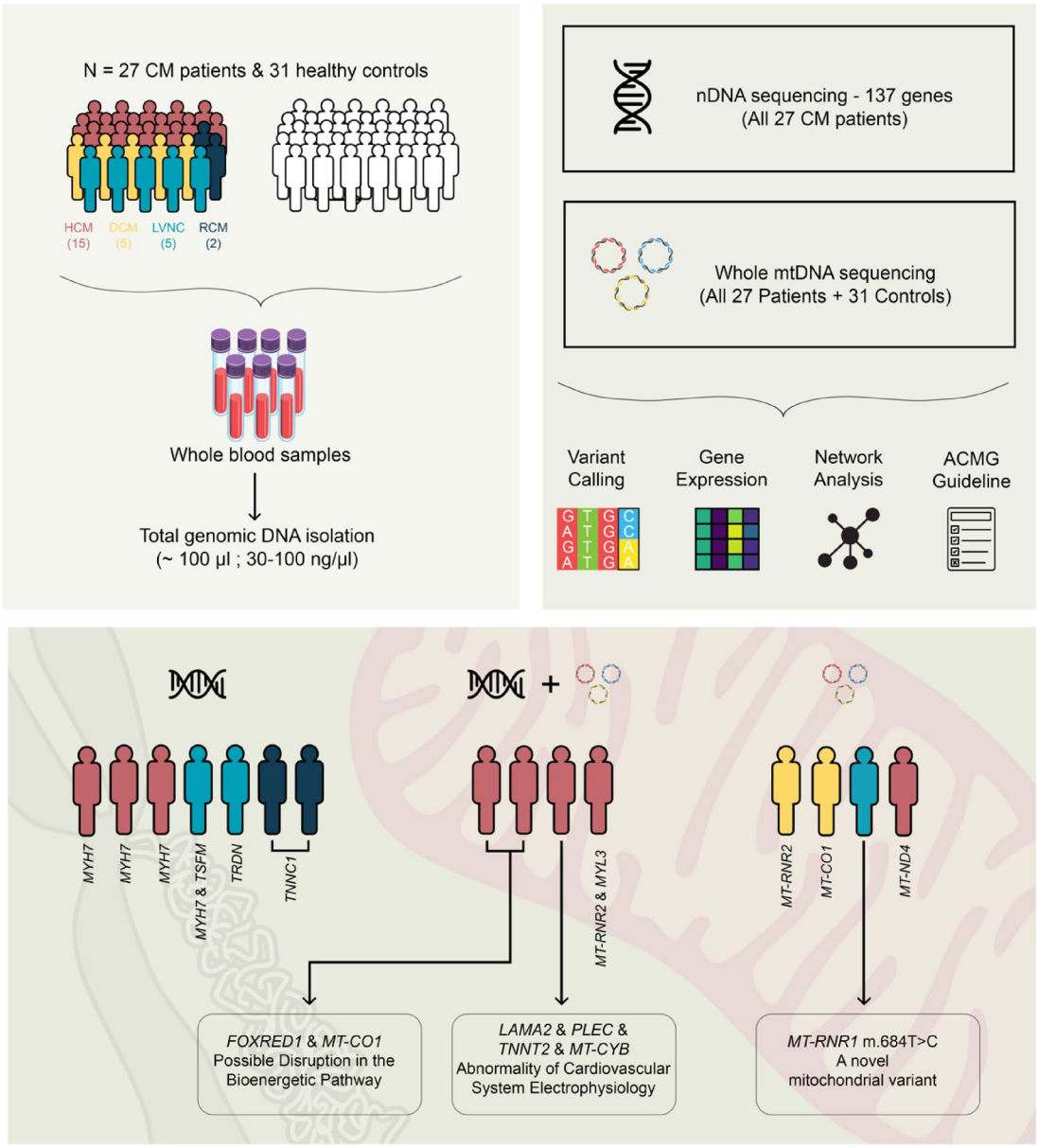
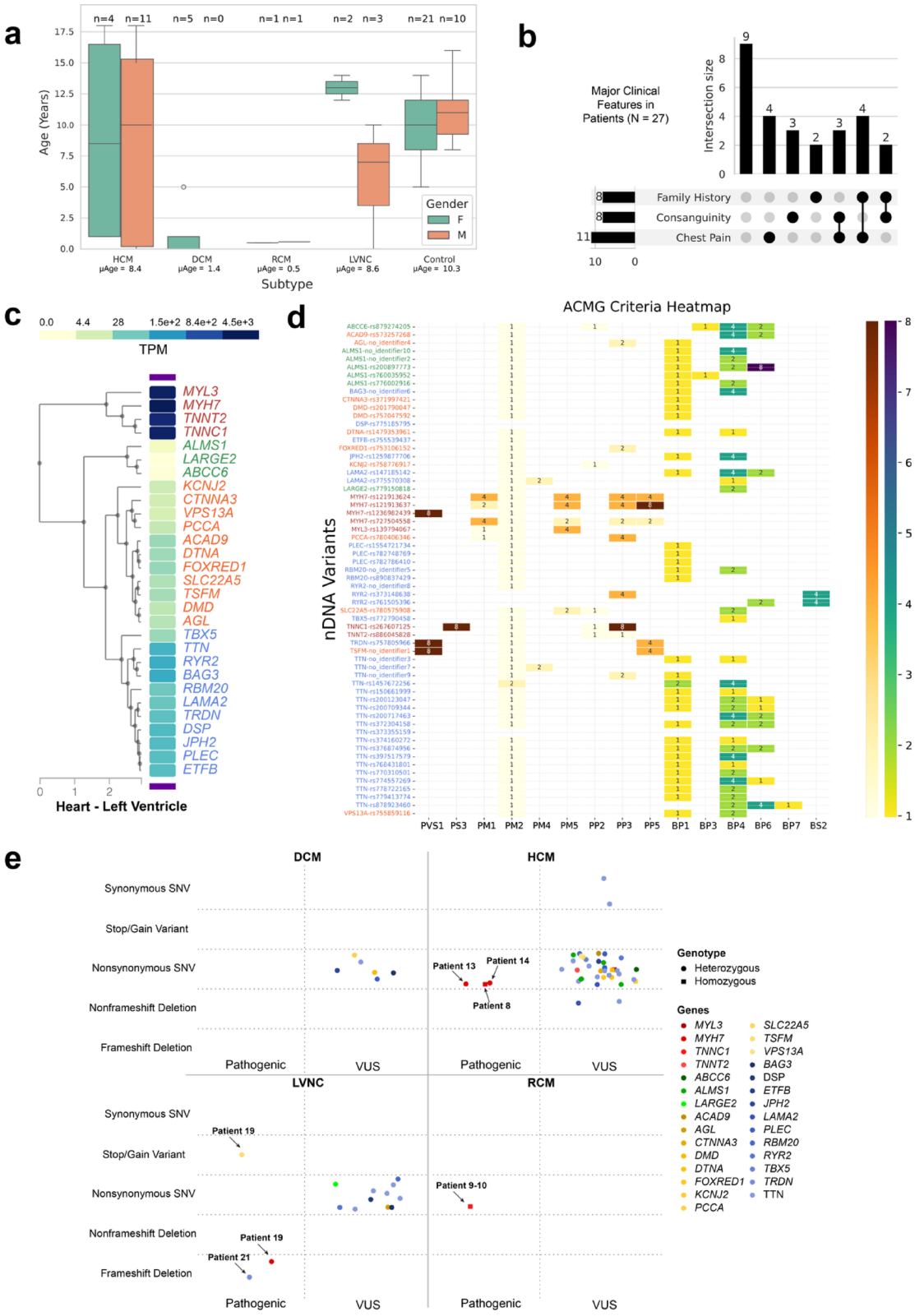


