Defining the cardiac alpha-tropomyosin: Genotype-phenotype correlations on inherited heart diseases.

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Dedication page

This work is dedicated to all those who contributed to the scientific development, especially my colleagues who wore the colors of Galicia and Health in Code.

To the patients and collaborating physicians who made their, respectively, pain and sweat an opportunity for learning and building knowledge.

To my family, who always supported me in my most distant goals with great care and wisdom.

To Existence, gift of life and evolution.

Thesis acknowledgment

A doctoral thesis is always the end of a stage. The end of this stage with specific acknowledgments would certainly be something unfair, as we always forget some names on this journey, but everyone who somehow supported my work and our research group will be eternally in our hearts. To those who have participated with a good feeling and honesty, we desire the most sincere wishes for success and happiness in their personal and professional projects. Thank you very much for every word, action and intention.

However, it is possible to be more particular, after all these great moments that we have lived in La Coruña. Starting with my work team in Health in Code SL, thank you very much for sharing knowledge, coffees and conversations. You know that was sincere, and those were memorable years for all those who shared sunny or cold days at El Fortín.

We also want to say thanks to all the medical colleagues who made this thesis possible. With your collaboration in several stages, sending clinical information, and providing discussions that were fundamental for the presentation of this study. To those whom I met in Congresses, work trips, whom I had the opportunity to witness working in the clinic and as scientists, thank you. It would be impossible not to mention the patients and their families, thank you so much for believing in the health services. I hope that this thesis translates into improving

health care for you, your descendants and also for other families with similar phenotypes.

I want to say thank you to Karla, who is a unique, lovely and always very friendly person. We are not together, but your support will be here in the memories of each page of this work.

Many thanks to the Universidade da Coruña and the people from Galicia. For allowing me to know a unique, fair, beautiful and so determined culture. These characteristics make this ancient place such a well-kept cradle that will echo for thousands of years to come in the hearts of many. Thanks to my kitesurfing mates for making me a Galician sailor from the rías baixas to the northern seas. Ultreya!

We have passed through this journey with health, work, victories and defeats. Hard times are now learning, and the moments of joy, good memories.

Abstract

Background: Cardiac alpha-tropomyosin (encoded by *TPM1* gene) is one priority sarcomere gene; nonetheless, most of the carriers are restricted to a single family or few index cases by each variant. *TPM1* has been associated with the development of different forms of inherited cardiomyopathy, and congenital heart defects.

Objective: To analyze the genotype-phenotype correlation in carriers of *TPM1* variants.

Methods: Genotype-phenotype correlation analysis was performed based on medical records from several European centers. Pathogenicity and molecular characteristics on each variant, clinical features, pedigrees, age of diagnosis, and prognosis were analyzed.

Results: Data on 257 *TPM1* variants was systematized, allowing changes in the pathogenicity of 87 variants, and the identification of functional domains overrepresented by phenotype. A *novel* cohort with 380 unpublished *TPM1* patients, including the founder variant p.Arg21Leu shows in general a favorable prognosis, and late-onset manifestations. An earlier age of diagnosis is observed in families with dilated/left ventricle noncompaction cardiomyopathy.

Conclusions: Genetic results from families with inherited heart diseases associated with *TPM1* variants can be useful for clinical decision making process and genetic counseling.

Resumen

Antecedentes: La alfa-tropomiosina cardíaca (codificada por el gen *TPM1*) es un gen sarcómero prioritario; sin embargo, la mayoría de los portadores están limitados a una sola familia o pocos casos índice por cada variante. *TPM1* se ha asociado con el desarrollo de diferentes formas de miocardiopatía hereditaria y cardiopatías congénitas.

Objetivo: Analizar la correlación genotipo-fenotipo en portadores de variantes en *TPM1*.

Métodos: Se ha realizado un análisis de correlación genotipo-fenotipo basado en registros médicos de varios centros europeos. Se analizaron la patogenicidad y las características moleculares de cada variante, las características clínicas, la edad de diagnóstico y el pronóstico en familias portadoras.

Resultados: Se sistematizaron datos de 257 variantes en *TPM1*, lo que permitió cambios en la patogenicidad de 87 variantes y la identificación de regiones sobrerrepresentadas por fenotipo. Una nueva cohorte con 380 pacientes no publicados en *TPM1*, incluyendo la variante fundadora p.Arg21Leu, muestra en general un pronóstico favorable y manifestación tardía. Se observa una edad más temprana de diagnóstico en familias con miocardiopatía dilatada/no compactada del ventrículo izquierdo.

Conclusiones: Los resultados genéticos de familias con cardiopatías hereditarias asociadas *TPM1* pueden colaborar para la toma de decisiones clínicas y el consejo genético.

Resumo

Antecedentes: A alfa-tropomiosina cardíaca é codificada polo xen *TPM1* que é sarcomérico prioritario; non obstante a maioría dos portadores están limitados a unha soa familia ou hai poucos casos índice por cada variante. A *TPM1* asociouse co desenrolo de distintas formas de miocardiopatías hereditarias e cardiopatías conxénitas.

Obxectivo: Análise da correlación xenotipo-fenotipo dos portadores de variantes en *TPM1*.

Métodos: Realizouse unha análise de correlación xenotipo-fenotipo baseado nos rexistros médicos de varios centros europeos. Analizouse a patoxenicidade e as características clínicas, idade de diagnóstico e o prognóstico nas familias portadoras.

Resultados: Sistematizáronse datos de 257 variantes en *TPM1*, o que permitiu o cámbeo na patoxenicidade de 87 variantes e a identificación de rexións sobrerepresentadas por fenotipo. Unha nova cohorte con 380 pacientes non publicados en *TPM1*, incluíndo a variante fundadora p.Arg21Leu, amosa en xeral un prognóstico favorable e unha manifestación tardía. Asemade obsérvase unha idade mais temperá de diagnóstico en familias con miocardiopatía dilatada/non compactada do ventrículo esquerdo.

Conclusións: Os resultados xenéticos de familias con cardiopatías hereditarias asociadas a *TPM1* poden coadxuvar cara a toma de decisións clínicas e no consello xenético.

Prelude

When I started my career in medical genetics in 2011, I could not have imagined writing this doctoral thesis about inherited heart diseases and cardiovascular genetics a decade later. Medical genetics is a broad field with a wide variety of rare and challenging diseases. In the first years, naturally I became interested in non-syndromic and syndromic congenital heart diseases affecting children, and later I did contact with “autosomal dominant arrhythmias” that could cause sudden cardiac death through generations in different families.

This interest also impulsed me to realize a medical residency in cardiology with the aim of a better understanding on the diagnosis, management and treatment of these hereditary heart conditions. When I was almost finalizing my residency at the end of 2015, I went to the Brazilian Congress of Cardiac Arrhythmias in São Paulo, and for the first time I attended a lecture on cardiovascular genetics as I would really like to work; pedigrees, genetic sequencing and risk stratification. The speaker was Dr Lorenzo Monserrat, director of this thesis. After his presentation we talked in person, he showed me the work of Health in Code and invited me to be part of his team in Galicia from 2016. That was a special day.

The first contacts with a new culture and languages were not easy, but it was fascinating to participate in a reference center for cardiovascular genetic sequencing in Europe. The company, born as a spin-off of the University of La Coruña, was growing fast, with a new generation sequencing analysis system and database that

allowed intense genotype-phenotype correlation investigations. Our work took place in an intense scientific environment with a generation of numerous hypotheses and possibilities for many scientific publications. The reporting model developed by the company for genetic results with clinical information is certainly a model of precision medicine that must be followed because it generates science, and at the same time allows a more consistent clinical decision making process.

In the middle of the daily work, we identified two elderly patients from a Portuguese hospital with the *TPM1* p.Arg21Leu variant in a homozygous state. That atypical result made us review all the families previously identified with this variant in our center, and the need to describe the associated phenotype. The hypothesis was raised that it would be a variant of favorable prognosis, and possibly a founder effect in Galicia. The occurrence of this variant in Portugal, and also an isolated report of this variant from Brazil in the ClinVar database made the object of this study part of my own life, as a Brazilian researcher, bringing soul to the academic research that is many times so formal. In this way, in my second year living in Spain, I started my PhD project with the aim of establishing the phenotype and prognosis associated with variants in the *TPM1* gene. Would the prognosis associated with the *TPM1* p.Arg21Leu variant be the same associated with the other variants identified in this gene? Genotype-phenotype correlations analysis on *TPM1* carriers could be useful for genetic interpretation and clinical management of these patients?

This doctoral thesis is therefore the result of all the scientific and technological development carried out at Health in Code over the years. The work of several people, even before my arrival, was fundamental for the first hypotheses

and data analysis. The preliminary results of this investigation were presented at the Iberian Meeting of Inherited Cardiomyopathies in Óbidos, Portugal. Together with Portuguese and Spanish colleagues, we added new patients carrying the p.Arg21Leu variant, while at the same time motivating us to expand the study to all variants in the same gene.

Although it is a priority gene for the development of hypertrophic cardiomyopathy, the scientific articles until then in the literature were almost always restricted to a single family, which did not allow an overview from a large cohort of patients with variants in cardiac tropomyosin. Other forms of cardiomyopathy were also analyzed in this process. We believe that the main value of this thesis is to gather available information about this gene, analyze the clinical data, adding the genotype and medical information of more than 300 patients unpublished in the literature with genetic variants in this gene.

Clinical data previously published in the literature were reviewed and are described in this thesis. Pathogenic variants in the *TPM1* gene have been associated with the development of different forms of cardiomyopathy and congenital structural heart diseases. Although this research is not a laboratory bench study, the analysis of functional studies already published on tropomyosin allowed us to better characterize the variants and phenotypes observed. Variants in tropomyosin are mainly functionally characterized with distinct sensitivities to calcium levels, and their respective effect on myocardial contraction.

Detailed characterization of pedigrees carrying the p.Arg21Leu variant was a task that allowed a deep genotype-phenotype correlation, providing diverse insights into the interpretation of genetic results in risk stratification of patients with hypertrophic cardiomyopathy. This work also evidenced the p.Arg21Leu variant as a Luso-Galician mutation due to historical components that founded this population and territories.

The different genetic variants in the *TPM1* gene were collected in our center (with the collaboration of several hospitals) and in different public databases. The systematization of these data allowed us to recategorize the pathogenicity's classification of several of these variants, adding to them power of clinical decision and benefits to patients. It was observed that the genetic variants are predominantly of the missense type, and it was not possible to categorize the radical variants. The results suggest that tropomyosin truncating-type variants are possibly not the cause of a pathology. We also have observed that the different functional domains of the protein are associated with the overrepresentation of variants associated with distinct phenotypes, such as hypertrophic cardiomyopathy vs dilated cardiomyopathy/non-compacted left ventricle.

