Targeted panel sequencing in adult patients with left ventricular non-compaction reveals a large genetic heterogeneity

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For Review Only

Targeted panel sequencing in adult patients with left ventricular non-compaction reveals a large genetic heterogeneity

Running title: Genetic complexity of adult left ventricle non compaction

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ABSTRACT

Left ventricular non-compaction (LVNC) is a cardiomyopathy that may be of genetic origin, however few data are available about the yield of mutation, the spectrum of genes and allelic variations. The aim of this study was to better characterize the genetic spectrum of isolated LVNC in a prospective cohort of 95 unrelated adult patients through the molecular investigation of 107 genes involved in cardiomyopathies and arrhythmias.

Fifty-two pathogenic or probably pathogenic variants were identified in 40 patients (42%) including 31 patients (32.5%) with single variant and 9 patients with complex genotypes (9.5%). Mutated patients tended to have younger age at diagnosis than patients with no identified mutation. The most prevalent genes were *TTN*, then *HCN4*, *MYH7*, and *RYR2*. The distribution includes 13 genes previously reported in LVNC and 10 additional candidate genes.

Our results show that LVNC is basically a genetic disease and support genetic counseling and cardiac screening in relatives. There is a large genetic heterogeneity, with predominant *TTN* null mutations and frequent complex genotypes. The gene spectrum is close to the one observed in dilated cardiomyopathy but with specific genes such as *HCN4*. We also identified new candidate genes that could be involved in this sub-phenotype of cardiomyopathy.

KEY WORDS: Left ventricular non compaction, cardiomyopathy, molecular genetic, next generation sequencing

INTRODUCTION

Left ventricular non-compaction (LVNC, OMIM300183) is a relatively rare cardiomyopathy, with or without LV dysfunction, characterized by excessively prominent trabeculations and associated deep recesses that communicate with the ventricular cavity¹. LVNC is part of unclassified cardiomyopathies according to the European Society of Cardiology² and to genetic cardiomyopathies by the America Heart Association³.

The prevalence of LVNC was estimated at 0.014% to 1.3% depending on the age of patients^{4,5}. Multiple imaging techniques are usually useful for the diagnosis of LVNC, with variable echocardiographic or magnetic resonance imaging diagnostic criteria but no clear consensus so that the positive diagnosis may be challenging⁶. The phenotypic expression and evolution of isolated LVNC is highly variable, and clinical features can range from asymptomatic to symptomatic, with a relatively stable course over several years or an evolution towards severe complications including congestive heart failure, ventricular arrhythmia and sudden cardiac death, atrial arrhythmias and systemic embolic events⁶.

LVNC is supposed to be related to a premature arrest of compaction of the loose myocardial meshwork during fetal embryogenesis, with persistent trabeculated myocardium, but the precise pathophysiology remains poorly understood. A family history is noticed in a significant proportion of patients and predominant mode of inheritance is autosomal dominant, with some cases with an X-linked transmission⁷.

Several genes have been identified as LVNC disease causing. The first reported genetic cause of isolated LVNC was described by Bleyl et al. with mutations in the X-linked *TAZ* gene, also responsible for Barth syndrome⁸. The sarcomere-encoding genes (*MYH7*, *ACTC1*, *TNNT2*, *MYBPC3*, *TMP1*, and *TNNI3*) appear to account for 17 to 30% of LVNC^{9,10} but other genes such as *DTNA* (α -dystrobrevin), *NKX-2.5*, Z-line protein-encoding *ZASP/LDB3*, lamin A/C (*LMNA*) genes have been also associated with LVNC¹¹. Recently, the *TTN* gene was also reported as involved in this disease¹²⁻¹⁴, with a highly heterogeneous

prevalence, as high as 19% and the first responsible gene for LVNC in a German study of 68 index cases¹³ to 7% in adults but 0% in children from a Dutch cohort of 327 patients¹⁴.

Several studies have analyzed the spectrum of genes in LVNC⁹⁻¹⁶ but with heterogeneous strategies (Sanger or Next-generation sequencing), usually in retrospective cohorts without imaging core-lab, and usually with a relatively small panel of genes (45 genes in the Dutch study¹⁴, exome sequencing in the recent German study¹³. Therefore, the exact spectrum of LVNC-causing variants, their prevalence and their impact in genetic counseling remain poorly understood. Furthermore, the unique specificity of LVNC as an independent nosology entity has been questioned and LVNC has been suggested as an overlapping phenotype with hypertrophic or dilated cardiomyopathy⁹. To explore these issues, a prospective French national research program was launched and focused on consecutive adult patients with a recent diagnosis of isolated LVNC. The general aim was to better characterize the allelic and genetic spectrum of LVNC through a large panel of genes previously reported in the various cardiac hereditary diseases. The objective was also to identify new potential candidate genes that could be involved in the phenotype of LVNC.

METHODS

The present study was conducted as part of the Programme Hospitalier de Recherche Clinique (PHRC Ref: 2011-A - 00987-34, coordinator Pr. G. Habib, Marseille) aimed at describing the clinical spectrum of LVNC and at characterizing the genetic spectrum of LVNC through a next-generation sequencing (NGS) strategy in a new prospective cohort.

Patients, inclusion criteria

The study included unrelated patients with a minimal age at inclusion of 18 years old, enrolled between 2012 and 2013 in 13 French centers for inherited cardiac diseases. Collected data included clinical data (initial presentation, first symptoms, and data from cardiac and neurologic examination), family history, and tests including ECG, echocardiography, MRI, CT scan, Holter monitoring when available, as well as follow-up

data. Only patients with a recent diagnosis of isolated LVNC (maximum in the last 6 months before inclusion) were enrolled. All echocardiographic documents were sent and reviewed by a core lab (Marseille) to confirm the diagnosis. Diagnosis of isolated LVNC was considered definite when several criteria were present in left ventricle (LV): (i) multiple trabeculations with deep endomyocardial recesses, (ii) two-layer myocardial structure with a thin compacted (C) and a thick non compacted (NC) layer, (iii) color Doppler evidence of perfused intertrabecular recesses, (iv) systolic NC / C ratio > 2 (parasternal short-axis view); (v) no associated heart disease¹⁷. Cardiac MRI was also frequently performed in our series, with a NC/C ratio of >2.3 in diastole as the recommended threshold for the diagnosis of LVNC using this technique¹⁸. However, for the purpose of the current study, only the echocardiographic criteria were used as inclusion criteria. Only patients with a diagnosis validated by an imaging core lab confirming a definite diagnosis of LVNC were included. Informed consent, blood samples, and clinical evaluations were obtained from all patients, with a protocol approved by the Ethics Committee of AP-HM (Assistance Publique- Hôpitaux de Marseille).

Genetic analysis

Targeted gene enrichment, high-throughput sequencing: Patients' DNAs were extracted from peripheral blood with **QIAasymphony**® (Qiagen, Hilden, Germany) and qualitatively checked using Tape Station DNA genomic array (Agilent, Santa Clara, USA). Custom targeted gene enrichment and DNA library preparation were performed using the **Nimblegen** EZ choice probes® and Kappa HTP Library preparation kit® according to the manufacturer's instructions (Nimblegen®, Roche Diagnostics, Madison, USA). The targeted regions include all coding exons and **+/- 50 base pairs** of flanking intronic regions of 107 genes known to be involved in cardiomyopathies (77 genes) and arrhythmias (30 genes) (**Suppl. Table 1**). The targeted regions were sequenced using the Illumina MiSeq platform on a 500 cycle Flow Cell (Illumina, Santa Cruz, USA) and MiSeq Software generates FASTQ format files after demultiplexing patients' sequences.