Fig. 1. Genetic profiling workflow and key findings in pediatric cardiomyopathies. This figure illustrates the comprehensive genetic profiling approach used in this study, including sequencing cardiomyopathy-related nuclear genes and the whole mitochondrial DNA in pediatric patients with cardiomyopathy. Two-stage sequencing was performed after obtaining patient consent. The sequencing of nDNA genes alone can diagnose seven patients. However, the analysis of these genes in conjunction with mtDNA may offer a greater number of diagnoses, given the possible influence of modifier and polygenic effects. All elements are original and were created using Adobe® Illustrator®.

region, while the remaining three were in the hypervariable segment. These coding region variants are m.525 C > AC (62%) detected in patient-19 with LVNC, m.3918G > A detected in patient-22 with HCM (61%), and m. 9053G > A and m.10045 T > C (87.5% and 92.3%, respectively) detected in patient-25 with HCM. Additionally, the mitochondrial haplogroups of patients and controls included in the study were examined (see Fig. 3b). The most prevalent major haplogroups among patients were H, J, and U, whereas the most common haplogroups among controls were H and U. No statistically significant differences were observed between the control and patient groups regarding haplogroups. Furthermore, all variants identified in both the patient and control groups, as well as solely in the control group, have been included in Supplementary Fig. 3a-b. The functional consequences and disease subtypes of filtered mtDNA variants are presented in Fig. 3c. A total of 33 variants were identified in two rRNA and ten protein-coding genes following an analysis of the mitochondrial loci. The 16 S (*MT-RNR2*) locus exhibited the greatest degree of variation, with six variants identified. This was followed by *MT-COI*, which demonstrated five variants, and 12 S (*MT-RNR1*) and NADH-ubiquinone oxidoreductase chain 5 (*MT-ND5*), which exhibited four variants each. The number of variants observed in each gene was



as follows: ATP synthase Fo subunit 6 (*MT-ATP6*), Cytochrome c oxidase subunit III (*MT-CO3*) and *MT-CYB* had three variants each; NADH-ubiquinone oxidoreductase chain 4 (*MT-ND4*) and NADH-ubiquinone oxidoreductase chain 4 (*MT-ND6*) had two variants each. Ultimately, a single variant was identified in three genes (NADH-ubiquinone oxidoreductase chain 2; *MT-ND2*, NADH-ubiquinone oxidoreductase chain 3; *MT-ND3* and NADH-ubiquinone oxidoreductase chain 4 L; *MT-ND4L*). In addition, m.684 T > C in *MT-RNR1* in patient-20 appeared to be a novel variant.

The expression levels of the mtDNA genes (in which the variants detected), derived from the GTEx database, were evaluated across cardiac muscle, skeletal muscle, and whole blood tissues, and are presented in Fig. 3d-i.

◀**Fig. 2**. General demographic features of the patients and the study highlights. **(a)** A boxplot shows the age distribution of patients and control group, their frequencies, and mean age over the subtypes. There are four distinct primary cardiomyopathy group (HCM; hypertrophic cardiomyopathy, DCM; dilated cardiomtyopathy, RCM; restrictive cardiomyopathy, LVNC; left ventricular non compactiomn). **(b)** Some basic indications of the patient group according to their medical history. **(c)** Expression level of VUS and pathogenic variant-detected genes in heart tissue. The graph was generated using Genotype-Tissue Expression (GTEx) website. The gene names were colored according to their expression level clusters (Very high expression; red, high expression; blue, moderate expression; yellow, low expression; green). **(d)** The representation of ACMG criteria strength for all 61 variants is provided. The lane on the right indicates benign supporting criteria, while the lane on the left indicates pathogenic supporting criteria. The following scale is used to quantify the strength of the evidence: 8 indicates very strong, 4 indicates strong, 2 indicates moderate, and 1 indicates supporting. The coloring of the variants is continued as in c. **(e)** Representation of VUS and pathogenic variants over disease subtypes and genotypes. Each gene was colored according to their corresponding expression clusters.

At the 99% confidence level ($p < 0.01$), comparisons between all groups were found to be statistically significant. Moreover, the expression of all genes was observed to be higher in cardiac muscle. The highest expression of *MT-ND4* and *MT-CO1* were observed in the left ventricle, compared to all other tissues in the body. *MT-CYB* is the second most highly expressed, while *MT-RNR1*, *MT-RNR2* and *MT-CO3* are the third most highly expressed in the heart in comparison to other tissues.

Haplogroup subtypes as well as a comprehensive list of all unfiltered mtDNA and nDNA variants with their pathogenicity predictions were provided in Supplementary Table 2 A-C and the classification of these variants in accordance with the ACMG pathogenicity criteria is provided in Supplementary Table 2D.