Finally, we analyzed our cohort of patients with cardiomyopathies carrying genetic variants in this gene, which allowed analysis on the penetrance of these variants and associated prognosis. Alongside our founder variant and patients who also carry the p.Asp175Asn variant, a founder effect in Finland, we observed another highly prevalent variant in this gene, p.Met281Leu, which will also require a founder effect study in Spain. Other variants seem to be associated with dilated or

left ventricle non-compaction cardiomyopathy. Heart defects were observed with different frequencies in association with these cardiomyopathies.

The analysis of genotype-phenotype correlations with affected families with variants in the *TPM1* gene will allow a more assertive interpretation of genetic results, and risk stratification within the scope of cardiology and medical genetics. Genetic counseling and the clinical management of these patients will become more precise with the results of our work and will allow the construction of health policies for these patients. Throughout this doctoral thesis, we have participated in other relevant publications on cardiovascular genetics, allowing that our analysis and discussions were performed with the existing learning in our research group at INIBIC and the collaboration of several European centers. This thesis can be considered a relevant step, but it will not be the last description about this gene, as it is essential to advance in the number of genotyped patients, since many variants still lack a greater number of carriers. A research becomes relevant as it is able to raise new study questions from its results. Nevertheless, the way to future answers is probably the method presented here.

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1.1: Tropomyosin: molecular aspects and function

1.1.1: Alpha-tropomyosin protein structure

Cardiac alpha-tropomyosin, encoded by the *TPM1* gene, is a 284-amino acid residue protein homodimer, a 33-kDa α-helical coiled-coil protein, that associates with actin filaments to constitute the thin sarcomeric filaments (Gupte et al, 2015). The tropomyosin protein sequence has characteristic heptad repeats (*abcdefg*) stabilized by hydrophobic core residues at the *a* and *d* positions, which interact to mediate dimerization (Perry SV et al, 2001). The *b, c, e, f* and *g* heptad positions are most occupied by ionic or polar residues; the coiled coil can be stabilized by salt bridges between *e* and *g* positions, and *b, c* and *f* residues may interact with actin or troponin (Redwood and Robinson, 2013).

Several authors have proposed different functional domains in the literature. The most considered structural description for the tropomyosin protein sequence can be divided in seven periods (or functional domains) with approximately 40 amino acids each. Even though each period has similar length, they have specific functions and are not quasi equivalents (Hitchcock-DeGregori SE et al, 2001; Hitchcock-DeGregori SE et al, 2002). For example, these authors have demonstrated that the deletion of period 5 (residues 166-207), and especially deletion or replacement of residues 166-188, a constitutively expressed region encoded by exon 5, had more severe consequences on actin affinity and cooperative myosin

S1-induced binding to actin than other periods. Period 6, residues 208-242, part of the troponin binding site, is required for full inhibition of the actomyosin ATPase in the absence of calcium. They concluded that the effect of each period can depend on its context, suggesting that the sequence alone is not the only factor important for function.

The head-to-tail overlapping region between two subsequent tropomyosin molecules (Figure 1.1) forms a continuous super-helical strand that matches the contours of F-actin, and imparts cooperativity to regulatory interactions (Muramaki et al, 2008; Matyushenko et al, 2019). This region is composed by N-terminal (amino acids 1-25) and C-terminal regions (amino acids 253-284), generating an overlapping region that also interacts with troponin T to control the state of the regulated thin filament. Several *TPM1* variants also have been described in this region between two subsequents tropomyosin molecules (Hershberger et al, 2010; Otsuka et al, 2015).

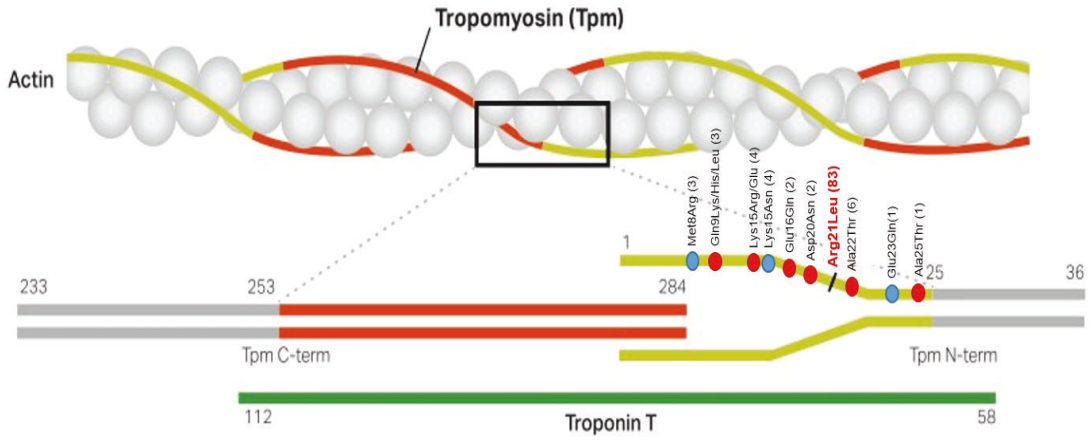


Figure. 1.1: Head-to-tail overlapping region between two subsequent tropomyosin molecules. **At the top:** αTpm.1 molecules across the actin's groove (one tropomyosin is represented in yellow, and the subsequent one in orange), with the N-terminal:C-terminal junction highlighted with a black rectangle. **Below:** The junction has been also reported as a tail-head overlapping region; 1-25 amino acids in the N-terminal region. Hypertrophic cardiomyopathy-related variants are pointed out in red circles, and dilated/left ventricle non compaction cardiomyopathy-variants in blue circles (*n* = number of carriers). The junction is one of the two αTpm.1's binding-sites with troponin T (in green). Adapted from Murakami et al, 2008).

Two alpha-tropomyosin regions are also described as troponin T binding sites, amino acids 175-190 and amino acids 258-284. The first one corresponds partially to the fifth period, which is considered a relevant functional domain, and the second one is interacting with the head-to-tail overlapping regions where also several cardiomyopathies variants have been reported.

Nevzorov and Levitsky (2011) reviewed the tropomyosin structure, focussing on the flexible central domain (amino acids 89-208). In general, the tropomyosin protein seems to be composed of two rather stable domains (C-terminal and N-terminal) joined by a linker region – the flexible central part, exhibiting the lowest stability. Based on the tropomyosin's regulatory mechanism in the sarcomere, they suggest that this molecule is a rod with high conformational mobility revealed in appearance of bends in some regions in response to external factors and different amino acids clusters. Further, there is no unambiguous answer to explain the complexity of tropomyosin, taking into account the effect of each amino acid, variant's effect and domain functionality.

Another central region has been described as middle-region or mid-region in the literature. It is composed by the intermediary actin-binding periods, approximately the third and fourth periods (amino acids 102-165) (Brown JH et al, 2005).

Other tropomyosin binding-sites have been described in association with other sarcomere proteins. Leiomodin (encoded by *LMOD2* gene) and tropomodulin (*TMOD1* gene) are two similar actin-capsular proteins located at the pointed end of

the thin filaments. Leiomodin-3 variants have been associated with lethal nemaline myopathy in humans, and leiomodin-2–knockout mice demonstrated dilated cardiomyopathy phenotype (Ly T, 2016). Disruptions in tropomodulin result in improper thin filament lengths, and its overexpression in mice leads to dilated cardiomyopathy. Further, absence of tropomodulin in differentiating embryonic stem cells leads to delayed myofibril assembly, and in mice, it leads to prominent heart defects, including aborted development of the myocardium as the primary defect (Tsukada T et al, 2010). The N-terminal tropomyosin-binding site in leiomodin is highly homologous to the first tropomyosin-binding site in tropomodulin. Leiomodin interacts with only one tropomyosin molecule, while tropomodulin interacts with both tropomyosin molecules (considering that tropomyosin protein is a homodimer). Leiomodin and tropomodulin proteins interact with tropomyosin at amino acids 1-21, and amino acids 1-14 respectively (Colpan et al, 2017).

To date only missense variants in *TPM1* have been consistently considered disease causing, and there is no genotype-phenotype correlation for the cardiomyopathy specific form to affect residues within a particular tropomyosin domain or a specific residue in the heptad repeat (Redwood C, Robinson P., 2013; Gupte TM et al, 2015). Several patients with hypertrophic cardiomyopaty were identified carrying *TPM1* variants in the troponin T-binding regions (175–190 and 258–284) (Jagatheesan et al. 2004), and two alter residues (Asp58 and Glu62) described as having direct interaction with actin protein (Li et al. 2012). *TPM1* p.Met8Arg and p.Lys15Asn, as well p.Met281Thr and p.Ile284Ala variants affect residues at the extreme N and C-terminus, respectively, that are involved in

head-to-tail interactions of neighboring tropomyosins (Murakami et al. 2008); additionally, these last variants are adjacent to the Ser283 phosphorylation site, and these mutations may affect Ser283 modification (Nixon BR et al, 2013).

1.1.2: Alpha-tropomyosin function

At the sarcomeric level, contractility is brought about by Ca^{2+} regulation of the cross-bridge cycle. Cross-bridges establish between myosin heads from thick filaments and the regulated thin filament, constituted by F-actin, tropomyosin, and the troponin complex. It responds to Ca^{2+} presence with a series of conformational changes. The three-state model is widely accepted like the scheme linking Ca^{2+} and muscle contraction like. This physiological process was explained by Gupte et al, 2015. In the absence of Ca^{2+}, tropomyosin protein physically closes the myosin-binding sites on actin superficie to produce the blocked state. Muscle contraction is triggered by the influx of Ca^{2+} into the sarcoplasm, where this ion binds to troponin C and initiates a sequence of conformational changes through troponin I and troponin T. The conformational change of the troponin T and its interaction with tropomyosin results in azimuthal switch of tropomyosin and uncovering of the myosin-binding sites of actin, which is called the closed state. Myosin binding to F-actin stimulates the contractile force in association with phosphate release, causing the movement of thin filaments by myosin heads, and so sarcomere contraction. The binding of myosin displaces tropomyosin, producing the open state. Sarcomere relaxation is produced by the dissociation of Ca^{2+} from troponin C and the ATP-induced dissociation of myosin from actin. Thus, muscle

contraction is brought about by coordination between Ca^{2+}-induced changes in the thin filament and the ATPase cycle of myosin.