Bioinformatics pipeline: In presence of overlapping paired-end reads, these were merged with Flash¹⁹. Merged single reads and paired-end reads were then aligned on Hg19 human reference genome using BWA-MEM²⁰. This was further followed by a local realignment around insertion and/or deletions and a quality base recalibration by using of the GATK program²¹. PCR and optical duplicates were highlighted with the MarkDuplicates Picard tool (<http://broadinstitute.github.io/picard>) and were further removed using samtools²². Resulting .bam outputs from merged single reads and properly paired-end reads were then combined into a unique .bam file. Variant calling was performed using the GATK Haplotype Caller program²¹ simultaneously on all sequenced samples. Detected variants were then annotated using ANNOVAR²³ and CADD²⁴ tools. Coverage statistics were produced using the HsMetrics Picard tool. Detected variants with sequencing depth greater than 30X and with at least 20% of reads supporting the alternative allele were kept for analysis. Detection of copy number variation (CNV) was performed after coverage normalization, by computing the ratio of a target's coverage of a given individual over the mean coverage of this target across all patients of the same sequencing run.

Variants interpretation: Pathogenicity of variants was determined according to current ACMG guidelines²⁵ that recommend classifying variants into 5 categories: pathogenic, likely pathogenic, unknown significance, likely benign and benign. A recent publication dedicated to cardiomyopathies recommended the use of a frequency threshold of 0.01%²⁶. Variants were filtered out according to their allele frequency as reported in the GnomAD database (<http://gnomad.broadinstitute.org/>). We then evaluated each variant considering a careful review of the literature, the location of the variant in the gene and the resulting corresponding protein, the *in silico* prediction tools (Polyphen2, SIFT, GVG D and Mutation Taster for missense variants and SpliceSiteFinder like®, MaxEntScan®, NNSPLICE®, GeneSplicer® and Human Splicing Finder® for splicing variants) and functional studies when available. Additionally, we looked at a local database of pathogenic variants related to our experience on the molecular diagnosis of cardiomyopathies. In practice, we considered as “pathogenic”

(class 5), a variant with confirmed pathogenicity criteria and already proved as responsible for cardiomyopathies or a novel nonsense variant with a frequency below 0.01%. We considered as “likely pathogenic” (class 4), unpublished variants with a frequency below 0.01% and unknown in our database, located in a functional domain of the protein and with pathogenicity prediction tools mainly (at least 3 out of 4 tools) in favor to a strong effect. “Variants of unknown significance” were new variants with no evidence for predicted deleteriousness and published variants with a frequency over 0.01%. Such variants were not considered in this work until proof of pathogenicity but are presented in supplemental data (Suppl. Table 2). In *TTN* gene, only null variants (consensus splice sites, stop codons, insertions and deletions leading to a shift in the reading frame) were considered as pathogenic according to a recent publication on *TTN* mutations in cardiomyopathies²⁷, and we excluded other variants, especially *TTN* missense variants. All variants considered as pathogenic and probably pathogenic have been confirmed by a second independent method (Sanger sequencing or MLPA)

Statistical analyses were performed with Fisher Exact test or chi2, for binary variables and Student t tests for continuous variables, when appropriate.

RESULTS

Patients

Ninety-five unrelated patients ranging from 19 to 81 years-old were included in the study with a mean age at diagnosis/inclusion of 46.3 (±15) years old. This cohort was composed of 56 males and 39 females. At the time of inclusion, 46 patients had a NYHA score >1, mean ejection fraction at inclusion was 42.5% (±14.5) and mean heart rate was 68.5 bpm (±15.6).

Performance of the custom panel

The Miseq sequencing run yielded an output of 1.8 to 2.2Gb per sample, with a mean sequencing depth per sample of 265 reads (SD:35.3). On average, 99.7% of selected targets (1740/1745) were covered over 30X and 98.5% (1720/1745) over more than 100X.

Allelic Spectrum

Cohort analysis led to the identification of 52 confirmed or highly suspected pathogenic variants (class 5 or 4) including 42 novel ones located in 23 different genes. Among these mutations, 50 were found in 22 cardiomyopathies related genes (*ACTC1*, *BAG3*, *DSC2*, *DSP*, *FLNC*, *HCN4*, *HEY2*, *LDB3*, *LMNA*, *MYBPC3*, *MYH6*, *MYH7*, *MYLK2*, *MYPN*, *NEXN*, *NKX2.5*, *PDLIM3*, *PKP2*, *RBM20*, *RYR2*, *TMEM43* and *TTN*) and 2 were observed in *ANK2* gene, known to be responsible for long QT Syndrome (Table 1).

Among the 22 cardiomyopathy genes, the most prevalent ones were *TTN* (19%, 10 variants), followed by *HCN4* and *MYH7* genes (10 %, 5 variants each), followed by *RYR2* (8%, 4 variants) then *MYH6* and *ACTC1* (6%, 3 variants each), then *MYBPC3*, *LDB3*, *MYLK2* and *NEXN* (4%, 2 variants). The 12 other genes were found mutated only once. Among the arrhythmias genes, *ANK2* was mutated in 2 patients (4%, 2 variants) (Fig. 1).

The 10 *TTN* truncating variants included 8 variants located in the A-band (80%) and two located at the end of the I-band at the junction with A-band. In the 22 remaining genes, 42 mutations were identified including 35 missense variants, 1 in-frame deletion, 5 null mutations (3 frame shifts, 1 splice and 1 non-sense mutations), and a CNV consisting in a complete deletion of *RYR2* exon 3 (Table 2).

In the second prevalent gene *HCN4*, 5 different missense variants were found including one already published in LVNC. These variants were located in transmembrane domain 4 (c.1123C>T, p.Arg375Cys), in the pore (c.1403C>T, p.Ala468Val, c.1438G>T, p.Gly480Cys and c.1444G>A, p.Gly482Arg) before the transmembrane domain 5 (c.1231C>G, p.Leu411Val). (Table 2)

In order to classify genes and variants according to their function in the cardiomyocytes, we defined 5 cellular “compartments” (Fig.1). The distribution of the 52 variants in the 23 genes showed that 52% of variants (N=27) were located in sarcomeric genes, 21% (N=11) were in ion channel or related genes, 8% (N=4) were in genes involved in the cellular structure, 6%

(N=3) were located in desmosome genes. In addition, 12% of variants (N=6) were found in transcription factors genes or genes involved in other structures or functions (eg. *NKX2-5*). Finally, upon the 52 identified variants, 40 were located in the 13 already known LVNC genes (77%) and 12 were located in the 10 additional candidate genes (23%)(Tables 1 & 2).

Multiple mutations in patients

In the cohort, 9 patients (9.5%) presented a complex genotype feature with the presence of more than one pathogenic variant. Seven patients harbored two disease-causing variants in cardiomyopathy genes (Fig.2) and 2 patients carried at least 3 pathogenic variants: one with *BAG3*, *MYH7*, and *NKX2-5* variants and the second with *ACTC1*, *ANK2*, *LDB3* and *MYLK2*. Regarding the *TTN* gene, 8 patients were carrying a unique *TTN* variant and 2 patients carried a *TTN* mutation associated with another gene variant (*MYH6*, *NEXN*).

Mutations, patients and phenotype

According to our variant selection criteria, a pathogenic variant was identified in 40 patients of the cohort (20 males and 20 females; 50%), including 31 patients (32.5%) with single variant and 9 patients with complex genotypes (9.5%). *TTN* mutations are predominant and identified in 10 patients of the cohort (10.5%). In 55 patients (58%), no genetic cause was identified. Fifteen patients had a known family history of LVNC, 55 patients were sporadic cases and family history was not available for 25 patients. Among these groups, the mutation rate is 53%, 46% and 28% respectively. The difference between familial cases and sporadic cases is not significant (p-value : 0.77).

An analysis was performed regarding the age at diagnosis, ejection fraction, presence of dyspnea (NYHA>1) and heart rate comparing the groups of mutated patients vs patients with no identified genetic cause. Mutated patients tended to be younger at diagnosis (43.0 vs 48.7 years, p=0.07) but systolic dysfunction showed no significative difference between groups

(Table 3). Interestingly, the mutation yield was higher in youngest patients <65 years old (38/84, 45%) as compared to oldest patients >65 years (2/11, 18.2%, p-value: 0.11).

Patients with complex genotypes (≥ 2 mutations), as compared to patients with single variants, tended to be younger at diagnosis and to have a decreased ejection fraction although differences were not significant (Table 3). Finally, in patients carrying a single variant, we observed that the LV mean ejection fraction in patient with a mutation in sarcomeric genes (N=18) was lower than in patients mutated in non-sarcomeric genes (N=13) (43.8% vs 51.6%, p-value: 0.26).