Discussion

Initially, the genetic architecture of cardiomyopathies (CMs) demonstrates both overlap and divergence between pediatric and adult populations[1,9,10,23–25]. Core sarcomeric genes—*MYH7*, *MYBPC3*, *TNNT2*, and *TNNI3*—are implicated in both groups across HCM, DCM, and RCM[1,24]. Pediatric studies report a higher frequency of de novo and compound heterozygous variants, especially in *MYH7* and *TNNI3*[24,25]. In adult cohorts, variants in *TTN* and *LMNA* are more prominently represented, whereas pediatric cases often reveal additional genes involved in mitochondrial translation and metabolic regulation[23–25]. LVNC is genetically heterogeneous in both age groups, frequently involving *MYH7*, with some pediatric cases showing biallelic mutations or mitochondrial gene involvement[23–26].While there is a shared genetic basis, pediatric cardiomyopathy shows broader gene involvement, including rarer and potentially more severe variant configurations. In our pediatric cohort, we identified pathogenic *MYH7* variants in three HCM cases, as well as in patients with LVNC, including one with both *MYH7* and *TSFM* variants and another with a *TRDN* variant. These findings align with previous observations that pediatric cases often show multiple gene involvement. Apart from sarcomeric genes, we also found mitochondrial DNA variants in HCM, DCM, and LVNC patients—such as in *MT-ND4*, *MT-RNR1*, and *MT-CO1*—highlighting the broader range of genes contributing to disease in children. Compared to adults, where single dominant variants in single genes are more common, pediatric cardiomyopathy appears to involve more diverse and sometimes overlapping genetic mechanisms.

In this study, we examined the demographic and clinical genetic characteristics of pediatric cardiomyopathy patients by analyzing both the genes associated with the disease and mtDNA variants. The objective was to identify the underlying causes of the disease in cases where the etiology was unclear, with the aim of providing a useful insight into its pathogenesis. Based on the distribution of clinical and demographic characteristics among the patients, we see parallel results with a comprehensive study conducted among pediatric cardiomyopathy patients in 2022[27]. The 29,6% positive family history (8/27; Fig. 2b) among our patients nearly correspond to the 36% rate in that study. In addition, the mean age was higher in our patient group compared to that study again. This may be attributed to the fact that asymptomatic patients receive a diagnosis during routine check-ups or due to other illnesses. Moreover, another study revealed positive association between cardiomyopathy subtype and age at diagnosis[24]. Similarly, the diagnosis of HCM was at later ages compared to the other subtypes in our study. However, the relatively small number of patients with non-syndromic isolated cardiomyopathy and the rarity of some subtypes may have influenced the observed differences in mean age at diagnosis in DCM, LVNC, and RCM.

We started our analysis by assessing the pathogenicity of nDNA variants. Out of 15 HCM patients, either a VUS or pathogenic variant was detected in all of them. In three HCM patients (20% of HCM patients), a pathogenic variant was detected in the *MYH7* gene, which is consistent that the variants found in HCM children are most commonly in this gene[23]. Patient-21 with LVNC has a pathogenic frameshift deletion (*TRDN*:p.Glu142fs) which is several times reported in cardiovascular conditions[27,28]. Also, this patient has a VUS in ryanodine receptor 2 encoding (*RYR2*, OMIM: 180902) gene. These together can contribute to early onset of this phenotype because *TRDN* is a protein that connects the *RYR2* with other mitochondrial cardiometabolic proteins[29] and functioning in regulating the release of calcium in the sarcoplasmic reticulum in heart[30]. In patient-19 with LVNC, the presence of pathogenic variants in both *TSFM* and *MYH7* is responsible for the observed phenotype. *TSFM* has previously been associated with the extremely rare combined oxidative phosphorylation deficiency type 3, and hypertrophy of the heart muscle has also been reported among the symptoms[31,32]. Patients-9 and patient-10, who are RCM and cousins on their mother's side, whose pedigree is given in Supplementary Fig. 4a, were found to have a homozygous pathogenic variant: *TNNC1*:p.8 A > V. The genetic etiology of RCM involves mutations in sarcomeric genes (e.g., *TNNI3*, *TNNT2*, *TNNC1*) and cytoskeletal non-sarcomeric proteins (e.g., *DES*, *FLNC*)