Nonetheless, the three-state model could not explain so clearly the regulatory mechanisms on cardiac contractile activity. Borovikov et al (2011) provided a strong evidence suggesting that the regulation of actin-myosin interaction by tropomyosin is realized not only via a simple movement of the tropomyosin strands from a block to open position, but also via an allosteric mechanism. They consider possible that during the ATPase cycle the myosin-dependent shift of the native or wild type strands from the periphery to the center on the thin filament increases the interplay between actin and myosin. This group have suggested that *TPM1* p.Asp175Asn and p.Glu180Gly hypertrophic cardiomyopathy-variants increase this effect by shifting the tropomyosin strands further to the center, and increasing the binding of actin to tropomyosin stronger.

1.1.3: *TPM1* functional studies

Several functional studies involving *TPM1* variants have been described in the literature (see Table 1.1). In fact, the number of publications with functional studies on *TPM1* variants is greater than the clinical descriptions of carriers in this gene. Some functional investigations were carried out to complement the clinical descriptions of some variants, in order to produce evidence supporting their pathogenic role. Other publications approaching one or several *TPM1* variants were dedicated to demonstrate how each variant could impact on cardiac physiology and disease development. Most of these investigations are *in vitro* assays; however,

animal models and, more recently, approaches through human-induced pluripotent stem cells (hiPSC) were also performed.

The most frequent functional studies evaluating the tropomyosin regulatory process have carried out assays measuring the Ca^{2+}-sensitivity changes. *In vitro* tropomyosin mutants have been reported in different Ca^{2+} concentrations regarding their motility, flexibility, stability, binding-affinity and actomyosin ATPase levels (force generation). *TPM1* variants previously described in hypertrophic cardiomyopathy patients, such as p.Ala63Val, p.Lys70Thr, p.Val95Ala, p.Asp175Asn and p.Glu180Gly, have been shown to increase thin filament Ca^{2+}-sensitivity with elevated filament sliding or ATPase activity at intermediate activating concentrations of Ca^{2+} (Bing et al. 1997; Lakdawala et al. 2010; Heller et al. 2003; Karibe et al. 2001). The degree of change in Ca^{2+}-sensitivity in the *in vitro* motility assay varied among the different *TPM1* variants, according to these same authors.

On the other hand, functional studies on *TPM1* variants identified in dilated cardiomyopathy patients suggested that these variants had the opposite effect of thin filament regulatory mechanism, decreasing Ca^{2+} sensitivity. Reduced Ca^{2+} sensitivity could be a consistent trend for all dilated cardiomyopathy variants in thin filament proteins (Gupte et al, 2015); nevertheless, some groups have suggested that although reduced Ca^{2+} sensitivity is true of the majority of dilated cardiomyopathy *TPM1* variants, there are significant exceptions (Memo et al. 2013; Marston 2011). Further, an apparently common functional impact generated by all dilated cardiomyopathy-associated tropomyosin variants is the inability to decrease thin filament Ca^{2+} sensitivity in response to phosphorylation of troponin I.

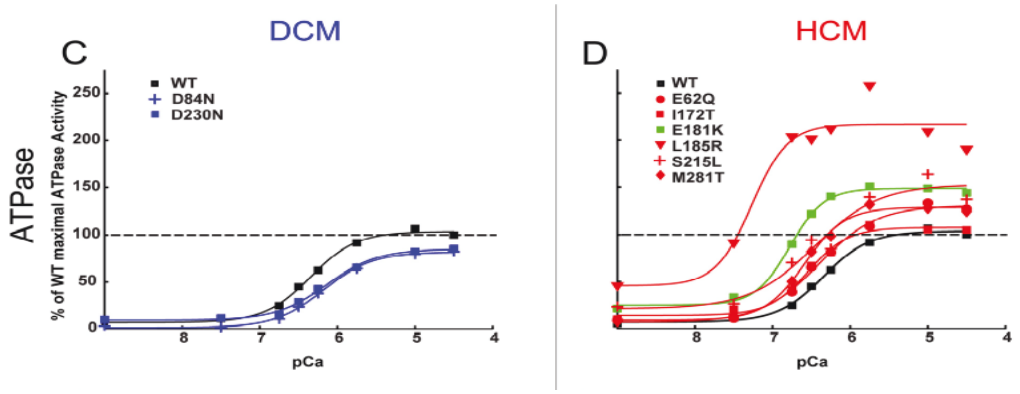


Figure. 1.2: Several functional studies have evaluated the TPM1 variant's impact based on Ca2+-sensitivity hypothesis. This image from Gupte et al (2015) could summarize the results observed in these *in vitro* assays: hypertrophic cardiomyopathy and DCM-associated variants have shown opposite effects ATPase activity across different calcium concentrations (pCa).

We should highlight that almost all *TPM1* variants have been described as autosomal dominant disease, with affected carriers with one normal and one mutated allele resulting in a likely mixture of wild type and mutant protein present in the thin filaments. Redwood and Robinson (2013) have cited several publications which have reconstituted thin filament either with a wild type/mutant mixture or defined as heterodimers. These studies described frequently the expected intermediate molecular phenotype although in some cases the heterodimer effects were similar to either wild type or mutant alone, suggesting that modeling the mutation effects simply using mutant protein alone is not a completely reliable guide.

TPM1 variants' impact on the contractility of intact sarcomeres has been investigated using cardiomyocytes transfected with adenoviral vectors and using demembranated myofibre or myocyte preparations, either reconstituted *in situ* or from patient samples and transgenic animal models (adult rats, mouse, and bovines). *TPM1* variants associated with hypertrophic cardiomyopathy (p.Ala63Val, p.Lys70Thr, p.Val95Ala and p.Glu180Gly) demonstrated a higher Ca^{2+} sensitivity and

force generation in comparison with the wild-type. However, some exceptions have been reported, such as p.Asp175Asn was found to behave as wild type. They speculated that this variant behavior could explain why in p.Asp175Asn families frequently had mild disease and those with other *TPM1* variants several cases of sudden cardiac death (Michele et al, 1999; Bai et al 2011) Here, we would like to open a parenthesis because it correspond to a old hypothesis, probably discarded by other recent publications.

Measurement of the kinetics of cardiomyocyte contractility upon field stimulation revealed that the p.Ala63Val variant caused significant slowing of relaxation (Michele et al, 2002). By contrast, *TPM1* variants identified in dilated cardiomyopathy patients have shown opposite effects, including lower Ca^{2+} sensitivity and force generation, as well as faster relaxation than hypertrophic cardiomyopathy variants.

Table 1.1: Main TPM1 variants and functional studies published to date (NP_001018005.1).

Protein name	c.DNA name	Heptad position	Phenotype	Reference
p.Met8Arg	c.23T>G	*a*	DCM	Michele et al (2002) Greenfield et al (2002) Matyushenko et al (2019)
p.Lys15Asn	c.45G>C	*a*	DCM	Colpan et al (2017) Matyushenko et al (2018)
p.Arg21Gly	c.61C>G	*g*	HCM	Ly et al (2017)
p.Ala22Thr	c.64G>A	*a*	HCM	Muramaki et al (2008)
p.Glu40Lys	c.118G>A	*e*	DCM	Chang et al (2005) Mirza et al (2005) Mirza et al (2007) Robinson et al (2007) Ochala et al (2008) Borovikov et al (2009) Borovikov et al (2011) Bai et al (2012) Memo et al (2013) Kopylova et al (2016)
p.Glu54Lys	c.160G>A	*e*	DCM	Mirza et al (2005) Chang et al (2005) Mirza et al (2007)

				Robinson et al (2007) Warren et al (2008) Borovikov et al (2009) Borovikov et al (2009) Bai et al (2012) Memo et al (2013) Kopylova et al (2016)
p.Glu62Gln	c.184G>C	*f*	HCM	Gupte et al (2015) Dorsch et al (2021)
p.Ala63Val	c.188C>T	*g*	DCM	Michelle et al (1999) Michelle et al (2002) Heller et al (2003) Hilario et al (2004)
p.Lys70Thr	c.209A>C	*g*	HCM	Michele et al (1999) Heller et al (2003) Zheng et al (2016)
p.Asp84Asn	c.250G>A	*g*	DCM	Bai et al (2013) Gupte et al (2015)
p.Ile92Thr	c.275T>C	*a*	DCM	Sliwinska et al (2018) Kuruba B et al (2021)
p.Val95Ala	c.284T>C	*d*	HCM	Karibe et al (2001) Tobacman et al (1999) Bai et al (2011) Sliwinska et al (2018) Zheng et al (2016) Kuruba B et al (2021)
p.Ile172Thr	c.515T>C	*d*	HCM	Gupte et al (2015) Matyushenko et al (2017)
p.Asp175Asn	c.523G>A	*g*	HCM	Bing et al (1997) Golitsina et al (1997) Bottinelli et al (1998) Michele et al (1999) Spindler et al (1999) Muthuchamy et al (1999) Wernicke et al (1999) Bing et al (2000) Kremneva et al (2004) Lakdawala et al (2010) Borovikov et al (2011) Li et al (2012) Ly et al (2012) Janco et al (2013) Schulz et al (2013) Spindler (2013) Kopylova et al (2019)
p.Glu180Gly	c.539A>G	*e*	HCM	Bing et al (1997) Golitsina et al (1997) Michele et al (1999) Spindler et al (1999) Muthuchamy et al (1998) Wernicke et al (1999) Bing et al (2000) Prabhakar et al (2001) Burkart et al (2003) Kremeneva et al (2004) Chang et al (2005) Pena JR (2006) Boussouf et al (2007) Peña et al (2010) Gaffin et al (2011) Bai et al (2011) Li et al (2012) Ly & Lehrer (2012) Janco et al (2012) Loong et al (2012) Lehrer et al (2014) Prasad et al (2014) Zheng et al (2016) Sewanan et al (2016)
p.Ala183Val	c.548C>T	*a*	HCM	Hitchcock-DeGregori and Singh (2006) Hitchcock-DeGregori et al. (2002)

p.Leu185Arg	c.554T>G	*c*	HCM	Chang et al (2005) Gupte et al (2015) Zheng et al (2016)
p.Glu192Lys	c.574G>A	*c*	LVNC/HCM	Zheng et al (2016) Sewanan et al (2021) Cha YJ et al (2022)
p.Thr201Met	c.602C>T	*e*	DCM	Dorsch et al (2021)
p.Ser215Leu	c.644C>T	*e*	HCM	Gupte et al (2015)
p.Asp219Val	c.656A>T	*b*	HCM	Tsaturyan et al (2022)
p.Asn230Asp	c.688G>A	*f*	DCM	Lakdawala et al (2010) Bai et al (2013) Gupte et al (2015) Lynn et al (2017)
p.Lys248Glu	c.742A>G	*c*	LVNC/DCM	Bai et al (2013)
p.Met281Thr	c.842T>C	*a*	HCM	Dorsch et al (2021)

DCM: Dilated cardiomyopathy, HCM: Hypertrophic cardiomyopathy, LVNC: Left ventricle non-compaction.