DISCUSSION

We present here the results of the genetic analysis of a cohort of 95 independent patients (index cases) with LVNC in order to evaluate the yield of mutation screening and to assess the allelic and genetic spectrum of the disease. The design of our study has some characteristics that may differ from previous studies since our project was a prospective study performed in newly diagnosed consecutive patients (diagnosis less than six months) with a validation of the cardiac diagnosis by an expert centralized imaging core-lab. This design was conceived to limit potential inclusion bias and strengthen the representativeness of the cohort. We also focused on isolated LVNC, without associated congenital heart defects, in adult patients in order to have a more homogeneous population. Next generation sequencing was performed with a panel of 107 genes involved in cardiomyopathies and some arrhythmias, which represents the most comprehensive genetic analysis published (exome sequencing was performed by Sedagast-Hamedani *et al* but [only selected genes known to be involved in the phenotype were reported](#))¹³.

When considering pathogenic or likely pathogenic variants, we identified a mutation in 42% of the patients, which represents a proportion slightly higher than previously reported in this disease^{9,10,13,14,16}, but relatively similar to features observed in other cardiomyopathies²⁸. The distribution of genes revealed a high degree of genetic heterogeneity with putative or

confirmed pathogenic mutations identified in 23 different genes. The distribution includes 13 genes previously published as associated with the phenotype of LVNC (77% of variants) and 10 additional candidate genes (9 cardiomyopathy and 1 arrhythmia genes) that were never reported before as associated with LVNC (Table 1). Despite the stringent selection criteria of variants, *TTN* represents the most prevalent gene in the cohort (19% of variants or 10.5% of patients) including 8 variants located in the A-band (80%). As previously observed in patients with DCM, *TTN* truncating mutations in the A-band region of the protein were over represented²⁷.

The following most prevalent genes were *HCN4* and *MYH7*, followed by *MYH6*, *RYR2* and *ACTC1*. Considering others published reports^{13,14}, some discrepancies were observed in the gene distribution especially regarding *TTN*, *MYH7*, *HCN4* and *LMNA*. Differences in distribution may be related in part to the variable characteristics of the cohorts (isolated LVNC or not, age at diagnosis, incident or prevalent cases). We observed a relatively high proportion of patients with *HCN4* pathogenic variants as we found 5 different variants, located in S4, S5 and pore domains of the protein (Table 2). Among these patients 3/5 presented bradycardia (one patient was implanted by a pace maker) but no valvular disease has been reported in any of them^{29,30}. This suggests that this recently published gene²⁹ constitutes an important disease-causing gene in LVNC. The prevalence of *HCN4* did not appear as such in previously published cohorts, possibly due to the absence of this gene in some studied panels^{9-11,14,15} or a difference in the cohort recruitment^{13,16}. For other genes, a higher rate of *MYH7* variants and a lower rate of *LMNA* variants were found in our cohort as compared with the study of Sedaghat-Hamedani *et al.*¹³ while frequency of *TTN* and *MYBPC3* variants were consistent. In the work of van Waning *et al.*¹⁴, the proportion of *MYH7* and *MYBPC3* in the group of adult patients were consistent with us but a lower rate of *TTN* variants was observed. In older publications^{9,10} in which only sarcomeric genes were analyzed, the spectrum of genes showed that *MYH7* was the most prevalent gene then *TNNT2*, *TMP1*, *TNNI3*. These last three genes were not found mutated in our cohort, which

could be due to the fact that our cohort is composed by patients with an adult onset of the disease. Interestingly, our results also strengthen the involvement of recently published genes such as *HCN4*^{29,30} and *RYR2*³¹ and help to better estimate their prevalence. As a whole, our finding about the large genetic and allelic spectrum is helpful in refining the genes of interest for routine molecular diagnostic of patients with LVNC.

Additionally, we tried to determine if patients reporting a familial history of LVNC, were more frequently found with a mutation than patients presenting as sporadic cases. The cohort includes 15 familial form and 55 sporadic cases, the 25 remaining patients had no information's about their relatives. Quite the same rate of mutation identification was found in the group of familial forms and sporadic cases (53 % and 46% respectively, difference not significant)"

Apart from the report of mutations in genes previously associated with LVNC, an important finding of our study is that we identified mutations in 10 genes known to be involved in cardiac inherited diseases but not described until now as associated with this specific phenotype. Among these genes, 9 were previously reported as associated with other sub-types of cardiomyopathy such as HCM, DCM and ARVC (Table 1), suggesting an overlap between the various cardiomyopathies. Among these 10 genes, *MYLK2* and *NEXN* were identified each in 2 patients and 7 genes (*BAG3*, *FLNC*, *HEY2*, *MYPN*, *PDLIM3*, *TMEM43* and *DSC2*) were involved each in only one patient. Although these genes could be good candidates for being pathogenic, the definitive role of these genes for causing LVNC will require confirmation in further studies, especially through segregation analyses in families or functional studies. Similarly, in 2 patients with no particular ECG abnormalities, we identified variants in *ANK2* gene known to be involved in long QT syndrome. As *ANK2* was never reported before in LVNC, cautious is necessary before any conclusion about the causal role of these variants. However, *HCN4* was initially described in channelopathies and then involved in LVNC^{29,30}, with a significant proportion in our cohort, illustrating the fact that a given gene involved in arrhythmias does not preclude the potential role in LVNC.

Another observation emerging from this study is the high level of patients (9.5%) presenting complex genotypes with causative variants in two (or more) different genes. This feature about double mutated patients was not previously described as so high in adult patients with LVNC. In patients with complex genotypes, cumulative effect of variants have been associated with a higher severity of the disease in one study¹⁴ but was not analyzed in details in most of other studies. The hypothesis of a gene-dose- effect can be suspected as well as in other sub- morphotypes of cardiomyopathies, particularly in HCM. In the present cohort, patients with complex genotypes tended to have a same age at diagnosis (43.1 vs. 43.5 years old) but more symptoms (dyspnea > NYHA1: 67% vs 53%) and a lower ejection fraction (36% vs. 47%). However these differences were not statistically significant and must be confirmed in larger cohorts.

The global analysis of the distribution of genes observed in our cohort of adult patients with LVNC also provides useful information regarding the debated issue of whether or not LVNC is an independent nosological entity or a phenotype overlapping with other cardiomyopathy sub-types such as HCM or DCM^{28, 32-34}. The biggest cohorts published so far about HCM patients (including 3267 HCM patients sequenced on 16 genes and 874 patients sequenced on 20 genes) reported sarcomeric genes (especially *MYBPC3*) as the major genes^{26,32}. In patients with DCM, the *TTN* gene has been consistently reported as the most frequent mutated gene³³. In a study of 639 DCM patients sequenced on 84 genes, the highest prevalence observed was for *TTN* (13%), *PKP2*, *MYBPC3*, *DSP*, *RYR2*, *DSC2* and *SCN5A* genes³⁴. In the present cohort, we observed that the most prevalent genes are *TTN*, then *MYH7*, *HCN4*, *MYH6*, and *RYR2*. This distribution, and the fact that *TTN* is by far the most frequent gene we observed in LVNC, as well as the high level of complex genotypes, suggests that the genetic profile of LVNC patients is relatively similar to patients with DCM but not similar to patients with HCM^{26,28,32-37}. However, the distribution of LVNC patients presents some specific findings, such as the relatively high rate of *HCN4* gene mutations, which favor the possible specific role of some particular genes in this disorder.

Limitations. Our results were derived from a cohort of adult-onset patients with isolated LVNC. Therefore, results may not be applicable to a pediatric population or a population with syndromic LVNC. Even though probably pathogenic variants completed all the criteria for pathogenicity, we don't provide family segregation and functional analysis for now.