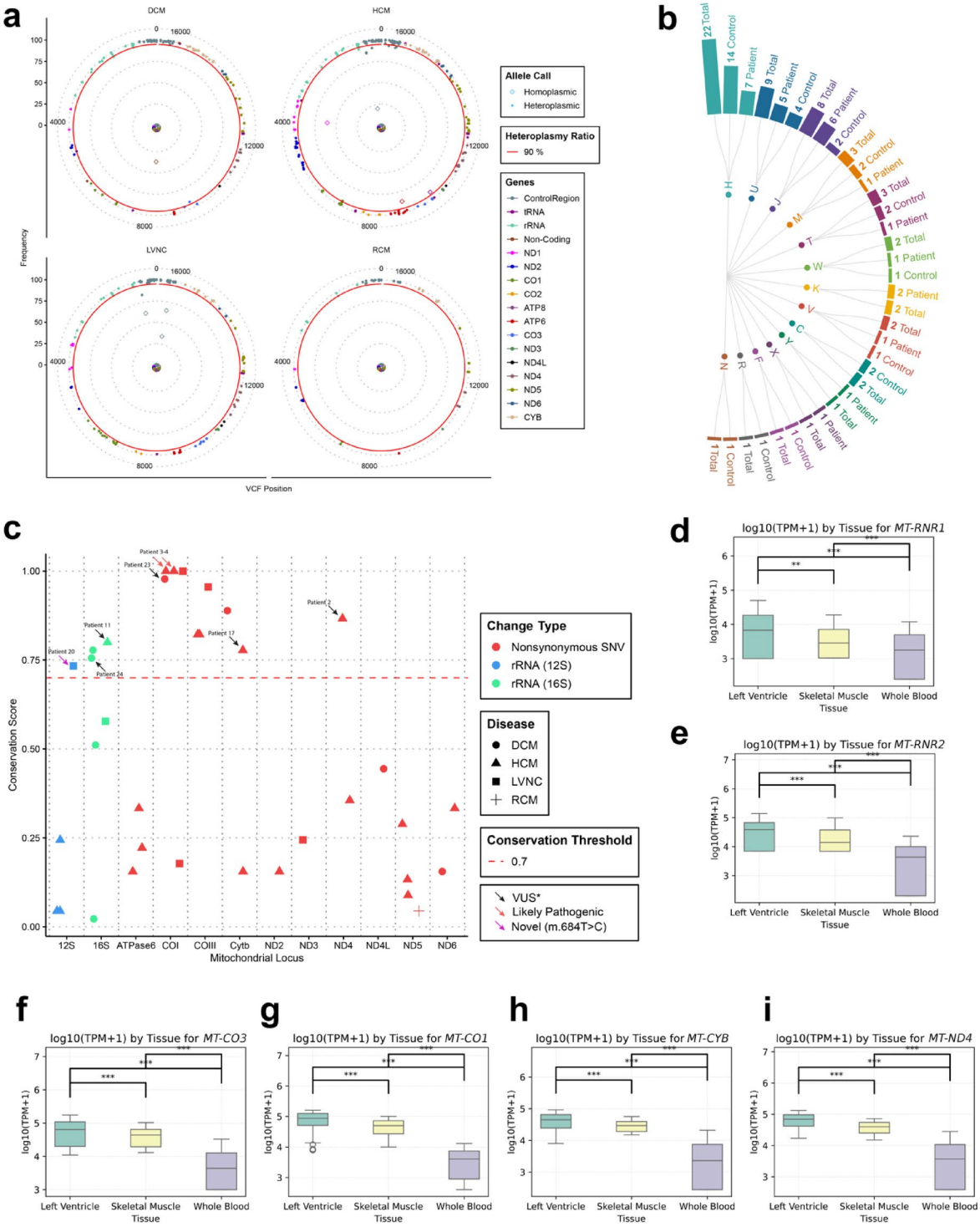


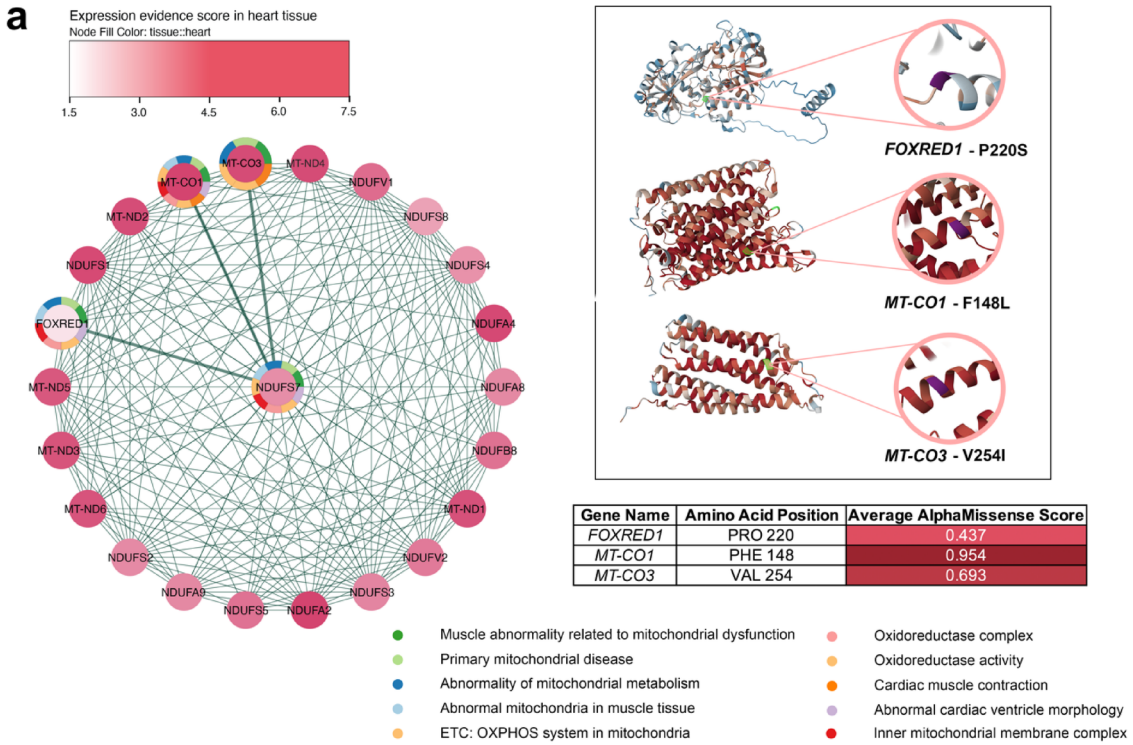
Fig. 3. Analysis of mtDA Variants, haplogroups and expression profiles. **(a)** Mitochondrial variants across cardiomyopathy subtypes from mtDNA sequencing are displayed on a solar plot. An identified variant is represented by each dot. According to the plot legend, the inner dashed rings of the plot represent the level of heteroplasmy and dots are colored according to genes at that region. The position of the mitochondrial base pair is shown on the X-axis. Red line represents a line for specified 90% heteroplasmy level. **(b)** mtDNA haplogroups distribution for the study cohort. **(c)** Filtered variants across mtDNA genes and functional consequences of these variants. Alteration type and the subtype of the patient determines the color and the shape, respectively. Conservation score line was given as red dashed line. Variants that are indicated by arrows are either pathogenic or likely pathogenic which are depicted as black or pink color, respectively. **d-i.** Expression levels of mtDNA genes with detected variants across cardiac muscle, skeletal muscle, and whole blood tissues, derived from the GTEx database. Statistical significance was observed between all tissue groups at the 99% confidence level ($p < 0.01$).

lately, leading to impaired myocardial relaxation and diastolic dysfunction[33]. Additionally, pathogenic variants of *TNNC1* have been associated with DCM and HCM in OMIM and in this gene support this association[34,35]. Although HCM is typically inherited in an autosomal dominant manner, the presence of homozygous situation in our study, with another mutation in the same gene, causing RCM in a case study presented by Ploski et al., suggests that pathogenic variants in this gene may have an autosomal recessive inheritance pattern for RCM[36]. On a side note, the variability of Ca2 + concentration, which affects myofilament function for each subtype, can create a difference for the expressional variability of cardiomyopathy. For example, high Ca2 + levels during contraction have been associated with hypertrophy in heart muscle, while low levels have been associated with dilation. Hence, examining the mitochondria regarding the storage of cytosolic Ca2 + in terms of the diagnosis and follow-up of the disease has been approved[37].