1.2: *TPM1* clinical information

1.2.1: Sarcomere proteins: where is alpha-tropomyosin?

Several sarcomeric proteins have been described in association with the development of inherited heart diseases. Cardiac alpha-tropomyosin is considered one of the main sarcomere proteins regarding the available information on its pathogenic role; however its participation in the distinct forms of cardiomyopathies is variable. In hypertrophic cardiomyopathy it is a relatively rare etiology, accounting for 1-5% of the cases with this phenotype (Elliott PM et al 2014). Some of the hypertrophic cardiomyopathy pedigrees described in the literature have cited patients with restrictive cardiomyopathy; nonetheless, these reports are very sporadic and could be considered as part of the hypertrophic cardiomyopathy clinical spectrum.

On the other hand, other publications in the literature have associated alpha-tropomyosin variants as the cause of dilated cardiomyopathy. Its contribution for this phenotype seems to be lower than in hypertrophic cardiomyopathy, and this association could be restricted to only a few variants. Lakdawala et al (2012) have suggested that these variants represent 1-2% of the dilated cardiomyopathy cases. Most of the cases have been reported in pediatric populations (Peña-Peña ML et al, 2020). Several works have demonstrated that alpha-tropomyosin pathogenic variants are also associated with the development of left ventricle non-compaction cardiomyopathy and congenital heart defects. Kayvanpour E et al (2019) have reported a prevalence of 2% of TPM1 variants among pediatric patients. These three phenotypes have been described affecting different individuals from the same pedigree, which could represent another clinical spectrum phenotype associated with these variants.

No alpha-tropomyosin variant has been associated with arrhythmogenic cardiomyopathy to date, including left ventricular-dominant arrhythmogenic cardiomyopathy.

1.2.2: Hypertrophic cardiomyopathy

First publication in the literature showing *TPM1* kindreds was reported by Thierfelder et al (1993). They reported the identification of a chromosomal 15q2 locus in two kindreds cosegregating with hypertrophic cardiomyopathy. *TPM1* gene had still not been mapped in this locus at that time. First family had 19 carriers across three generations affected by hypertrophic cardiomyopathy. Among them,

four young females (ages between 25-36 year-old) were considered unaffected carriers, and five individuals aged between 18 and 43 years were also considered to be affected based only in electrocardiogram minor changes suggesting hypertrophic cardiomyopathy. Most of the affected individuals had ages higher than 35 years old, with older individuals being more symptomatic. One patient evolved with left ventricle dilatation at the fifth decade of life. Two affected carriers manifested severe phenotypes at pediatric ages: both suffered a sudden cardiac death at six months and 8 year-old, respectively. This last individual had at this early age, 23 mm of left ventricle hypertrophy. The mean maximal left ventricle wall thickness for affected adults in this family was only 12.5 ± 4.7 mm.

The second kindred reported by Thierfelder et al (1993) had eight individuals with clear electrocardiogram and echocardiogram hypertrophic cardiomyopathy phenotype. Cardiac hypertrophy in affected members of this family was significantly greater (mean maximal left ventricle wall thickness = 20.3 ± 3.1 mm) than in those of the first family reported by them ($P < 0.0004$). A sudden cardiac death was registered in a 18 year-old male individual, and a myectomy in a 12 year-old female individual. Linkage analysis in these two pedigrees confirmed familial cosegregation of the disease with the 15q2 locus, taking into consideration a reduced penetrance (50%) for LOD score calculation. This publication from 1993 could be considered a relevant initial mark in genetics not only for *TPM1* gene, but also for hypertrophic cardiomyopathy genetics.

In the next year, the same group (Thierfelder et al, 1994) identified the two missense mutations (Asp175Asn; Glu180Gly) in the *TPM1* gene causing hypertrophic cardiomyopathy in these pedigrees linked to chromosome 15q2 (Figure 1.3).

Two years later, Nakajima-Taniguchi et al (1995) identified two *TPM1* missense variants in two unrelated probands among 50 Japanese patients with hypertrophic cardiomyopathy by PCR analysis, p.Ala63Val e p.Asp175Asn. The authors did not carry out any familial cosegregation study. First proband was diagnosed with hypertrophic cardiomyopathy at the age of 32, his electrocardiogram had ST segment depression and T wave inversion, and echocardiogram revealing asymmetrical left ventricular hypertrophy. This patient gradually progressed to left ventricle dysfunction and dilatation. He had an apparent family history of hypertrophic cardiomyopathy. His mother had died of heart failure resulting from hypertrophic cardiomyopathy, his grandmother of unspecified cardiac disease and his older brother suddenly in childhood. The other proband carrying the p.Asp175Asn variant was presenting heart failure symptoms, and had episodes of paroxysmal atrial fibrillation. He also had a positive family history of hypertrophic cardiomyopathy: his mother had died of hypertrophic cardiomyopathy, his sister and elder daughter were both diagnosed as hypertrophic cardiomyopathy, and his second daughter died suddenly at the age of 20 years. The authors have suggested that p.Asp175Asn would be a variant with a fatal prognosis.

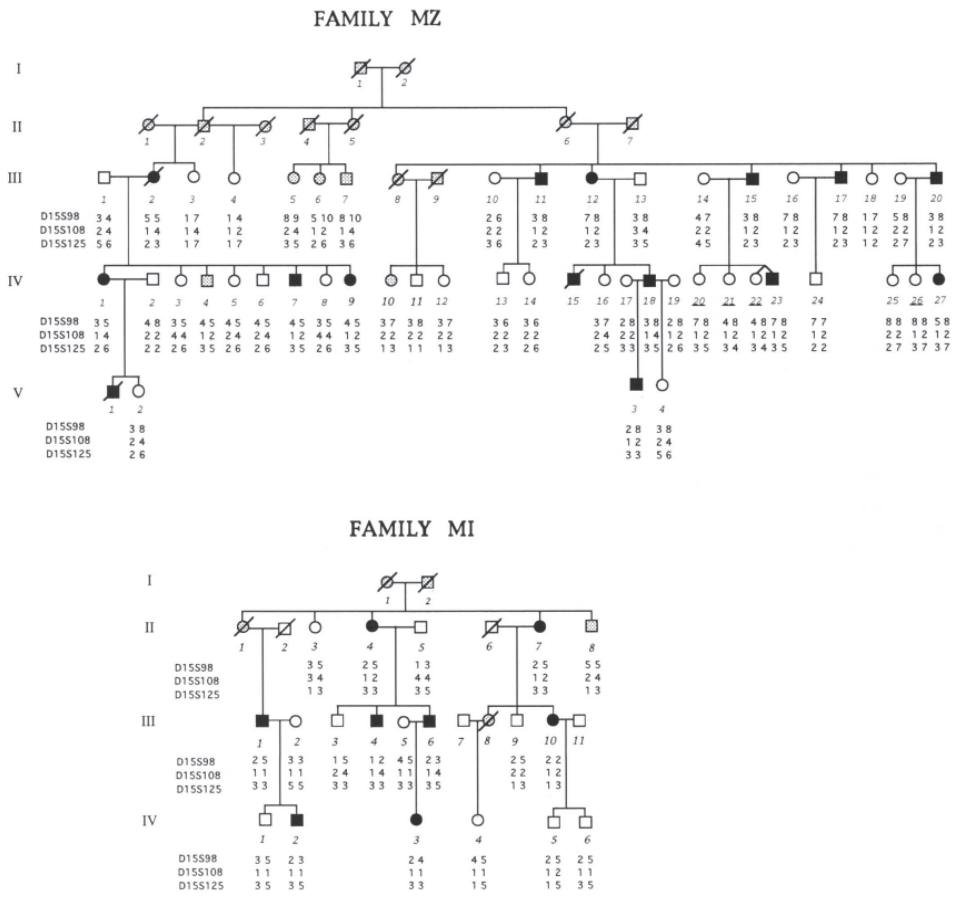


Figure 1.3: Two hypertrophic cardiomyopathy-pedigrees published by Thierfelder et al (1993), showing linkage analysis which identified 15q2 chromosomal locus where TPM1 gene was mapped later.

Based on this same Japanese hypertrophic cardiomyopathy-cohort, in the next year the same group (Yamauchi-Takihar et al 1996) reported the familial study of both above mentioned probands (p.Ala63Val e p.Asp175Asn), and an additional pedigree carrying the variant p.Lys70Thr. All pedigrees were small families with two or three carriers, including index cases. Sudden cardiac deaths cases in pediatric and young adult family members were reported in the three pedigrees; however, without genetic testing. The authors concluded that *TPM1* variants are not as rare as reported, accounting for about 5% of all cases in this small Japanese index cases cohort. These mutations are characterized by hypertrophy of the left ventricle which

then progresses to dilatation and a high incidence of sudden or disease-related death.

Watkins et al (1995) also reported the p.Asp175Asn variant in a 53 year old male proband with hypertrophic cardiomyopathy. He had syncopes, a ventricular tachycardia episode documented, and 18 mm of left ventricular hypertrophy. His 21 years old son, and his 12 years old daughter were diagnosed on screening as variant carriers with 14 and 12 mm of left ventricle hypertrophy, respectively. His genitors were deceased by non-cardiac causes, and their genetic status is unknown; however, family history did not suggest a familial hypertrophic cardiomyopathy, and the proband's variant was considered with *de novo* presentation.

Covielo DA et al (1997) described the clinical features of 21 p.Asp175Asn carriers (3 pedigrees) with hypertrophic cardiomyopathy. Phenotype expression exhibited by all affected individuals from these three families included a wide spectrum of clinical severity, but general clinical stability. Eighteen (85%) of the 21 affected individuals had mild or absent symptoms. Electrocardiograms frequently showed deep inferolateral Q waves, anterolateral T wave inversions and left ventricular hypertrophy, while echocardiograms pointed out to a non-obstructive hypertrophic cardiomyopathy profile in most individuals; a rest outflow tract obstruction was present in only 15% of individuals. No evolution to A subgroup of these carriers had longitudinal follow-up. They compared *TPM1* p.Asp175Asn with other sarcomere variants, and this variant showed a better survival curve with mortality rate low than 10% in advanced ages.