In conclusion, molecular analysis of 107 genes in 95 adult patients with isolated LVNC shows a mutation detection rate of 42%. These data, coming from the most comprehensive study available until now in terms of genes that were analyzed, show that LVNC is basically a genetic disease in most cases, with a large genetic heterogeneity. The global distribution of genes appears quite close to the profile observed in DCM patients, with *TTN* as the most frequent mutated gene, but with some specific genes such as *HCN4*. We found 9.5% of patients presenting a complex genotype with a disease causing variant in two different genes located on different chromosomes. This observation could explain part of intra-familial variable expressivity in case of bi-lineal inheritance, as some relative should be carrier of a single variant with a moderate phenotype. We also described mutations in 10 genes not described until now as associated with LVNC. Although these genes are putative good candidates for causing LVNC, the definitive causal role of these genes in this phenotype will require confirmation in further studies.

REFERENCES

1- Towbin JA, Lorts A, Jefferies JL. Left ventricular non-compaction cardiomyopathy. Lancet. 2015;386:813-25.

2- Elliott P, Andersson B, Arbustini E, *et al.* Classification of the cardiomyopathies: A position statement from the European Society Of Cardiology Working Group on Myocardial and Pericardial Diseases. Eur Heart J 2008; 29:270–276.

3- Maron BJ, Towbin JA, Thiene G, *et al.* American Heart Association; Council on Clinical Cardiology, Heart Failure and Transplantation Committee; Quality of Care and Outcomes Research and Functional Genomics and Translational Biology Interdisciplinary Working Groups; Council on Epidemiology and Prevention. Contemporary definitions and classification of the cardiomyopathies: An American Heart Association Scientific Statement from the Council on Clinical Cardiology, Heart Failure and Transplantation Committee; Quality of Care and Outcomes Research and Functional Genomics and Translational Biology Interdisciplinary Circulation Journal. Circulation 2006;113:1807–1816.

4- Sandhu R, Finkelhor RS, Gunawardena DR, Bahler RC. Prevalence and characteristics of left ventricular non compaction in a community hospital cohort of patients with systolic dysfunction. Echocardiography. 2008;25:8-12.

5- Pignatelli RH, McMahon CJ, Dreyer WJ, *et al.* Clinical characterization of left ventricular non compaction in children: a relatively common form of cardiomyopathy. Circ J. 2003;108:2672–2678.

6- Habib G, Charron P, Eicher JC, *et al*; Working Groups 'Heart Failure and Cardiomyopathies' and 'Echocardiography' of the French Society of Cardiology.. Isolated left ventricular non-compaction in adults: clinical and echocardiographic features in 105 patients. Results from a French registry. Eur J Heart Fail 2011; 13:177–85.

- 1
2
3 7- Sasse-Klaassen S, Gerull B, Oechslin E, Jenni R, Thierfelder L. Isolated non
4 compaction of the left ventricular myocardium in the adult is an autosomal dominant
5 disorder in the majority of patients. *Am J Med Genet A*. 2003; 119: 162–167.
6
7
- 8
9 8- Bleyl SB, Mumford BR, Thompson V, *et al*. Lethal non compaction of the left
10 ventricular myocardium is allelic with Barth syndrome. *Am J Hum Genet*. 1997;61:868-
11 72.
12
13
- 14
15 9- Klaassen S, Probst S, Oechslin E, *et al*. Mutations in sarcomere protein genes in left
16 ventricular non compaction. *Circulation*. 2008;117:2893–2901.
17
18
- 19
20 10- Probst S, Oechslin E, Schuler P, *et al*. Sarcomere gene mutations in isolated left
21 ventricular non compaction cardiomyopathy do not predict clinical phenotype. *Circ*
22 *Cardiovasc Genet*. 2011;4:367-74
23
24
- 25
26 11- Xing Y, Ichida F, Matsuoka T, *et al*. Genetic analysis in patients with left ventricular non
27 compaction and evidence for genetic heterogeneity. *Mol Genet Metab*. 2006; 88:71-7.
28
29
- 30
31 12- Hastings R, de Villiers CP, Hooper C, *et al*. Combination of Whole Genome
32 Sequencing, Linkage, and Functional Studies Implicates a Missense Mutation in Titin
33 as a Cause of Autosomal Dominant Cardiomyopathy With Features of Left Ventricular
34 Non compaction. *Circ Cardiovasc Genet*. 2016;7 :426-435.
35
36
37
- 38
39 13- Sedaghat-Hamedani F, Haas J, Zhu F, *et al*. Clinical genetics and outcome of left
40 ventricular non-compaction cardiomyopathy. *Eur Heart J*. 2017 ;38:3449-3460.
41
42
- 43
44 14- van Waning JI, Caliskan K, Hoedemaekers YM, *et al*. Genetics, Clinical Features, and
45 Long-Term Outcome of Non compaction Cardiomyopathy. *J Am Coll Cardiol*. 2018
46 20;71:711-722
47
48
- 49
50 15- Tian T, Wang J, Wang H, *et al*. A low prevalence of sarcomeric gene variants in a
51 Chinese cohort with left ventricular non-compaction. *Heart Vessels*. 2015; 30:258-64.
52
53
54
55
56

16- Wang C, Hata Y, Hirono K, *et al.* A Wide and Specific Spectrum of Genetic Variants and Genotype-Phenotype Correlations Revealed by Next-Generation Sequencing in Patients with Left Ventricular Non compaction. J Am Heart Assoc. 2017 Aug 30;6(9).

17- Jenni R, Oechslin E, Schneider J, Attenhofer Jost C, Kaufmann PA. Echocardiographic and pathoanatomical characteristics of isolated left ventricular non-compaction: a step towards classification as a distinct cardiomyopathy. Heart 2001;86:666–67.

18- Petersen SE, Selvanayagam JB, Wiesmann F, *et al.* Left ventricular non-compaction: insights from cardiovascular magnetic resonance imaging. J Am Coll Cardiol. 2005; 46:101–105.

19- Magoc T. and Salzberg S. FLASH: Fast length adjustment of short reads to improve genome assemblies. Bioinformatics, 2011; 27:21, 2957-63.

20- Li H and Durbin R. Fast and accurate short read alignment with Burrows-Wheeler Transform. Bioinformatics. 2009; 25:1754-60.

21- McKenna A, Hanna M, Banks E, *et al.* The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. Genome Research. 2010; 20:1297-303.

22- Li H., Handsaker B, Wysoker A, *et al.* The Sequence alignment/map (SAM) format and SAMtools. Bioinformatics, 2009; 25: 2078-9.

23- Wang K, Li M, Hakonarson H. ANNOVAR: Functional annotation of genetic variants from next-generation sequencing data. Nucleic Acids Research. 2010; 38:16, e164.

24- Kircher M, Witten DM, Jain P, O’Roak BJ, Cooper GM, Shendure J. A general framework for estimating the relative pathogenicity of human genetic variants. Nature Genetics, 2014; 46: 310-5.

- 25- Richards S, Aziz N, Bale S, *et al.* Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. *Genet Med.* 2015; 17: 405-424.
- 26- Walsh R, Buchan R, Wilk A, *et al.* Defining the genetic architecture of hypertrophic cardiomyopathy: re-evaluating the role of non-sarcomeric genes. *Eur Heart J.* 2017;0:1-8
- 27- Ware JS, Cook SA. Role of titin in cardiomyopathy: from DNA variants to patient stratification. *Nat Rev Cardiol.* 2018 ;15:241-252
- 28- Walsh R, Thomson KL, Ware JS, *et al.* Reassessment of Mendelian gene pathogenicity using 7,855 cardiomyopathy cases and 60,706 reference samples. *Genet Med.* 2017;19:192-203.
- 29- Milano A, Vermeer AM, Lodder EM, *et al.* HCN4 mutations in multiple families with bradycardia and left ventricular non compaction cardiomyopathy. *J Am Coll Cardiol* 2014;64:745–56.
- 30- Schweizer PA, Koenen M, Katus HA, Thomas D. A Distinct Cardiomyopathy: HCN4 Syndrome Comprising Myocardial Noncompaction, Bradycardia, Mitral Valve Defects, and Aortic Dilation. *J Am Coll Cardiol.* 2017;69:1209-1210.
- 31- Ohno S, Omura M, Kawamura M, *et al.* Exon 3 deletion of RYR2 encoding cardiac ryanodine receptor is associated with left ventricular non-compaction. *Europace.* 2014;16:1646-54.
- 32- Lopes LR, Syrris P, Guttman OP, *et al.* Novel genotype-phenotype associations demonstrated by high-throughput sequencing in patients with hypertrophic cardiomyopathy. *Heart.* 2015;101:294-301.
- 33- Herman DS, Lam L, Taylor MR, *et al.* Truncations of titin causing dilated cardiomyopathy. *N Engl J Med.* 2012;366: 619-28.