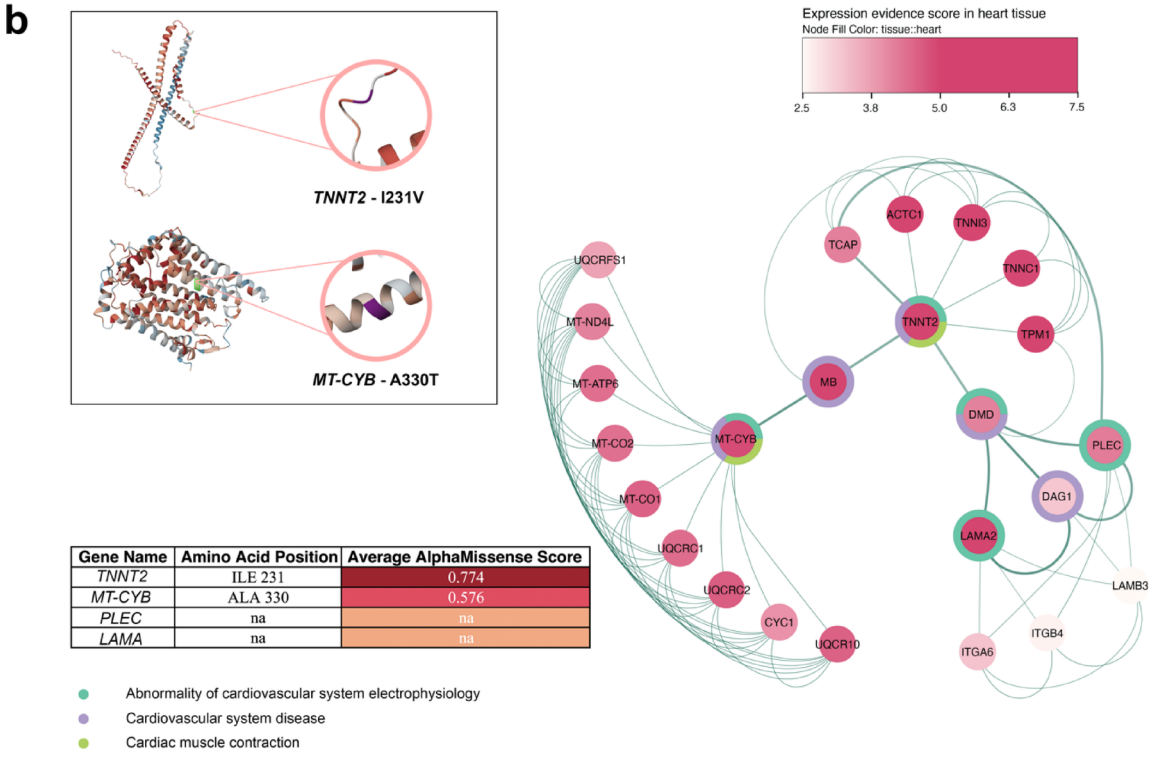
Furthermore, the PM2 (Absent from controls) and BP1 (Missense variant in a gene for which primarily truncating variants that are known to cause disease) criteria within the ACMG guidelines are overly broad, leading to frequent classification of variants as VUS (see Fig. 2d). A revision is necessary to enhance their specificity and improve variant interpretation accuracy. Nonetheless, this study identified nine novel VUS in four genes that may be directly or indirectly associated with cardiomyopathy (see Supplementary Table 2D).

After the evaluation of the variants in the nuclear genome, we continued our analysis by integrating and evaluating mtDNA variants. Specifically, patient-3 and patient-4, who are siblings, were found to have heterozygous missense variant, *FOXRED1*:p.Pro220Ser (OMIM: 613622). Furthermore, these siblings also have highly conserved mitochondrial variants in *MT-CO1* and *MT-CO3* (m.6345 T > C and m.9966G > A, respectively) and former one with the conservation score of 100% is concluded as likely pathogenic according to in silico analyses. Next, we looked at the pathways and structural pathogenicity of the variants of the three genes with their interactors in these two siblings to see if we could find evidence that the co-occurrence of these variants has an effect. The results of the gene enrichment analysis conducted on the first common interactor (NADH: ubiquinone oxidoreductase core subunit S7 encoding *NDUFS7* (OMIM: 601825), a subunit of one of the complexes that forms the mitochondrial respiratory chain) of all three genes, following the network analysis performed on the String platform, are presented in Fig. 4a along with structural positions of the variants. *MT-CO1* variant has the highest alpha missense pathogenicity score (0.954), followed by variant in *MT-CO3* (0.693). The network reveals a multi-protein interaction that is involved as many subunits in the NADH: oxidoreductase complex. A functional study in heart-specific Ndufs4-null mice demonstrated a reduction of approximately 50% in complex I activity in the heart, resulting in severe hypertrophic cardiomyopathy[38]. It can be reasonably deduced that disruptions to this network are likely to be the underlying cause of HCM. Although the pathogenicity score of *FOXRED1* variant and the expression of the gene are lower than those of the other genes, it is noteworthy that the variant is located at the beginning of an alpha-alpha connection, which is the most commonly occurring structural families. Moreover, the expression of *FOXRED1* in the heart is higher than in the skeleton and whole blood, although it is not as elevated as that of the mitochondrial genes (Supplementary Fig. 5a). When analyzed individually, cytochrome c oxidase, also known as Complex IV, is identified as the enzyme in the electron transport chain that transfers electrons from cytochrome c to molecular oxygen. The enzyme is composed of 14 subunits in humans, with only two (*MT-CO1* and *MT-CO2*) exhibiting catalytic activity. *MT-CO3*, however, lacks this function. Additionally, the gene *FOXRED1*, which encodes structural subunits and assembly factors, is associated with mitochondrial Complex-I deficiency and linked to cardiomyopathy[39]. It has been also noted that *MT-CO1* plays a role in Complex IV formation and is important in the context of cardiomyopathies[40]. Additionally, gene enrichment analysis has revealed interactions in which these four genes co-occur. Those identified through platforms such as Monarch Phenotype, Gene Ontology (GO), and Wikipathways indicate a potential defect due to a mitochondrial abnormality in the cardiac muscle. Consequently, the co-existence of these two variants in *FOXRED1* and *MT-CO1* could explain the early-onset etiology of HCM in these siblings whose father has also has late-onset HCM and pedigree is given in Supplementary Fig. 4b. Altogether, the variants in *MT-CO1* and *FOXRED1* genes can be associated with early-onset cardiomyopathy, emphasizing their relevance within mitochondrial diseases in children, especially in the context of complex I disorders[41].