Karibe A et al (2001) analyzed a large *TPM1* four generations-pedigree with the new p.Val95Ala: family members carriers were examined. Hypertrophic cardiomyopathy diagnosis was defined as an echocardiographic finding of >13 mm of left ventricle hypertrophy. The individuals included all 14 members positive for the mutation, 11 additional members who died of the disease, and an affected family member for whom no DNA was available. Maximum left ventricle wall thickness was 16 mm (range 8 to 27 mm). No left ventricle outflow obstruction was documented. The electrocardiogram was abnormal in 11 (79%) of 14 affected family members, but only 6 (43%) showed left ventricle hypertrophy by voltage criteria. Eight (57%) of the 14 affected members showed ST-T–wave abnormalities. Disease penetrance and survival prognosis was evaluated using the graphs that we can observe in the figures 1.2 and 1.3 (see next page), respectively.

Jongbloed RJ et al (2003) reported the sixth *TPM1* variant in the literature. p.Glu62Gln variant was identified in a large hypertrophic cardiomyopathy-pedigree affecting five generations. Nine affected carriers were identified, and other nine sudden cardiac deaths were reported (four in teenagers presumed carriers, and five older obligated carriers). Carriers were described individually (only two had severe hypertrophy), and deceased carriers with available data from autopsy showed septal hypertrophy.

Based in a family where an 8 years old female suffered a sudden cardiac death, Van Driest SL et al (2002) described a small kindred with two cases of sudden cardiac death in young individuals. Autopsy revealed hypertrophy in the index case. Familial screening identified three affected carriers of the variant *TPM1*

p.Leu185Arg: the 38 years old father was asymptomatic, and had 19 mm of left ventricle hypertrophy. The second child was the 11 years old brother who had 9 mm left ventricular maximal wall thickness (upper limits of normal for age), and the youngest brother was diagnosed with hypertrophic cardiomyopathy at the age of 2 years and 6 months. Five years of follow-up of this family showed an arrhythmogenic profile in the youngest brother: he evolved with ventricular fibrillation episodes documented by cardiac defibrillator. At the age of 6 years old he had 15 mm and suffered a sudden cardiac death after several ventricular fibrillation episodes despite his cardiac device, and medical therapy with amiodarone and atenolol. Father and oldest brother also received an implantable cardioverter defibrillator and they were asymptomatic at the end of follow-up. Earing MG et al (2003) documented in this family the diastolic ventricular dysfunction as the only echocardiographic finding able to identify positive carriers among the family members.

TPM1 p.Asp175Asn variant profile was revisited in the literature by Jääskeläinen P et al (2004). In this paper, they suggested that the *TPM1* variant and other truncating *MYBPC3* variant could be founder variants in Finland. So the authors compared both variants: *TPM1* variant presented a higher penetrance (91%) than the *MYBPC3* variant (67%; p value <0,05), and almost all affected carriers in both groups are symptomatic. Pathological Q waves were more common in the *TPM1* carriers (50%) than *MYBPC3* carriers (10%; p < 0,001); however, short bursts of ventricular tachycardia were more common in the *MYBPC3* group than *TPM1* carriers (20% versus 0%, respectively, p < 0,001). Mean maximal left ventricle wall

thickness was intermediary in both groups (14-25mm), and finally no left ventricle outflow tract obstruction was observed. They discussed that, although some reports in the literature had indicated that *TPM1* p.Asp175Asn as a variant having a benign prognosis, some sudden cardiac deaths were reported in this Finland cohort which could represent an intermediary prognosis profile.

Posteriorly, the same group recharacterized these both founder variants in *TPM1* and *MYBPC3* (Jääskeläinen P et al, 2013). Comparing the groups of index patients (*TPM1, MYBPC3*, no mutation), p.Asp175Asn carriers had the highest rate (75%) of family history of hypertrophic cardiomyopathy (15 of 20 versus 12 of 35 versus 56 of 251, p = 0.001). Myectomy was also more common in patients with p.Asp175Asn than in those carrying the truncating *MYBPC3* variant (p = 0.048). No significant differences were observed in left ventricle hypertrophy among the three groups. Only one (1/20) *TPM1* had heart failure signs, and 10% presented non-sustained ventricular tachycardia; half of them had positive family history of sudden cardiac death.

García-Castro et al (2009) also reported the *TPM1* p.Asp175Asn variant in a Spanish hypertrophic cardiomyopathy-cohort with 120 index cases. She was a 41 years old carrier with 32 mm of left ventricle hypertrophy and positive family history for hypertrophic cardiomyopathy.

Other Finland researchers also described inducibility of life-threatening ventricular arrhythmias, and cardiac resonance imaging findings in *TPM1* p.Asp175Asn. Ventricular arrhythmias were induced in one third of the patients.

Inducibility was associated with the maximum left ventricular thickness and the number of markers of sudden cardiac death, suggesting that in hypertrophic cardiomyopathy the susceptibility to ventricular arrhythmias is related to the cardiomyopathy phenotype (Hedman A et al, 2004). Further, these patients were also evaluated on myocardial contractile impairment. Hypokinetic segments in hypertrophic cardiomyopathy was strongly and independently related to left ventricle mass and maximal wall thickness in patients with hypertrophic cardiomyopathy attributable to the p.Asp175Asn mutation in *TPM1* (Sipola P, 2005).

Otsuka et al (2015) identified six carriers in four pedigrees carrying respectively the *TPM1* variants, p.Met281Thr and p.Asp175Asn, and two novel variants p.Ala22Thr and p.Ala107Thr. In these last two families, cases of sudden cardiac death were reported at the ages 9, 30 and 45 years. No additional clinical data on each carrier was available. Mean age of diagnosis was 46±19 years, older than the other sarcomere genes in the study and similar with the patients in which no genetic variant was identified. Left ventricle wall thickness was 16.3±4.9mm in *TPM1* carriers.

Vieria E et al (2016) did a communication to report the *TPM1* p.Ile130Le variant in a small family. There were three affected family members and an unaffected relative carrying the variant. There also was a case of sudden cardiac death at the age of 12 years. No detail about clinical expression of the variants in each carrier was provided.

Selvi Rani D et al (2015) reported two pedigrees with hypertrophic cardiomyopathy carrying the variant p.Ser215Leu. It's an interessant paper because they reported a digenic etiology in ventricular hypertrophy likely associated with a more severe phenotype. In the first pedigree, an additional likely pathogenic *MYH7* mutation was also identified in all affected individuals. They had prominent left ventricular hypertrophy, and other two cases of sudden cardiac death at early ages were reported. In the second pedigree, all the carriers had only the *TPM1* variant and no cardiovascular event was documented.

Tran Vu MT et al (2019) identified 9 index cases carrying *TPM1* variants in a hypertrophic cardiomyopathy cohort from Vietnam, corresponding to 20.5% of the patients. One patient had the variant, p.Met141Lys, and eight patients had the p.Met281Thr variant. Positive family history of hypertrophic cardiomyopathy and sudden cardiac death was reported in 55% and 33% of the *TPM1* carriers, respectively. No patient had syncope or apical aneurysm in this group. Non-sustained ventricular tachycardia was reported in 33% of the *TPM1* carriers, and extreme left ventricle hypertrophy (>30mm) in a single carrier (11%).

Carlus SJ et al (2020) reported a new *TPM1* variant, p.Gly3Arg, in three homozygous carriers. Two of them had congenital hypertrophic cardiomyopathy, and the third had patent ductus arteriosus, without left ventricle hypertrophy. Each genitor (a non-consanguineous Saudi couple) was an unaffected simple heterozygous carrier of this variant. Family history showed sudden cardiac deaths in two mother's cousins, at 20 and 27 years-old, respectively, siblings of a same consanguineous couple; two cousins died aged 20 and 27 years. The pedigree

suggested hypertrophic cardiomyopathy with a recessive autosomal inherited pattern.

Finsterer J et al (2021) described a female carrier of the variant *TPM1* c.425A>T underwent successful cardio-pulmonary resuscitation because of ventricular fibrillation at age 17 years. His echocardiogram at ages 24 years and 26 years revealed hypertrabeculation of the left ventricular apex, apical-lateral and apical-septal regions, with a two-layered myocardium of the inferolateral region of the left ventricle. Work-up revealed hypertrophic cardiomyopathy and a cardioverter defibrillator was implanted. Family history was positive for sudden cardiac death at age 30 years in her father, implantation of an cardiac defibrillator in one sister of the father because of ventricular arrhythmias, and implantation of an cardiac defibrillator in the son of the father's sister also after successful cardio-pulmonary resuscitation from ventricular fibrillation. No skeletal myopathy was detected. The proband's father was a carrier of the *TPM1* variant too.

Trachoo et al (2022) published the findings of a Thai cohort with dilated and hypertrophic cardiomyopathy patients. Among the carriers, they reported a 25 years-old male patient with obstructive hypertrophic cardiomyopathy carrying the *TPM1* p.Glu115Lys. Unspecified cardiac arrhythmias were reported in this patient, without syncopes or ICD implantation. He had a negative family history of cardiomyopathy. Finally, Tsaturyan et al (2022) reported a case of a 11 years-old girl that suffered a sudden cardiac death without any specific trigger factor during her usual daily activities. Post mortem evaluation identified an asymmetric nonobstructive hypertrophic cardiomyopathy. She was previously completely

asymptomatic, and her family history was unremarkable. Familial study showed a *de novo* presentation of the *TPM1* p.Asp219Val variant.