34- Haas J, Frese KS, Peil B, *et al.* Atlas of the clinical genetics of human dilated cardiomyopathy. *Eur Heart J.* 2015 May 7;36:1123-35a.

35- Waldmüller S, Schroeder C, Sturm M, *et al.* Targeted 46-gene and clinical exome sequencing for mutations causing cardiomyopathies. *Mol Cell Probes.* 2015;29: 308-14.

36- Charron P, Elliott P, Gimeno JR, *et al.* The Cardiomyopathy Registry of the EURObservational Research Programme of the European Society of Cardiology: baseline data and contemporary management of adult patients with cardiomyopathies. *Eur Heart J.* 2018 Jan 24. [Epub ahead of print]

37- Mademont-Soler I, Mates J, Yotti R, *et al.* Additional value of screening for minor genes and copy number variants in hypertrophic cardiomyopathy. *PLoS One.* 2017;12:e0181465.

38- K.R. Egan, J.C. Ralphe, L. Weinhaus, *et al.* Just sinus bradycardia or something more serious? *Case Rep Pediatr*, 2013, p. 736164.

39- L. Shan, N. Makita, Y. Xing, *et al.* *SCN5A* variants in Japanese patients with left ventricular noncompaction and arrhythmia *Mol Genet Metab*,2008;93:468-474.

40- Parent JJ, Towbin JA, Jefferies JL. Fibrillin-1 Gene Mutations in Left Ventricular Non-compaction Cardiomyopathy. *Pediatr Cardiol.* 2016;37:1123-6.

41- Arndt AK, Schafer S, Drenckhahn JD, *et al.* Fine Mapping of the 1p36 Deletion Syndrome Identifies Mutation of PRDM16 as a Cause of Cardiomyopathy. *Am J Hum Genet* 93:67–77.

42- Ramond F, Janin A, Filippo SD, *et al.* Homozygous PKP2 deletion associated with neonatal Left Ventricle Non Compaction. *Clin Genet.* 2017;91:126-130.

43- Williams T, Machann W, Kühler L, *et al.* Novel desmoplakin mutation: juvenile biventricular cardiomyopathy with left ventricular non-compaction and acantholytic palmoplantar keratoderma. *Clin Res Cardiol.* 2011;100 :1087-93.

Table 1: List of genes published in LVNC cardiomyopathy and potentially new genes identified in the present work.

Cellular structure	Gene (NM)	Protein	Phenotype	Ref. in LVNC	This Cohort
Sarcomere					
	MYH7 (NM_000257.2)	Myosin heavy chain	HCM, DMC, LVNC	9-10	yes
	TNNT2 (NM_001001430.1)	Troponin T2	HCM, DMC, LVNC	9-10	no
	ACTC1 (NM_005159.4)	Cardiac Actin	HCM, DMC, LVNC	9-10	yes
	MYBPC3 (NM_000256.3)	Cardiac C protein	HCM, DMC, LVNC	9-10	yes
	TPM1 (NM_001018005.1)	Alpha-tropomyosin	HCM, DMC, LVNC	9-10	no
	TNNI3 (NM_000363.4)	Troponin I3	HCM, DMC, LVNC	9-10	no
	DTNA (NM_001390.4)	Alpha-Dystrobrevin	DCM, LVNC	11-15	no
	MYH6 (NM_002471.3)	Myosin light chain	DCM, HCM, LVNC	15	yes
	ACTN2 (NM_001103.2)	Actinin	HCM, LVNC	32	no
	TTN (NM_001256850.1)	Titin	DMC, HCM, LVNC	12	yes
	MYLK2 (NM_033118.3)	Myosin Light chain kinase	DCM	This work	yes
	MYPN (NM_032578.2)	Myopalladin	DCM	This work	yes
	NEXN (NM_144573.3)	Nexilin	HCM, DCM	This work	yes
Structure					
	FLNC (NM_001458.4)	Filamin-C	HCM, DCM	This work	yes
	LDB3 (NM_007078.2)	LIM Domain Binding 3	DCM, LVNC	11-15	yes
	LMNA (NM_170707.2)	Lamine A/C	DMC, LVNC	11-15	yes
Ion channel and related genes					
	ANK2 (NM_001148.4)	Ankyrin 2	LQT	This work	yes
	HCN4 (NM_005477.2)	Hyperpolarization Activated Cyclic Nucleotide Gated Potassium Channel 4	ARVC, LVNC	29,30	yes
	RYR2 (NM_001035.2)	Ryanodin receptor 2	ARVC, LVNC, CPVT	31	yes
	CASCQ2 (NM_001232.3)	Calsequestrin 2	QTL, LVNC	38	no
	SCN5A (NM_198056.2)	Sodium channel, voltage-gated, type V, alpha subunit	LQT, Brugada, DCM, LVNC	39	no

Other				
Nkx2.5 (NM_004387.3)	NK2 Homeobox 5	DCM, LVNC	11-15	yes
TAZ (NM_000116.3)	Taffazin	Barth Syndrom, LVNC	8	no
FBN1 (NM_000138.4)	Fibrillin	Marfan Syndrom, LVNC	40	no
ABCC9 (NM_020297)	ATP Binding Cassette Subfamily C Member 9	DCM, LVNC	35	no
PDRM16 (NM_022114)	PR/SET Domain 16	LVNC	41	no
BAG3 (NM_004281.3)	BCL2 Associated Athanogene 3	DCM	This work	yes
HEY2 (NM_012259)	Hairy-Related Transcription Factor 2	DCM	This work	yes
PDLIM3 (NM_014476.4)	PDZ And LIM Domain 3	ARVC, HCM	This work	yes
RBM20 (NM_001134363.1)	RNA Binding Motif Protein 20	DCM	13	yes
TMEM43 (NM_024334.2)	TransmembraneProtein 43	ARVC	This work	yes
Desmosome				
PKP2 (NM_004572.3)	Plakophilin 2	ARVC, LVNC	42	yes
DSP (NM_004415.2)	Desmoplakin	ARVC, LVNC, DCM	43	yes
DSC2 (NM_024422.3)	Desmocollin	ARVC	This work	yes

Table 2: List of pathogenic and probably pathogenic variants identified in the cohort.

Position c., cDNA position; Position p., protein effect; Published: No or Yes; Associated phenotype for published variants; GnomAD correspond to the allelic frequency, and Htz corresponds to the allele count in GnomAD in all populations; GVGD, SIFT, Mutation taster and polyphen are algorithms corresponding to *in silico* Predictive Algorithms used for evaluation of missense variants. Range of scores for each are indicated in the title column; "Type" indicated the nature of the variant; MS: missense, NS: Nonsense, Del; deletion, Splice; mutation affecting splicing site. Column "interpretation", indicates conclusions about the pathogenicity of the variant: class 5 ; certainly pathogenic, Class 4; probably pathogenic. NA: not applicable