Patient-17 has missense variants in *TNNT2*, *PLEC*, and laminin subunit alpha 2 (*LAMA2*, OMIM: 156225), as well as in *MT-CYB* (m.15734G > A). The position of the variant has a high conservation rate (78%), and it is accompanied by a *MT-RNR1* variant (m.2060 A > G). Both genes are highly expressed in the left ventricle when compared to both skeletal muscle and whole blood and they are the third most highly expressed in the heart, after the brain and kidney. The expression of *PLEC* and *LAMA2* in the heart muscle is relatively low in comparison to other tissues, yet higher than that observed in skeletal muscle and whole blood (Supplementary Fig. 5a). While *LAMA2*, *PLEC*, *TNNT2* and *MT-CYB* have different roles in the cell, they are interlinked in cardiac muscle function (Fig. 4b). The structural position of the identified variants through AlphaMissense could only be analysed for *TNNT2* and *MT-CYB*, as the others were not found. The pathogenicity score evaluation for the *TNNT2*:p.Ile231 position is higher than for *MT-CYB* (0.774 and 0.576, respectively). Furthermore, gene set enrichment analysis of all four genes, including interactors, also suggested the presence of abnormal cardiac muscle electrophysiology. Moreover, *TNNT2* variant may correlate with asymmetric septal hypertrophy, impacting cardiac troponin T (cTnT) essential for regulating calcium and actin contractility, especially in the left ventricle. A study by Rippol-Vera et al. suggests that some *TNNT2* variants may interact with other variants associated with other cardiac diseases or pathways, influencing the way the disease develops and the prognosis[42]. It is essential that *MT-CYB* in the mitochondria produces energy correctly in its role in the ATP supply chain, which is needed by *TNNT2* and the cardiac muscle to function efficiently. In addition, plectin is a cytolinker protein that stabilizes cytoskeletal networks and connects intermediate filaments to membrane complexes, contributing to structural integrity. Like desmoplakin, which anchors desmosomes to intermediate filaments and is essential for intercellular adhesion in cardiac tissue, plectin supports cardiomyocyte stability under



Gene Name	Amino Acid Position	Average AlphaMissense Score
FOXRED1	PRO 220	0.437
MT-CO1	PHE 148	0.954
MT-CO3	VAL 254	0.693



Gene Name	Amino Acid Position	Average AlphaMissense Score
TNNT2	ILE 231	0.774
MT-CYB	ALA 330	0.576
PLEC	na	na
LAMA	na	na

mechanical stress[43]. Therefore, the co-occurrence of these variants has the potential to provide an explanation for the observed phenotype.

Lastly, patient-11 has one homozygous variant, *MYL3*:p.Ala57 Asp, with an AlphaMissense pathogenicity score that is high (0.860, see Supplementary Fig. 5b). Furthermore, the high expression of *MYL3* in the left ventricle (Fig. 2c) and the presence of a highly conserved mitochondrial variant in *MT-RNR2* (m.2060 A > G) in patient-11 may contribute to the phenotype because of their potential cumulative effect. *MYL3* encodes a component of the myosin complex, essential for muscle contraction. The combined dysfunction of the 16 S subunit may result in the onset of cardiac and muscle-related symptoms, particularly affecting energy-intensive

◀**Fig. 4**. PPI network analysis for affected both nDNA and mtDNA genes and structural findings. **(a)** Network analysis of *FOXRED1*, *MT-CO1*, and *MT-CO3* in siblings with HCM. This network, consisting of 22 interconnected nodes, highlights the interactions among these genes. The thick green line indicates the first common interactor. Expression evidence scores are provided by StringDB. The accompanying boxes and tables showcase the positions of variants within the 3D structures of the proteins, along with their corresponding AlphaMissense pathogenicity scores. The colors surrounding the nodes represent the pathways in which the proteins are involved. **(b)** A PPI network was constructed for *PLEC*, *LAMA2*, *TNNT2*, and *MT-CYB*, revealing 24 interconnected nodes in patient-17 with HCM. AlphaMissense scores were not available for variant positions in *PLEC* and *LAMA2*. The colors surrounding the nodes indicate the pathways in which these proteins are involved. For AlphaMissense values above 0.7 indicate a high pathogenicity indicator, while those between 0.3 and 0.7 suggest a moderate pathogenicity indicator, and values below 0.3 indicate a low pathogenicity indicator (In this context, we have adopted 0.693 as a proxy for 0.7).

tissues such as the heart and skeletal muscles[44]. This could potentially elucidate the clinical presentation of the patient.

A study by Zaragoza et al., which examined the relationship between mtDNA and cardiomyopathy, classified a variant on *MT-ND4* as highly likely to be pathogenic for HCM/DCM[45]. In silico analysis demonstrated that the variant (m.11084 A > G) on *MT-ND4* in patient-2 is likely pathogenic. Additionally, its AlphaMissense prediction score is also high (0.797, see Supplementary Fig. 5b). Given that this gene is highly expressed in the left ventricle among tissues (Fig. 3i), it is possible that this variant may have resulted in HCM for this patient. Another patient-23 with DCM has a VUS in *MT-COI* and its association with mitochondrial cardiomyopathies is already known[40] therefore this variant can explain the phenotype for patient-23 considering its high expression rate in left ventricle even though its AlphaMissense pathogenicity score is moderate (0.389).

Next, patient-24 has an insertion (m.3159 AA > T) in *MT-RNR2*, 16 S locus, which is a very rare variant with a high conservation score. Two variants in *MT-CO3* (m.9948G > A) and *MT-RNR1* (12 S locus, m.684 T > C, relatively highly conserved), were detected in patient-20 with LVNC. The latter one seems a novel homoplasmic variant for this patient, whose pedigree is provided in Supplementary Fig. 4c. This patient's one sibling died of an unknown cause and another sibling also manifests LVNC and some other dysmorphic findings caused by additional *de novo* microdeletion revealed by microarray analysis. Furthermore, the heteroplasmic m.525 C > AC insertion detected in *MT-RNR1* in patient-19 also suggests that this locus may be important for LVNC. It is suggested that mitochondrial rRNA mutations can affect mitochondrial translation and can cause or modify the phenotype of cardiomyopathy[44,46,47]. In a study that investigates the *MT-RNR1* encoding the 12 S subunit of mitochondrial rRNA, it was stated that LVNC may be associated some variants in this locus. However, they also suggested that the 12 S rRNA, would have a more smaller effect than other mutations found that changed its conformation[48]. One study suggests that analyzing mtDNA sequence variants in myocardial tissue samples could provide useful information for understanding the causes of LVNC[49]. The study also suggests reduced mtDNA in LVNC patients, indicating potential mitochondrial dysfunction normally contributing to LVNC development. It is thereby evident that mitochondrial rRNA encoding variants should be examined in great elaboration regarding LVNC, given the existence of a potential link between the two.