Until this point, fifteen *TPM1* variants were clinically reported in association with hypertrophic cardiomyopathy (p.Gly3Arg, p.Ala22Thr, p.Glu62Gln, p.Ala63Val, p.Lys70Thr, p.Val95Ala, p.Ala107Thr, p.Glu115Lys, p.Ile130Leu, p.Asp175Asn, p.Glu180Gly, p.Leu185Arg, p.Ser215Leu, p.Asp219Val, p.Met281Thr) in the above mentioned publications. At least, 23 pedigrees were reported carrying these variants; nonetheless, it is difficult to establish the assertive number of *TPM1* index cases and pedigrees reported in these studies. Even though these articles have provided clinical information on carriers, some of the patients may be duplicated among these publications previously cited above, especially those reporting cohorts. Moreover, the same can be occurring with other studies where the same authors are participating as co-authors (Van Driest SL et al, 2002; Erdman et al, 2003; Andersen et al, 2009; Fokstuen et al, 2011; Núñez L et al, 2013; Wilson KD et al, 2015; Walsh R et al, 2017; Micheu MM et al, 2020), in which, other 34 *TPM1* missense-variants (p.Glu16Gln, p.Asp20Asn, p.Arg21His, p.Asp28Asn, p.Asp28His, p.Lys37Glu, p.Asp84Glu, p.Arg101Pro, p.Ala102Asp, p.Glu115Lys, p.His153Asp, p.Ile154Thr, p.Asp159Asn, p.Lys161Glu, p.Glu180Val, p.Ala183Val, p.Glu187Gln, p.Glu192Lys, p.Asp219Asn, p.Lys226Gln, p.Lys226Arg, p.Lys233Asn, p.Ala239Thr, p.Leu249Trp, p.Asp254Gly, p.Asp254Glu, p.Asp258Glu, p.Lys264Glu, p.Lys266Arg, p.Ala277Thr, p.Asn279His, p.Met281Val, p.Thr282Ser, p.Ile284Val) were reported, totalyzing 49 *TPM1* variants identified in patients with hypertrophic cardiomyopathy phenotype in the literature to date.

1.2.3: Dilated Cardiomyopathy and left ventricular non-compaction

Olson et al (2001) have sequenced 350 unrelated patients with sporadic and familial dilated cardiomyopathy, and they have identified two missense-type variants in *TPM1*. First proband was clinically evaluated at the age of 17 years because his father and paternal uncle had died from heart failure at the age of 27 and 49 year old, respectively. At his first evaluation he was asymptomatic, but he evolved with shortness of breath, edema and non-sustained ventricular tachycardia at the age of 26. No sign suggesting hypertrophic cardiomyopathy or ischemic cardiomyopathy was detected, and myocardial biopsy was compatible with idiopathic dilated cardiomyopathy. Despite the medical therapy and an implantable cardiodesfibrillator he died a year later while waiting for cardiac transplant. He was a carrier of the variant *TPM1* p.Glu54Lys. Second family carrying the variant *TPM1* p.Glu40Lys was diagnosed starting with a symptomatic female proband at the age of 3 months which was transplanted with 10 years old. His mother was also a carrier and was diagnosed with dilated cardiomyopathy at the age of 33 years old, and she remains asymptomatic at the age of 40 years old. His cardiac evaluation did not reveal any other cause of dilated cardiomyopathy (coronarography and myocardial biopsy were normal). Family history showed maternal grandfather, father and siblings with unspecified heart disease in their 50's. The authors have discussed that until this time, only four *TPM1* variants had been reported in the literature, always in hypertrophic cardiomyopathy-pedigrees, although some carriers had developed end-stage hypertrophic cardiomyopathy. These were the first *TPM1* pedigrees

presenting dilated cardiomyopathy at the moment of the diagnosis. Both variants had the same amino acid change (Glu >> Lys) at the same *TPM1* heptad position *e*.

Lakdawala et al (2010) performed genetic testing and clinical evaluation in 264 patients with dilated cardiomyopathy, and described the *TPM1* p.Asp230Asn variant carriers in detail. Familial cosegregation was documented in two large Caucasian families, and *in vitro* studies demonstrated major inhibitory effects on sarcomere function with reduced Ca^{2+}- sensitivity, maximum activation, and Ca^{2+} affinity compared to wildtype *TPM1*. Early presentation (from infancy to adolescence), was not uncommon in both families, and it was associated with severe, sometimes lethal outcomes, including sudden cardiac death and refractory heart failure leading to death or transplantation. In contrast, a mild course was seen in relatives diagnosed in adult ages. In the family one, there were three individuals that suddenly died at ages 3 years, 13 months, and 5 months respectively, without prior evidence of heart disease. Other two family members presented with advanced heart failure at 5 months of age, and were given the presumptive diagnosis of myocarditis. In one of them, adenovirus DNA was detected by polymerase chain reaction on endomyocardial biopsy. Both had substantial recovery of left ventricle systolic function and resolution of symptoms after receiving medical therapy at the time of presentation. Other two carriers developed refractory end-stage heart failure in adolescence. Another relative was asymptomatic when diagnosed with dilated cardiomyopathy at 7 year old during family screening, but developed severe left ventricular systolic dysfunction at age 17 and he underwent transplant at 18 years of age. On the other hand, four *TPM1* p.Asp230Asn carriers

who presented in adulthood had mild clinical courses. Clinical studies revealed mild to moderate left ventricular systolic dysfunction and enlargement and symptoms resolved with medical management. Four variant carriers (at the ages 54, 16, 22, and 8 years, respectively), had asymptomatic systolic dysfunction and/or enlargement. Three adults were unaffected carriers.

In the second pedigree, young members of this family also presented with severe systolic dysfunction. Proband developed failure to thrive at 10 weeks of age and echocardiography showed severe ventricular dilatation and dysfunction (ejection fraction 15%). Cardiac transplantation was considered, but she had substantial recovery with medical therapy. By age 8 years, she was asymptomatic with only mild left ventricular systolic dysfunction. At age 20, another family member underwent cardiac transplantation shortly after presenting with refractory symptoms and severe left ventricular systolic dysfunction. A heart failure death was reported in a 12 years old individual; however, he had congenital cataracts and skeletal muscle weakness of undefined etiology. Post mortem examination showed cardiomegaly with marked endocardial and interstitial fibrosis. Skeletal muscle analyses showed chronic myopathic changes but normal dystrophin staining, and no inflammation, evidence of storage disease, tissue-specific atrophy, nor fiber type grouping. Similar to the first family, the clinical course of adult carriers was far less severe. Three adult relatives were asymptomatic (ages 38, 36 and 33 years, respectively), and another adult had unexplained syncope at age 56 years. Cardiac evaluation revealed ventricular tachycardia and dilated cardiomyopathy which improved with medical management in this last patient. After a pregnancy, a carrier

had worsening systolic dysfunction, improving to a normal range with the institution of an ACE inhibitor.

Later, the same group (Lakdawala et al, 2012) reported that in a dilated cardiomyopathy cohort, they identified three families with *TPM1* variants; p.Met8Arg (a single carrier - family study was not performed), p.Asp230Asn (the two families above mentioned totalizing 15 carriers), and p.Ala211Gly (one carrier). *TPM1* variants were considered responsible for 1.7% of the disease causing variants in this cohort.

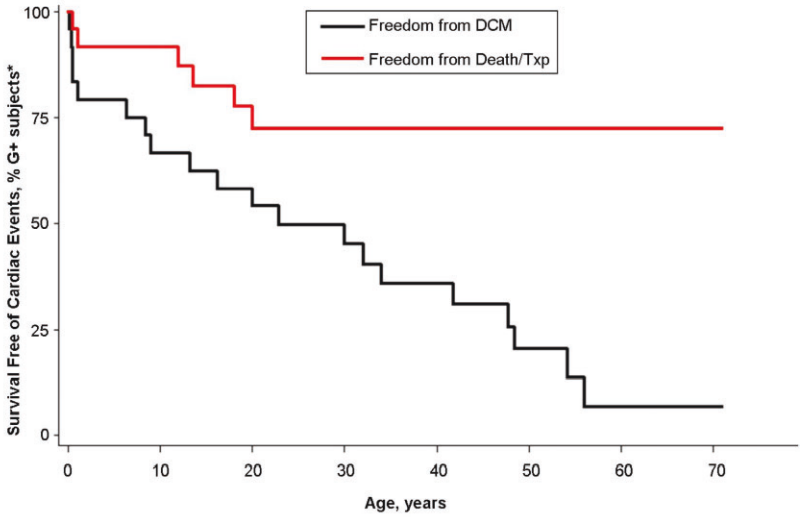


Figure 1.4: Survival (red) and diagnosis (black) curves from the classic study of Lakdawala et al (2013) with p.Asp230Asn carriers describing the differences in TPM1 dilated cardiomyopathy phenotype by age.

Hershberger RE et al (2010) identified five *TPM1* variants in a cohort with 312 patients (181 with familial and 131 with idiopathic dilated cardiomyopathy). *TPM1* p.Ser16Ile, p.Ala239Thr and p.Ala277Val variants were identified in sporadic cases of dilated cardiomyopathy, while this last variant was identified in a severely

affected heart failure patient requiring transplant at age 13. This proband was also a carrier of the p.Glu244Asp in troponin T (*TNNT2* gene). Other two variants, p.Lys15Asn and p.Ile92Thr were reported cosegregating with the disease, respectively in three and two family members. No specific phenotype features on carriers were described.

Rampersaud et al (2011) described four pediatric dilated cardiomyopathy index cases carrying *TPM1*. First case was a female proband healthy until 2 months when she presented with progressive difficulty breathing. Cardiac evaluation revealed dilated cardiomyopathy, and she underwent heart transplant at 18 months. She was a carrier of the variant *TPM1* p.Lys15Asn. Cardiac evaluation was also carried out in her paternal half brothers, ages at 8 and 10 years. They were asymptomatic carriers of the variant, and had dilated cardiomyopathy - the older half brother presented improvement in his echocardiographic findings with angiotensin converting enzyme inhibitors A. The father was also a carrier and had an electrocardiogram at age 39, revealing a left anterior hemiblock and slow R wave progression. He underwent an echocardiogram at age 46 which revealed left ventricular hypertrophy with systolic function at the lower limits of normal. Second case is a female identical twin, born at 34 weeks gestation. She presented at 3 months of age with cardiorespiratory shock. Her identical twin subsequently underwent cardiac screening and was diagnosed with the same phenotype. Both twins underwent heart transplants, at 5 months and 8 months, respectively. They were carriers of the *TPM1* p.Ile92Thr variant. The variant was identified in their mother and maternal uncle, both had adult-onset dilated cardiomyopathy at ages

43 and 34, respectively. Their maternal grandmother, who also carried this variant, developed dilated cardiomyopathy at age 59 post doxorubicin chemotherapy for lymphoma. Third family was a male carrier of two variants; *TNNT2* p.Glu244Asp and *TPM1* p.Ala277Va. He was diagnosed at age 13 years with severe dilated cardiomyopathy after a four day history of respiratory distress, low energy and exercise intolerance. At this same age he required a left ventricular assist device and subsequently received a heart transplant. Cosegregation analysis of the variant in the family was not performed, but his mother underwent echocardiographic screening at age 39 that was normal; she had nonspecific ST-T wave changes on electrocardiogram. Last case was another female proband diagnosed with dilated cardiomyopathy at age 39 soon after her daughter's diagnosis of dilated cardiomyopathy at age 13 years, after an episode of syncope. She had a heart transplant at age 14. The mother had a son who did asymptomatic cardiac screening at age of 2 years, and subsequently was diagnosed with asymptomatic dilated cardiomyopathy on echocardiogram at 7 years old. Genetic information showed the proband carrying the variant *TPM1* p.Ala239Thr; however, genotyping from additional relatives was not available.