Gene	Position c.	Position p.	Published No/Yes	Associated phenotype	GnomAD, Htz	GVGD (C65-C0)	SIFT (0-1)	Mutation Taster (1-0)	Polyphen (1-0)	Type	Interpretation
ACTC1	c.670G>T	p.Asp224Tyr	N	NA	/	65	0	1	0.994	MS	Class 4
ACTC1	c.281A>G	p.Asn94Ser	N	NA	4.061e-6, 1	45	0	1	0.615	MS	Class 4
ACTC1	c.623G>A	p.Arg208His	N	NA	4.061e-5, 10	25	0	1	0,01	MS	Class 4
ANK2	c.11150T>A	p.Ile3717Asn	N	NA	1.083e-5, 3	45	0	0,995	0.865	MS	Class 4
ANK2	c.9145C>T	p.Arg3049Trp	N	NA	8.155e-6, 2	65	0	0,93	0.999	MS	Class 4
BAG3	c.465_466insGCG	p.Ala155delinsAlaAla	N	NA	/	NA	NA	NA	NA	MS	Class 4
DSC2	c.1448A>T	p.Asn483Ile	N	NA	/	15	0	NA	0.905	MS	Class 4
DSP	c.3035delA	p.Asp1012fs	N	NA	/	NA	NA	NA	NA	Del	Class 5
FLNC	c.1933_1935del	p.645del	N	NA	3.969e-5, 11	NA	NA	NA	NA	Del	Class 4
HCN4	c.1403C>T	p.Ala468Val	N	NA	4.065e-6, 1	65	0	1	0,95	MS	Class 4
HCN4	c.1123C>T	p.Arg375Cys	N	NA	4.061e-6, 1	65	0	1	0,99	MS	Class 4
HCN4	c.1231C>G	p.Leu411Val	N	NA	/	25	0	1	0,99	MS	Class 4
HCN4	c.1444G>A	p.Gly482Arg	Y	NCVG	/	65	0	1	1	MS	Class 5
HCN4	c.1438G>T	p.Gly480Cys	N	NA	/	65	0	1	1	MS	Class 4
HEY2	c.683C>T	p.Thr228Met	N	NA	3.231e-5, 2	0	0,05	1	0,45	MS	Class 4
LDB3	c.625G>C	p.Glu209Gln	N	NA	/	25	0	1	0,94	MS	Class 4
LDB3	c.608C>T	p.Ser203Leu	Y	CMD	2.538e-5, 7	65	0	1	0,88	MS	Class 5

<i>LMNA</i>	c.738delG	p.Gln246fs	N	NA	/	NA	NA	NA	NA	Del	Class 5
<i>MYBPC3</i>	c.532G>A	p.Val178Met	Y	HCM	/	0	0,01	1	0,992	MS	Class 5
<i>MYBPC3</i>	c.1504C>T	p.Arg502Trp	Y	HCM	5.411e-5, 15	65	0	1	0,484	MS	Class 5
<i>MYH6</i>	c.1793dupA	p.Asn598fs	N	NA	8.122e-6, 2	NA	NA	NA	NA	Dup	Class 5
<i>MYH6</i>	c.4828C>T	p.Arg1610Cys	N	NA	3.247e-5, 1	0	0	1	0,988	MS	Class 4
<i>MYH6</i>	c.50G>T	p.Arg17Leu	Y	Cardiac septal defect	/	0	0	1	0,55	MS	Class 5
<i>MYH7</i>	c.379C>A	p.Pro127Thr	N	NA	/	0	0	1	0,98	MS	Class 4
<i>MYH7</i>	c.3830G>C	p.Arg1277Pro	N	NA	/	35	0	1	0,842	MS	Class 4
<i>MYH7</i>	c.3586C>T	p.His1196Tyr	N	NA	/	15	0	1	0,613	MS	Class 4
<i>MYH7</i>	c.2419C>G	p.Arg807Gly	N	NA	/	25	0,02	1	0,85	MS/Splice	Class 4
<i>MYH7</i>	c.4588C>T	p.Arg1530X	N	NA	/	NA	NA	NA	NA	NS	Class 4
<i>MYLK2</i>	c.1754T>A	p.Ile585Asn	N	NA	/	45	0	1	0,921	MS	Class 4
<i>MYLK2</i>	c.1658G>A	p.Arg553His	N	NA	1.276e-5, 3	0	0,15	0,92	0,004	MS	Class 4
<i>MYPN</i>	c.3457G>A	p.Gly1153Arg	N	NA	1.219e-5, 3	65	0	1	1	MS	Class 4
<i>NEXN</i>	c.2012T>C	p.Ile671Thr	N	NA	2.541e-5, 7	0	0	1	0,936	MS	Class 4
<i>NEXN</i>	c.1396A>C	p.Lys466Gln	N	NA	/	0	0	1	0,996	MS	Class 4
<i>NKX2-5</i>	c.604C>G	p.Leu202Val	N	NA	/	25	0	1	0,07	MS	Class 4
<i>PDLIM3</i>	c.742C>T	p.Arg248Cys	N	NA	8.153e-6, 2	0	0	1	1	MS	Class 4
<i>PKP2</i>	c.2018G>A	p.Gly673Asp	Y	ARVC	/	65	0	1	1	MS	Class 5
<i>RBM20</i>	c.1907G>A	p.Arg636His	Y	DCM	/	0	0	1	0,99	MS	Class 5
<i>RYR2</i>	c.13936G>C	p.Asp4646His	N	NA	/	0	0	1	0,99	MS	Class 4
<i>RYR2</i>	c.6180G>T	p.Gln2060His	N	NA	/	15	0	1	0,89	MS	Class 4
<i>RYR2</i>	c.878A>C	p.Gln293Pro	N	NA	/	65	0	1	0,756	MS	Class 4
<i>RYR2</i>	c.169- ? c.273+?del ?	/	Y		/	NA	NA	NA	NA	Del	Class 5
<i>TMEM43</i>	c.317A>G	p.Tyr106Cys	N	NA	2.031e-5, 5	65	0	1	1	MS	Class 4
<i>TTN</i>	c.93376delA	p.Arg31126fs	N	NA	/	NA	NA	NA	NA	Del	Class 4
<i>TTN</i>	c.93376_93377del 1	p.Arg31126fs	N	NA	/	NA	NA	NA	NA	FS	Class 4

TTN	c.82724delA	p.Asn27575fs	N	NA	/	NA	NA	NA	NA	FS	Class 4
TTN	c.98039_98040insTCAA	p.Asn32680fs	N	NA	/	NA	NA	NA	NA	Ins	Class 4
TTN	c.64100_64101insTTGA	p.Asp21368X	N	NA	/	NA	NA	NA	NA	Ins	Class 4
TTN	c.53947C>T	p.Arg17983X	N	NA	/	NA	NA	NA	NA	NS	Class 4
TTN	c.61961G>A	p.Trp20654X	N	NA	/	NA	NA	NA	NA	NS	Class 4
TTN	c.44248C>T	p.Arg14750X	N	NA	/	NA	NA	NA	NA	NS	Class 4
TTN	c.80845C>T	p.Arg26949X	Y	CMD	/	NA	NA	NA	NA	NS	Class 4
TTN	c.43360C>T	p.Arg14454X	Y	CMD	/	NA	NA	NA	NA	NS	Class 4

Table 3: Summary of clinical data according to the genetic status

	Mutated Patients (N=40)	Not mutated patients (N=55)		Single mutation (N=31)	Complex genotype (N=9)		Sarcomeric gene (N=18)	Non Sarcomeric gene (N=13)	
Mean Age (years)	43.0±15.5	48.7±15.2	p=0.07	43.5±14.5	43.1±15.3	p=0.94	42.1±15.7	45.5±12.9	p=0.78
NYHA>1 (%)	48	47	p=1.00	41	77	p=0.12	44	31	p=0.48
Mean Heart rate (bpm)	71±18	66±13	p=0.18	70.5±20	73±11	p=0.60	72±17	67±24	p=0.50
Patients with Ejection Fraction <50% (%)	57	71	p=0.19	52	67	p=0.47	61	46	p=0.48

FIGURE LEGENDS

Figure 1: Distribution of genes according to their number of identified pathogenic variants and visualization of their cellular location and function.

Figure 2: Representation of genes association in patients carrying two pathogenic variants. Gene symbol were indicated on the right and left scales. For each of the seven patients carrying 2 mutations, the two mutated genes are connected by a straight line.

For Review Only

Suppl Data- Table 1: List of genes analyzed in this cohort.