Conclusion

Our study is the first whole mtDNA sequencing study in the region, contributing to the country's mtDNA haplogroup map and providing foundational data for understanding regional genetic diversity and its potential impact on complex disease associations. Indeed, as stated by the ACMG, interpreting mitochondrial variants is complex and challenging[50]. Hence, this study was conducted to elucidate the significance of polygenic effects, including those of mtDNA variants and other variants with unknown effects, in pediatric cardiomyopathy patients. Rather than relying solely on large panels, more targeted and robust analyses tailored to specific subtypes could provide deeper insights[51]. In our study, integrating mtDNA sequencing provided crucial insights that would otherwise remain unknown, highlighting the importance of this approach in understanding pediatric diseases involving bioenergetic pathways, such as cardiomyopathies.

Limitations

It is important to note that a significant proportion of the evaluated variants are found within the *TTN* and *PLEC* gene alone (see Supplementary Fig. 2). Given the large size of these genes, this is not an unusual finding. However, there is a clear need for studies that demonstrate the interaction with other genes in cardiac disorders, as VUSs in these genes alone is insufficient to explain the diseases. However, this study did not evaluate the effect of these variants in combination with others in patients with *TTN* VUSs. Nevertheless, investigating *TTN* mutations remains crucial[52], particularly for understanding their role in inherited cardiomyopathies manifesting at a young age[53]. Furthermore, there is currently no tool available to evaluate human *MT-RNR1* and *MT-RNR2* variants in silico, which is an important limitation given the growing number of such variants. Some combined approaches, such as Heterologous Inferential Analysis (HIA)[54], have been proposed to address this gap, but more research is needed. Finally, identifying isolated cases of non-syndromic pediatric primary cardiomyopathy is inherently challenging due to their rarity, which contributed to the limited sample size of this study. As the cohort was intentionally restricted to these isolated group, findings may not be generalizable to all forms of pediatric cardiomyopathy, particularly syndromic or secondary types.

Methods

Ethics declarations

This study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Clinical Practice Ethics Committee of the Faculty of Medicine at ESOGU (#25403353-050-99-E68845/2019-205). Informed consent was obtained from the parents or legal guardians of the probands, and each participant provided informed consent.

Diagnosis

All patients were evaluated in pediatric cardiology department. Transthoracic echocardiogram (TTE) and a 12-lead electrocardiogram (ECG) was used as initial modalities a nd, 24- to 48-hour ambulatory electrocardiographic monitoring, exercise TTE, intraoperative transesophageal echocardiogram (TEE), cardiac magnetic resonance imaging (MRI) was used when necessary.

Children with positive family history and left ventricular wall thickness more than 2 standard deviations (SD) from the predicted mean and without family history, more than 2.5 SDs were diagnosed with hypertrophic cardiomyopathy (HCM) according to 2020 American Heart Association/American College of Cardiology (AHA/ACC) guidelines[55]. Children with reduced left ventricular ejection fraction (LVEF) (< 50%) with enlarged left ventricle volume in the absence of other cardiac findings including coronary artery disease, primary valvular heart disease in addition other systemic and extrinsic reasons including hypothyroidism, toxins and medications were diagnosed with dilated cardiomyopathy (DCM)[56]. There were only two having restricted cardiomyopathy (RCM) who were children of first-degree cousins. They had reduced ventricular filling with normal/near normal ejection fraction and myocardial thickness and mildly reduced diastolic volumes. These patients were diagnosed with RCM in the presence of all these findings according to ESC[57]. The LVNC group was diagnosed according to morphological diagnostic criteria stated by Jenni et al.[58]; multiple trabeculations in echocardiographic imagining, multiple communicating ventricular cavities and intertrabecular recesses in color Doppler imaging, recesses within the middle and apical side of the ventricle, and a noncompacted to compacted ratio more than 2.0, in addition to the endocardium with two layered constructions. While the AHA classifies LVNC as a genetic cardiomyopathy[15], the European Society of Cardiology (ESC) considers it unclassified[14] due to ongoing controversy regarding its clinical significance[59]. Diagnostic uncertainty is further complicated by methodological variability and the absence of a gold standard. Jenni et al.[58] proposed a widely adopted echocardiographic criterion based on an NC/C ratio >2 in systole, though interobserver variability remains a concern[60]. Recent studies suggest that prominent trabeculation alone may not correlate with adverse outcomes in all populations, particularly in the absence of left ventricular dysfunction[61]. This study focused solely on non-syndromic, primary genetic cardiomyopathies as defined by the AHA[15], using Jenni criteria for consistent LVNC diagnosis.

The subjects were then elaboratively evaluated by clinical geneticists to exclude secondary causes of cardiomyopathy, including RASopathies, muscular dystrophies, Barth syndrome and other metabolic disorders. They were evaluated for neurodevelopmental milestones, intellectual capability, and underwent a systemic examination with anthropometric measurements, dysmorphic feature inspection, organomegaly examination, and neurological examination. Perinatal history and pedigree analysis were also assessed, and ophthalmological and auditory examinations were done if needed. For control group, there were 32 participants, but one of them voluntarily withdrew from the study. They had no history of heart problems or familial heart and syndromic findings, as confirmed by cardiology and genetics clinic.

Study population

This study included only isolated cases of non-syndromic pediatric primary cardiomyopathy. Syndromic cases, secondary cardiomyopathies, and systemic diseases were excluded to focus on primary presentations without known extrinsic causes. All have been followed-up patients, and they were born in the Central Anatolia region, except for one individual whose family had relocated to the region. There are a total of 27 patients 12 of whom were female and 15 were male. The control group included 31 individuals, 21 of whom were female and 10 were male. Detailed demographic and clinical summary of the study participants can be found in Supplementary Table 1 A.

Sample collection and DNA isolation

Approximately 400 µl of peripheral whole-blood samples from 58 individuals were collected into EDTA containing tubes. MagPurix robotic system and its isolation kit were used in accordance with the manufacturer's instructions. Around 100 µl of total genomic DNA was isolated at a concentration range of 30–100 ng/µl measured and stored at −20 °C.