Chang B et al (2011) identified a novel *TPM1* variant, p.Lys37Glu, in a family with left ventricular noncompaction and sudden cardiac death. Pedigree analysis showed three generations affected with left ventricle non-compaction cardiomyopathy. The 8 years old female proband was evaluated after her older brother died suddenly at 9 years of age during physical activity. Although she was asymptomatic, her electrocardiogram showed ST-T abnormalities and

echocardiography revealed numerous trabeculations in the apex consistent with a diagnosis of isolated non-compaction cardiomyopathy. Left ventricle systolic function was normal. Her mother also had ST-T changes on electrocardiogram, and her echocardiogram revealed numerous trabeculations in the apex and lateral and posterior walls of the left ventricle consistent with the same phenotype. Nonetheless, left ventricle systolic function was depressed. The maternal aunt had an episode of atrial flutter at 33 years old, and his echocardiogram revealed numerous trabeculations in the apex also diagnosing non-compaction cardiomyopathy. His systolic function was also depressed. The maternal grandmother of the proband died suddenly of unknown cause, at 45 years of age, as had her two sisters.

Another description with a variant causing the same amino acid change (Asp >> Asn) and similar clinical profile that *TPM1* p.Asp230Asn was reported in four-generation family with dilated/non-compaction cardiomyopathy (van de Meerakker JB et al, 2013). *TPM1* p.Asp84Asn was identified in eight carriers with ages from five months to 52 years old. The proband started with heart failure symptoms at the age of 5 months. Echocardiogram demonstrated an dilated left ventricle with severely reduced function. No abnormalities in heart catheterization and muscle biopsy was found, and the girl was diagnosed as having an idiopathic dilated cardiomyopathy. Later, echocardiographic imaging also revealed non-compaction cardiomyopathy. She died at home during her sleep at the age of 5 years and 7 months. The proband's father was at the time of the diagnosis of his daughter of 29 years old and asymptomatic, and remains stable over the past

decade. Her paternal aunt and paternal grandmother were carriers of the *TPM1* variant and had dilated cardiomyopathy. The proband's great-grandmother had possibly heart failure at the age of 75 years old. She was an obligated carrier, because there was another relationship in which her children were also affected carriers. One family member died suddenly at the age of 41, post mortem pathological examination revealed cardiac hypertrophy and dilatation. Further, two female relatives have also been diagnosed with dilated cardiomyopathy, where both received an implantable defibrillator at the age of 51 and 52 years old, respectively. In subsequent years she had one appropriate cardioverter defibrillator discharge and progressive heart failure developed. She died at the age of 68 years waiting for a heart transplantation. Another relative was reported having an unspecified cardiac death at the age of 6 years.

Nijak et al (2018) described a family with left ventricular non-compaction with Ebstein anomaly attributed to a *TPM1* variant, p.Leu113Val. Three patients had left ventricular non-compaction diagnosis and two patients had the overlapping of this diagnosis with Ebstein anomaly. Index case was diagnosed with non-compaction and mitral valve insufficiency as a neonate. Delivery was induced at the 30th week of pregnancy, because of premature closure of the ductus arteriosus and hydrops fetalis. Subsequently, he was also diagnosed with non-compaction cardiomyopathy, progressive mitral valve insufficiency and pulmonary hypertension. He underwent a mitral valve replacement at age 3.5 years. She was born at 39 weeks and neonatal echocardiography revealed also the presence of non-compaction. At 2 years of age, echocardiography demonstrated an abnormal left ventricle with pronounced apical

and posterior-lateral trabeculation as well as reduced contractile function, dilatation of the left atrium, venae pulmonales and progressive pulmonary hypertension. The tricuspid valve demonstrated an abnormal septal leaflet with grade 2/4 insufficiency. Owing to progressive heart failure, she was placed on a mechanical biventricular assist. Awaiting her heart transplant she died at age 3.5 years, due to acute respiratory distress and right ventricular failure. Their father had left ventricular non-compaction, and at the age 33 his cardiac imaging showed also a mildly dilated left atrium. His left ventricular function was normal. All family members were carriers of the *TPM1* variant.

Eighty two Japanese patients with left ventricle non-compaction were investigated by Takasaki A et al (2018). Seven sarcomere genes were sequenced in this cohort. Twenty-seven patients were identified with causal variants, including a poor prognosis in carriers with *TPM1, ACTC1* and *TNNC1.* These authors performed a functional study of the *TPM1* variant, p.Arg178His. They showed in human-induced pluripotent stem cells (hiPSCs) that this variant could cause pathological changes, with mislocalization of tropomyosin 1, disruption of the sarcomere structure in cardiomyocytes, and impaired calcium handling. Microarray analysis indicated that the *TPM1* mutation resulted in the down-regulation of the expression of numerous genes involved in heart development, and positive regulation of cellular processes, especially the calcium signaling pathway.

Liu et al (2018) discussed the importance of preconception and prenatal genetic evaluation in heart transplant individuals and fetal and postnatal cardiac monitoring in their offspring. This discussion was based on a case report of a 24

years old female proband with idiopathic dilated cardiomyopathy which post heart transplant gave birth to a healthy term female infant. At 5 months of age, the infant was diagnosed with severe left ventricular dysfunction with an ejection fraction of 18% and moderate non-compaction of the left ventricle. She received a heart transplant at 7 months of age. Genetic testing revealed a likely pathogenic variant in the *TPM1* p.Arg160His.

Sousa A et al (2019) reported a 40 years old female proband with dilated cardiomyopathy diagnosed at the age of 17 years. Her electrocardiogram was unremarkable, and an echocardiogram showed left ventricular end-diastolic diameter of 60 mm with left ventricular ejection fraction of 38%. She was carrier of the variant *TPM1* p.Ile253Thr. No familial data was provided.

Yao Q et al (2019) investigated the correlation between the 3' untranslated region (3'UTR) single nucleotide polymorphisms (SNPs) of the *TPM1* gene and dilated cardiomyopathy. In a case-control study with 245 dilated cardiomyopathy patients and 245 healthy controls, they analyzed four *TPM1* SNPs: rs12148828, rs11558748, rs707602, rs6738, rs7178040, and the plasma miR-21 level. The risk of dilated cardiomyopathy development in the rs6738 locus G allele carriers was 1.69 times more than A allele carriers (95% CI: 1.22-2.33, p=0.001). Age and gender had no effect on the association of *TPM1* gene SNPs with dilated cardiomyopathy risk ($p > 0.05$), suggesting the relevance of this genotype. The plasma miR-21 level of *TPM1* gene rs6738 locus in homozygous carriers was significantly higher than that of the simple heterozygous and wild-type genotypes ($p < 0.001$). The *TPM1* rs6738 is associated with dilated cardiomyopathy, which may be related to the abnormal

increase of miR-21 level in patients with this phenotype; nonetheless, further investigations could be needed to prove the causal relationship between miR-21 level and the development of dilated cardiomyopathy phenotype.

Kayvanpour E et al (2019) carried out a meta-analysis study and systematic review of the literature in left ventricle non-compaction, totalizing 7598 patients. *TPM1* variants were identified in 2% of the patients (417 individuals). They did not report any details about each carrier.

Hirono K et al (2020) studied 53 pediatric probands (age range 0 - 14 years; median: 0.3 months) with left ventricle non-compaction and congenital heart disease. Twenty-eight probands had a pathogenic genetic variant, eight in *MYH7* and two with the same *TPM1* variant (p.Arg238Gln). Other genes were mutated in only a single proband. They described clinical features and prognosis of these patients collectively, and only the prognosis was detailed by each carrier. No cardiovascular event was described in *TPM1* carriers. Posteriorly, the same group (Hirono K et al, 2021) also published these two carriers in another paper about left ventricle non-compaction with fetal-onset, suggesting a neonatal diagnosis of carriers with *TPM1* mutations. Over again, they did not specify details about clinical features of these newborns.

Franaszczyk M et al (2020) discussing clinical relevance and *de novo* presentation of sarcomere variants, reported a dilated/non-compaction cardiomyopathy proband carrying the variant *TPM1* p.Lys205Arg, in addition to *MYH7* p.Ile201Thr The family study revealed that the *MYH7* variant was *de novo*,

while the *TPM1* variant was inherited from the asymptomatic proband's father. His left ventricle ejection fraction was 26%, and the ratio of non-compaction to compacted myocardial layer was 3.2:1. After standard heart failure medical therapy, he had partial recovery of the left ventricle function (ejection fraction 37%).

Sun et al (2021) evaluated a large newborn population with a total of 37 fetal patients with left ventricle non-compaction (incidence 0.07%). Biventricular non-compaction cardiomyopathy was identified in 43% of the fetuses, and 38% only observed in the left ventricle. 51% had additional heart defects. 47% of the cases had positive genetic testing, with non-sarcomere gene variants corresponding for the vast majority. In contrast, only one sarcomere gene variant was observed in *TPM1* (p.Arg133Gln). The carrier had no cardiac malformations, and familial analysis revealed a *de novo* presentation.

Man Y et al (2022) described a 40 years old male Chinese index case with dilated cardiomyopathy. His electrocardiogram showed ST-T wave changes and nodal tachycardia, and echocardiography revealed a left ventricular dilatation and an ejection fraction of 43.1%, and his mother had the same symptoms of chest tightness, dyspnoea, and abdominal distension and died of dilated cardiomyopathy at the age of 50 years. The *TPM1* p.Glu114Gln variant was identified in the proband.