Gene	Reference Sequence	Chromosome
AARS2	NM_020745.2	chr6
ABCC9	NM_020297	chr12
ACAD9	NM_014049	chr3
ACTA1	NM_001100_	chr1
ACTC1	NM_005159.4	chr15
ACTN2	NM_001103.2	chr1
AGK	NM_018238.3	chr7
AKAP9	NM_005751.4	chr7
ANK2	NM_001148.4	chr4
ANKRD1	NM_014391.2	chr10
BAG3	NM_004281.3	chr10
C2orf64(COA5)	NM_001008215.1	chr2
CACNA1B	NM_000718.2	chr9
CACNA1C	NM199460.2	chr12
CACNA2D1	NM_000722.2	chr7
CACNB2	NM_201596.2	chr10
CALR3	NM_145046.3	chr19
CASQ2	NM_001232.3	chr1
CAV3	MN033337.2	chr3
COX10	NM_001303.3	chr17
COX15	NM_078470.4	chr10
CSRP3	NM_003476.3	chr11
CTNNA3	NM_013266.2	chr10
DES	NM_001927.3	chr2
DSC2	NM_024422.3	chr18
DSG2	NM_001943.3	chr18
DSP	NM_004415.2	chr6
DTNA	NM_001390.4	chr18
EMD	NM_000117.2	chrX
EYA4	NM_004100.4	chr6
FBN1	NM_000138.4	chr15
FHL1	NM_001159702	chrX
FLNC	NM_001458.4	chr7
GAA	NM_000152.3	chr17
GJA5	NM_005266.5	chr1
GLA	NM_000169.2	chrX
GPD1L	MN_015141.3	chr3
HCN4	NM_005477.2	chr15
HEY2	NM_012259	chr6
JPH2	NM_020433.4	chr20
JUP	NM_002230.2	chr17

<i>KCNA5</i>	NM_002234.2	chr12
<i>KCND3</i>	NM_004980.4	chr1
<i>KCNE1</i>	NM_000219.3	chr21
<i>KCNE1L</i>	NM_012282.2	chrX
<i>KCNE2</i>	NM_172201.1	chr21
<i>KCNE3</i>	NM_005472.4	chr11
<i>KCNH2</i>	NM000238.2	chr7
<i>KCNJ2</i>	NM000891.2	chr17
<i>KCNJ5</i>	NM_000890.3	chr11
<i>KCNJ8</i>	NM_004982.2	chr12
<i>KCNQ1</i>	NM_000218.2	chr11
<i>KRAS</i>	NM_004985.3	chr12
<i>LAMP2</i>	NM_002294.2	chrX
<i>LDB3</i>	NM_007078.2	chr10
<i>LMNA</i>	NM_170707.2	chr1
<i>MRPL44</i>	NM_022915.3	chr2
<i>MYBPC3</i>	NM_000256.3	chr11
<i>MYH6</i>	NM_002471.3	chr14
<i>MYH7</i>	NM_000257.2	chr14
<i>MYL2</i>	NM_000432.3	chr12
<i>MYL3</i>	NM_000258.2	chr3
<i>MYLK2</i>	NM_033118.3	chr20
<i>MYOM1</i>	NM_003803.3	chr18
<i>MYOZ2</i>	NM_016599.3	chr4
<i>MYPN</i>	NM_032578.2	chr10
<i>NEBL</i>	NM_006393.2	chr10
<i>NEXN</i>	NM_144573.3	chr1
<i>NKX2-5</i>	NM_004387.3	chr5
<i>NPPA</i>	NM_006172	chr1
<i>PDLIM3</i>	NM_014476.4	chr4
<i>PKP2</i>	NM_004572.3	chr12
<i>PLN</i>	NM_002667.3	chr6
<i>PRDM16</i>	NM_022114	chr1
<i>PRKAG2</i>	NM_016203.3	chr7
<i>PSEN1</i>	NM_000021.3	chr14
<i>PSEN2</i>	NM_000447.2	chr1
<i>PTPN11</i>	NM_002834.3	chr12
<i>RAF1</i>	NM_002880.3	chr3
<i>RANGRF</i>	NM_016492.4	chr17
<i>RBM20</i>	NM_001134363.1	chr10
<i>RYR2</i>	NM_001035.2	chr1
<i>SCN1B</i>	NM_199037.3	chr19
<i>SCN2B</i>	NM_004588.4	chr11
<i>SCN3B</i>	NM018400.3	chr11
<i>SCN4B</i>	NM174934.3	chr11

SCN5A	NM_198056.2	chr3
SCO2	NM_001169109.1	chr22
SDHA	NM_004168.2	chr5
SGCD1	NM_000337.5	chr5
SLC25A4 (ANT1)	NM_001151.3	chr4
SNTA1	NM_003098.2	chr20
SOS1	NM_005633.3	chr2
SYNPO2	NM_133477.2	chr4
TAZ	NM_000116.3	chrX
TCAP	NM_003673.3	chr17
TGFB3	NM_003239.2	chr14
TMEM43	NM_024334.2	chr3
TMEM70	NM_017866.5	chr8
TMPO	NM_003276.2	chr12
TNNC1	NM_003280.2	chr3
TNNI3	NM_000363.4	chr19
TNNT2	NM_001001430.1	chr1
TPM1	NM_001018005.1	chr15
TTN	NM_001256850.1	chr2
TTR	NM_000371.3	chr18
VCL	NM_014000.2	chr10

Suppl. Data- Table 2: list of variants interpreted as VUS found in the cohort.

Gene name	HGVSc.	HGVSp.
AARS2	c.1752+13C>T	---
AARS2	c.44C>G	p.Ala15Gly
ABCC9	c.2424+10A>G	---
ABCC9	c.4212-31T>G	---
ABCC9	c.1981C>T	p.Arg661Cys
ABCC9	c.1165-7 1165-6delITT	---
ABCC9	c.-38C>A	---
ACAD9	c.244+7A>G	---
ACTN2	c.2057T>A	p.Ile686Asn
AGK	c.21G>A	p.Thr7Thr
AKAP9	c.11546+11G>C	---
AKAP9	c.11229G>A	p.Met3743Ile
AKAP9	c.11384A>G	p.Asn3795Ser
AKAP9	c.4246-4G>T	---
AKAP9	c.7212C>T	p.Thr2404Thr
ANK2	c.11032+45 11032+50delGTGTGT	---
ANK2	c.1288-40C>A	---
ANK2	c.2179-11A>G	---
ANK2	c.2024C>G	p.Thr675Arg
ANK2	c.5072A>G	p.Gln1691Arg
ANK2	c.7915C>G	p.His2639Asp
ANK2	c.2662C>A	p.Arg888Arg
ANK2	c.4710C>T	p.Thr1570Thr
ANK2	c.7161T>C	p.Ala2387Ala
CACNA1B	c.2268-23dupG	---
CACNA1B	c.3413+22T>C	---
CACNA1B	c.3711-41A>T	---
CACNA1B	c.4473+20G>C	---
CACNA1B	c.5777+34G>A	---
CACNA1B	c.274A>G	p.Thr92Ala
CACNA1B	c.282G>T	p.Trp94Cys
CACNA1B	c.4497C>T	p.Tyr1499Tyr
CACNA1B	c.5052C>T	p.Ala1684Ala
CACNA1B	c.6936C>T	p.Asn2312Asn
CACNA1C	c.1218-48C>T	---
CACNA1C	c.2531-39G>T	---
CACNA1C	c.5823-16G>A	---
CACNA1C	c.5930-33G>T	---
CACNA1C	c.3280A>G	p.Ile1094Val
CACNA1C	c.5519A>G	p.Glu1840Gly
CACNA1C	c.2460G>C	p.Lys820Asn
CACNA2D1	c.1516-10delT	---
CACNA2D1	c.728+29A>G	---
CACNB2	c.121 122insTTTTTT	p.Gln40_Ser41insPhePhe
CACNB2	c.1302+51_1302+52insCTTTTTTTTTTT	---
CACNB2	c.886-36dupT	---
CACNB2	c.1550A>C	p.Glu517Ala
CACNB2	c.1880G>A	p.Arg627His
CACNB2	c.1650C>T	p.Ser550Ser
COX15	c.507C>T	p.Tyr169Tyr
COX15	c.876C>G	p.Ser292Ser
COX15	c.999A>G	p.Ser333Ser
DES	c.1245-39G>A	---
DES	c.736-19G>A	---
DSC2	c.630+45G>A	---
DSC2	c.943-27A>G	---
DSC2	c.1448A>T	p.Asn483Ile
DSG2	c.1173C>A	p.Ser391Arg
DTNA	c.1138G>A	p.Ala380Thr
DTNA	c.549A>G	p.Glu183Glu
EMD	c.399+50C>T	---
FBN1	c.1148-33G>C	---
FBN1	c.1571C>T	p.Thr524Met
FBN1	c.3026C>T	p.Pro1009Leu
FBN1	c.165-7G>A	---
FBN1	c.1530G>A	p.Ser510Ser
FBN1	c.6402C>T	p.Pro2134Pro
FLNC	c.1048-11C>T	---
FLNC	c.1210+14delC	---
FLNC	c.4951+45T>C	---
FLNC	c.4301G>T	p.Arg1434Leu
FLNC	c.7546G>A	p.Glu2516Lys
FLNC	c.2733G>A	p.Lys911Lys
FLNC	c.492G>T	p.Arg164Arg
GAA	c.546+23C>G	---
GAA	c.2786G>A	p.Ser929Asn
GAA	c.2482-1G>T	---
GLA	c.427G>A	p.Ala143Thr
GLA	c.639+31C>G	---
HCH4	c.3081C>T	p.Pro1027Pro
HEY2	c.565T>A	p.Phe189Ile
HEY2	c.438G>T	p.Ser146Ser
HEY2	c.843C>G	p.Ser281Ser
JPH2	c.1836C>A	p.Pro612Pro
JPH2	c.1963C>A	p.Arg655Arg
KCNA5	c.1573C>T	p.Arg525Trp
KCND3	c.1269+18G>A	---
KCND3	c.1372-6dupT	---
KCNH2	c.431A>T	p.Asp144Val