Sequencing and initial bioinformatic analyses

Library was prepared according to Illumina Trusight Enrichment and clinical exome sequencing was performed in Illumina-NextSeq500 platform. Mitochondrial DNA was subsequently sequenced on IonTorrent-S5 platform. The library preparation was conducted using the Ion AmpliSeq™ Library Kit 2.0 and Precision-ID mtDNA Whole Genome Panel by following the manufacturer's guide. Sequenced output data (fastq files) were aligned to the related genes of human reference genome (UCSC hg19/GRCh37) by BWA-mem (v.0.7.12)[62] and quality assessments were performed by using FastQC[63]. After quality trimming and filtering the bad reads out, variant calling was performed by GATK (v.4.4)[64]. For mtDNA variant calling, mtDNA_rCRS (NC_012920.1) is used as a reference genome for alignment[65] and Torrent Variant Caller plugin v.5.2.1.39 was used for variant calling. For mtDNA variants, BAM files were converted to fasta files through MitoSuite, which would give the consensus

sequence of the reading for some further analyses. Confirmation of novel mtDNA variant (m.684 T > C) has been carried out using Sanger sequencing.

Variant filtering and in silico predictions.

All variants identified in 137 genes that are associated cardiomyopathy and in mtDNA were obtained in VCF format and initially filtered based on the following criteria: (1) Variants at the exonic regions with MAF < 0.01 (minor allele frequency) and read depth of at least 20x were included. (2) mtDNA variants were compared to the control group variants, and if they were found in the control group, they were not considered for evaluation. General data handling was performed with and "R" base functions and (version 4.4.0, [R Core Team 2024]), and R packages and Python 3.10; ggplot2 and matplotlib for plotting.

Mitochondrial variant analyses were performed on these 'fasta' files through Mitomap-MITOMASTER and population data and conservation scores were obtained (reviewed on April 2, 2025)[66]. For the mtDNA-phylogenetic tree, haplogroups were retrieved from Haplogrep (v.2) using PhyloTree17[67]. Mitochondrial variants were classified following the specifications of the ACMG/AMP standards for mtDNA variant interpretation outlined by McCormick et al. in 2020[68]. Frequency-based criteria were applied as follows: PM2 for variants with a population frequency < 0.0002, BS1 for variants between 0.005 and 0.01, and BA1 for variants ≥ 0.01. The PP3 criterion, indicating computational support for pathogenicity, was applied only when multiple in silico tools, including APOGEE1[69], APOGEE2[70], HmtVar[71], and MToolBox[72], predicted a deleterious effect without any conflicting benign predictions, fully in accordance with the guideline's requirement for concordance across multiple predictors. Likewise, BP4 was assigned when computational tools consistently predicted a benign effect, and none supported pathogenicity, as recommended in the same guideline. Conservation scores were considered only as supporting context and were not used as standalone criteria. For rRNA variants, conservation scores, the expression profile and PP3 was applied only if HmtVar prediction labeled them as pathogenic since no specific rRNA-dedicated prediction tool is officially recommended by the panel.

For nDNA variants, a comprehensive set of prediction tools, provided by Ensembl Variant Effect Predictor (VEP) developed was applied[73]. Based on these in silico predictions and inspired by the Varsome scoring system, a pathogenicity or benign scoring scheme on ACMG criteria was implemented[74]. Scores of 8, 4, 2, and 1 were assigned to variants with very strong, strong, moderate, and supporting evidence of pathogenicity, respectively. These results were cross-referenced with ACMG criteria obtained using InterVar[75]. In addition, minor allele frequency (MAF) data from both gnomAD v4.1.0 and the Million Exome[76] dataset were incorporated to assess population-based frequency criteria.

Additionally, we evaluated all variants, excluding those within the rRNA genes, using AlphaMissense, a more recently developed deep-learning model for pathogenicity prediction[77]. Figure 1 was created using Adobe® Illustrator®. Some icons and design elements were selected from Illustrator's built-in library and were customized to reflect the context of the study. All content is based on original data and interpretations. Pedigree integration and figure composition, including the merging of plots, were also carried out using Adobe Illustrator (https://www.adobe.com/products/illustrator.html). The mitochondrial solar plot shown in the figure was adapted from Stephen Turner's github account[78].

Gene expression and protein-protein interaction network analysis

The publicly available GTEx database (https://www.gtexportal.org/home) was used to determine the expression levels of our selected genes in healthy human left ventricle tissue samples. The STRING database (https://string-db.org) was utilized to construct a PPI network encompassing the selected genes. PPI network was further expanded by including additional interacting proteins with a minimum interaction score of 0.700 and we included all interaction types for three patients. Network visualization was achieved using Cytoscape software. Isolated nodes were excluded from the final visualization. Following the PPI network construction, significant pathways or structural interactions associated with the core network nodes were identified through enrichment analysis. These enriched pathways were then highlighted within the main network nodes for improved visualization.

Protein 3D structures and pathogenicity scores

Protein 3D structures were generated using AlphaFold. For each variant identified in the study, the pathogenicity score was determined using the AlphaMissense pathogenicity tool and these scores are given in corresping tables in the figures.

Statistical analysis

Non-parametric Mann-Whitney U tests were employed to compare age distributions between patient and control groups due to non-normal distributions and small sample sizes. Gene expression values obtained from the GTEx database were log2 transformed and subjected to ANOVA to assess differences across heart muscle, skeletal muscle, and whole blood tissues. Tukey's HSD post-hoc tests were used to identify specific pairwise differences. Chi-squared tests were conducted to compare the distribution of haplogroups between patient and control cohorts. Statistical significance was set at $p < 0.05$ for all tests.

Data availability

Sequencing data for cardiomyopathy-associated genes in VCF file except five patients who are not alive will be shared reasonable request to corresponding author. Moreover, aligned mtDNA-seq data for 27 patients and 31 individuals in control group are publicly available upon publication on Sequence Read Archive (SRA) under BioProject Number PRJNA1028644 on NCBI (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1028644). Not ably, unfiltered variants for both mtDNA and nDNA are provided in Supplementary Table 1B-D along with the information of 137 genes.

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Author contributions

M.A.T. and E.E.G. conceptualized and designed the project. Funding acquisition was done by E.E.G and O.C. M.A.T. and D.C. performed all wet-lab procedures. M.A.T. performed all bioinformatics analyses and visualized the data. M.A.T., E.E.G. and E.S. interpreted the results. P.K. and H.K. were involved in diagnosing the patients. M.A.T., E.E.G., and E.S. provided discussion. B.D.A., S.A. and O.C. supervised the project. M.A.T. wrote the manuscript with final approval.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

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