KCNH2	c.2592+3G>A	---
KCNH2	c.1263G>A	p.Thr421Thr
KCNH2	c.3258T>C	p.Pro1086Pro
KCNH2	c.3258T>C	p.Pro1086Pro
KCNJ8	c.-214C>A	---
KCNQ1	c.160_168dupATCGCGCCCC	p.Ile54_Pro56dup
KCNQ1	c.576C>A	p.Arg192Arg
LDB3	c.399_407delAGGCACCCC	p.Gly134_Pro136del
LDB3	c.668C>T	p.Ser223Leu
MRPL44	c.828-35A>G	---
MRPL44	c.792C>T	p.Thr264Thr
MYBPC3	c.2068-47C>G	---
MYBPC3	c.2994+37G>T	---
MYBPC3	c.2320G>A	p.Ala774Thr
MYBPC3	c.2602G>A	p.Gly868Ser
MYH6	c.1892-35A>G	---
MYH6	c.4828C>T	p.Arg1610Cys
MYH6	c.3978G>C	p.Lys1326Asn
MYH6	c.3979-9_3979-8delCC	---
MYH6	c.90C>T	p.Pro30Pro
MYH7	c.2419C>G	p.Arg807Gly
MYH7	c.5150A>T	p.His1717Leu
MYH7	c.571G>A	p.Val191Ile
MYH7	c.3246-3C>A	---
MYLK2	c.1658G>A	p.Arg553His
MYLK2	c.1754T>A	p.Ile585Asn
MYOM1	c.2795-26C>T	---
MYOM1	c.1615A>G	p.Ser539Gly
MYOM1	c.3283A>G	p.Ile1095Val
MYOM1	c.2274G>A	p.Ser758Ser
MYPN	c.2246G>A	p.Ser749Asn
MYPN	c.1899C>T	p.Asn633Asn
NEBL	c.2518+20C>A	---
NEBL	c.886A>G	p.Ser296Gly
PDLIM3	c.500C>T	p.Ala167Val
PDLIM3	c.742C>T	p.Arg248Cys
PKP2	c.634C>T	p.Arg212Cys
PKP2	c.2019C>T	p.Gly673Gly
PKP2	c.918C>T	p.Pro306Pro
PRDM16	c.3109+38_3109+41dupACAC	---
PRDM16	c.677-42G>A	---
PRDM16	c.885-14C>T	---
PRDM16	c.3091G>A	p.Glu1031Lys
PRDM16	c.561G>C	p.Gln187His
PRDM16	c.387+7G>A	---
PSEN1	c.549-15_549-14delGT	---
RAF1	c.771G>C	p.Ser257Ser
RYR2	c.11558-40T>C	---
RYR2	c.1292+39A>G	---
RYR2	c.1477-11delT	---
RYR2	c.2823-45C>T	---
RYR2	c.576+42A>C	---
RYR2	c.1454G>A	p.Arg485Gln
RYR2	c.13936G>C	p.Asp4646His
RYR2	c.14731C>A	p.Gln4911Lys
RYR2	c.4010A>G	p.Tyr1337Cys
RYR2	c.14808+7A>G	---
RYR2	c.11922G>A	p.Leu3974Leu
RYR2	c.12027C>T	p.Asn4009Asn
RYR2	c.3321G>A	p.Thr1107Thr
SCN2B	c.30T>C	p.Pro10Pro
SCN5A	c.1881G>A	p.Pro627Pro
SCN5A	c.4527C>T	p.Pro1509Pro
SDHA	c.-15G>A	---
SDHA	c.1894G>T	p.Val632Phe
SDHA	c.825C>T	p.Asp275Asp
SGCD	c.91C>T	p.Arg31Trp
SNTA1	c.160G>C	p.Gly54Arg
SOS1	c.3392-44T>C	---
SYNPO2	c.1070-13_1070-12dupTT	---
SYNPO2	c.1805C>T	p.Pro602Leu
SYNPO2	c.2818T>C	p.Ser940Pro
TAZ	c.331C>T	p.His111Tyr
TAZ	c.584-7delT	---
TGFB3	c.353-34C>G	---
TGFB3	c.517-47delG	---
TGFB3	c.755-28A>T	---
TMPO	c.280-20T>C	---
TMPO	c.1165G>A	p.Gly389Arg
TMPO	c.1235T>C	p.Ile412Thr
TMPO	c.1405C>G	p.Leu469Val
TMPO	c.1750A>G	p.Thr584Ala
TMPO	c.330A>G	p.Leu110Leu
TNNI3	c.116C>T	p.Ser39Phe
TNNT2	c.382-50G>A	---
TPM1	c.808A>G	p.Ile270Val
TTN	c.614_619delAGACAA	p.Lys205_Thr206del
TTN	c.10045A>G	p.Thr3349Ala
TTN	c.102116T>C	p.Phe34039Ser
TTN	c.107687C>T	p.Pro35896Leu
TTN	c.16985G>A	p.Gly5662Asp
TTN	c.18655G>A	p.Glu6219Lys
TTN	c.3295G>A	p.Val1099Met
TTN	c.343G>A	p.Val115Met

TTN	c.39289C>G	p.Pro13097Ala
TTN	c.42524T>G	p.Phe14175Cys
TTN	c.49664C>G	p.Pro16555Arg
TTN	c.52373T>C	p.Val17458Ala
TTN	c.55000T>C	p.Cys18334Arg
TTN	c.60607C>T	p.Pro20203Ser
TTN	c.60934G>A	p.Glu20312Lys
TTN	c.64165C>A	p.Pro21389Thr
TTN	c.84203G>C	p.Ser28068Thr
TTN	c.86887T>C	p.Trp28963Arg
TTN	c.90594T>A	p.His30198Gln
TTN	c.95410T>C	p.Ser31804Pro
TTN	c.98021G>A	p.Arg32674His
TTN	c.27608A>G	p.Glu9203Gly
TTN	c.106531+6T>C	---
TTN	c.104913C>T	p.Ala34971Ala
TTN	c.12780G>T	p.Ala4260Ala
TTN	c.52878C>T	p.Val17626Val
TTN	c.71340C>T	p.Thr23780Thr
TTN	c.98061T>C	p.Ala32687Ala
VCL	c.239+23T>C	---
VCL	c.1223T>C	p.Ile408Thr
VCL	c.2655C>T	p.Phe885Phe
VCL	c.2760C>T	p.Ala920Ala
VCL	c.804A>G	p.Arg268Arg

NB: For recessive gene variants were retain only if the patient is carrier of another variant in the same gene.