Around 13 dilated or noncompaction cardiomyopathy pedigrees were described in the literature to date. Here, some index cases were reported with detailed clinical data, unlike hypertrophic cardiomyopathy where there was a predominance of pedigrees greater than the description of probands. Nineteen

TPM1 variants were identified among these patients (p.Met8Arg, p.Lys15Asn, p.Ser16Ile, p.Lys37Glu, p.Glu40Lys, p.Glu54Lys, p.Asp84Asn, p.Ile92Thr, p.Leu113Val, p.Glu114Gln, p.Arg133Gln, p.Arg160His, p.Arg178His, p.Lys205Arg, p.Ala211Gly, p.Arg238Gln, p.Ala239Thr, p.Ile253Thr, p.Ala277Val). Further, other isolated dilated cardiomyopathy index cases carrying *TPM1* variants have been reported in different cohorts; however, no specific clinical information on each carrier has been described (van Spaendonck-Zwarts et al, 2013; Pugh TJ et al, 2014; Wilson KD et al, 2015; Ji Y et al, 2015; Walsh R et al, 2017). These articles reported the following 15 variants: p.Ala31Thr, p.Glu33Lys, p.Asp58Asp, p.Asp55Asn, p.Glu114Gly, p.Ala120Val, p.Ser123Thr, p.Glu139Val, p.Met141Ile, p.Thr201Met, p.Leu232Arg, p.Ala242Val, p.Ser245Leu, p.Ala277Gly and p.Thr282Ser. In total, 34 *TPM1* variants were described in the literature in association with dilated/noncompaction cardiomyopathy.

1.2.4: Congenital heart defects

In addition to the articles already described with congenital heart defects in association with cardiomyopathy, mainly left ventricle non-compaction phenotype, some other reports have endorsed their occurrence in *TPM1* carriers. Teekakirikul P et al (2002) reported a large pedigree with five generations (11 individuals) affected with atrial septal defects. Eight individuals had large atrial septal defects, requiring surgical patch repair at birth. One of the carriers had also transient dilated cardiomyopathy that resolved with medical therapy. Two of the affected family members suffered sudden cardiac death in mid- to late adult life. The variant showed complete penetrance in every generation. The heterozygous 3-base

in-frame deletion variant in *TPM1* (c.13_15del; p.Lys5del) causing loss of a highly conserved N-terminal lysine was identified cosegregating with the disorder in the family.

England J et al (2017) studied a cohort with 380 patients with congenital heart defects, reporting four variants in the *TPM1* gene; an intronic variant (IVS1+2T>C), two missense-type variants (p.Ile130Val, and p.Ser229Phe) and a polyadenylation signal site variant GATAAA/AATAAA. They have studied a cohort with 380 patients with congenital heart defects. The carriers had respectively Tetralogy of Fallot, pulmonary atresia with a hypoplastic right ventricle, and the last two variants were identified in patients with atrial septal defect. *In vitro* and *in silico* assays showed that the variants affected the transcription causing embryonary damage. No familial cosegregation study was performed by this group.

2. Hypothesis and objectives

2.1: Hypothesis

There is a genotype-phenotype correlation between *TPM1* variants, and clinical data of these carriers which can be useful for the medical making-decision process.

2.2: Objectives

2.2.1: Main objective: To analyze the genotype-phenotype correlation in carriers of *TPM1* variants.

2.2.2: Secondary objectives:

A. To perform a detailed *TPM1* p.Arg21Leu variant genotype-phenotype correlation, including pedigrees, familial cosegregation data, specific clinical information of each carrier and geographical distribution.

B. To describe all genetic variants identified in the *TPM1* gene in our center and in the literature, according to their pathogenicity, phenotype of carriers, variant's characteristics, and functional domain.

C. To analyze the clinical phenotype in carriers of *TPM1* variants, including age of diagnosis, cardiovascular events, and prognosis.

3. Methods

This is a descriptive study with families carrying genetic variants in *TPM1* (NP_001018005.1 isoform). This study is in accordance with the principles of the Helsinki Declaration, and is part of the research line registered with the number 2012/139 in the Research Ethics Committee of Galicia, Spain.

From March 2008 to September 2021, the *TPM1* gene was sequenced in 21,671 consecutive index cases with different inherited heart diseases from different hospitals around the world referred to our center for molecular genetic diagnosis.

All exons and intronic boundary regions of 213 genes related to cardiomyopathies and sudden cardiac death were studied by Next Generation Sequencing*: AARS2, ABCC9, ACAD9, ACADM, ACADVL, ACTA1, ACTA2, ACTC1, ACTN2, ACVRL1, ADAMTSL4, AGK, AGL, AGPAT2, AKAP9, ALMS1, ANK2, ANK3, ANKRD1, APOA5, APOB, APOC3, ATPAF2, BAG3, BMPR1B, BMPR2, BRAF, BSCL2, CACNA1C, CACNA1D, CACNA2D1, CACNB2, CALM1, CALM2, CALR3, CAPN3, CASQ2, CAV1, CAV3, CAVIN4, CBL, CBS, CETP, COL1A1, COL1A2, COL3A1, COL5A1, COL5A2, COQ2, COX15, COX6B1, CRELD1, CRYAB, CSRP3, CTF1, CTNNA3, DES, DLD, DMD, DNAJC19, DOLK, DSC2, DSG2, DSP, DTNA, ELN, EMD, ENG, EYA4, FAH, FBN1, FBN2, FHL1, FHL2, FHOD3, FKRP, FKTN, FLNA, FLNC, FOXD4, GAA, GATA4, GATA6, GATAD1, GDF2, GFM1, GJA1, GJA5, GLA, GLB1, GNPTAB, GPD1L, GUSB, HCN4, HFE, HRAS, JAG1, JPH2, JUP, KCNA5, KCND3, KCNE1, KCNE2, KCNE3, KCNE5, KCNH2, KCNJ2, KCNJ5, KCNJ8, KCNK3, KCNQ1, KLF10, KRAS, LAMA2, LAMA4, LAMP2, LDB3, LDLR, LIAS, LMNA, LRP6,*

MAP2K1, MAP2K2, MIB1, MLYCD, MRPL3, MRPS22, MTO1, MYBPC3, MYH11, MYH6, MYH7, MYL2, MYL3, MYLK, MYLK2, MYOT, MYOZ2, MYPN, NEBL, NEXN, NKX2-5, NOTCH1, NOTCH3, NPPA, NRAS, OBSL1, PCSK9, PDHA1, PDLIM3, PHKA1, PITX2, PKP2, PLN, PLOD1, PMM2, PRDM16, PRKAG2, PRKG1, PSEN1, PSEN2, PTPN11, RAF1, RANGRF, RBM20, RYR2, SCN10A, SCN1B, SCN2B, SCN3B, SCN4B, SCN5A, SGCA, SGCB, SGCD, SHOC2, SKI, SLC22A5, SLC25A4, SLC2A10, SLMAP, SMAD1, SMAD3, SMAD4, SMAD9, SNTA1, SOS1, SPRED1, SURF1, TAZ, TBX1, TBX20, TBX5, TCAP, TGFB2, TGFB3, TGFBR1, TGFBR2, TMEM43, TMEM70, TMPO, TNNC1, TNNI3, TNNT2, TPM1, TRDN, TRIM63, TRPM4, TSFM, TTN, TTR, TXNRD2, and *VCL.*

The minimum read-depth obtained was >30 (average from 250x to 400x). DNA fragments that did not fulfil these criteria were sequenced by Sanger. A multidisciplinary team performed Bioinformatics analysis and clinical interpretation. The pathogenicity classification of the variants was in accordance with current recommendations (Richards S et al, 2015). Pathogenic/likely pathogenic genetic variants in any of the sequenced genes were described, as well as those of unknown clinical significance in the priority genes (see Table 3.1). Information regarding allelic frequency in the general population was considered based on Genome Aggregation Database (gnomAD) and TOPMed Program populations. Familial cascade genetic screening in relatives was performed using Sanger sequencing method. In addition, a call was made during the I Iberian Meeting of Cardiomyopathies (Óbidos, Portugal, March/2017) to identify especially *TPM1* p.Arg21Leu pedigrees from other hospital centers, since we have the hypothesis of that this variant is a founder effect in Portugal and Galicia, Spain.

Clinical data of index cases and family members were collected in a multivariable form. The diagnosis criteria and sudden cardiac death risk stratification model for hypertrophic and dilated cardiomyopathy, and left ventricle non-compaction followed the recommendations of the European Society of Cardiology (Elliott PM et al, 2014; Theresa A McDonagh TA et al, 2021; Nunez-Gil IJ and Feltes-Guzmán G, 2012). Sudden cardiac death risk score (ESC calculator) was obtained for carriers with available clinical data. Early onset was considered the diagnosis under the age of 35 years (Gray B et al, 2015). Other sudden cardiac death risk factors for hypertrophic cardiomyopathy described in the North American guidelines were also considered (Maron MS et al, 2019): left ventricular apical aneurysm, end-stage hypertrophic cardiomyopathy, and extensive late gadolinium enhancement (defined here as ≥3 affected cardiac segments) on MRI.

3.1: *TPM1* p.Arg21Leu variant

Especially for the *TPM1* p.Arg21Leu variant, we have carried out a more detailed analysis. We have designed the pedigrees evaluating *TPM1* p.Arg21Leu familial cosegregation. Two-point logarithm of the odds (LOD scores) was calculated in all families using the PARAMLINK package for R software. The model was set with $\theta = 0$, phenocopy rate = 0.005 and two different penetrance values: 0.80 and 0.95. An indeterminate status was assigned to family members that were reported only with a diagnosis suspicion, as well as to males younger than the age of 50 and females younger than age 55 who did not fulfil the clinical criteria for cardiomyopathy and could develop the disease afterwards. We use the model proposed by Nyholt DR (2000) to establish familial cosegregation.

We also analysed the factors related to penetrance and disease expression in hypetrophic cardiomyopathy and dilated cardiomyopathy: age, sex, genotype, clinical features and sudden cardiac death risk factors. We have questioned each *TPM1* p.Arg21Leu index case about the name of the province from where the family originated. Based on this information, we evaluated the number of families by province in Spain and Portugal, and the number of hypertrophic cardiomyopathy genetic studies requested by each province to estimate the relative frequency of the variant in different regions and the geographic distribution of the variant.

3.2: Survival and age of diagnosis analysis

The cumulative probability of occurrence of cardiovascular death (sudden cardiac death, appropriate defibrillator shock, heart failure death, stroke-related death and unspecified cardiac death) and heart transplant was estimated with the Kaplan-Meier method and factors were compared using the log-rank (Mantel-Cox) method. Survival was calculated from birth for all TPM1 carriers collectively, and separately for the following groups: *TPM1* p.Arg21Leu carriers, hypertrophic cardiomyopathy carriers and dilated cardiomyopathy/left ventricle noncompaction carriers. A two-sided p value <0.05 was considered to indicate statistical significance. *TPM1* carriers and all clinically affected first/second-degree relatives without genetic testing were included for this survival analysis. Cumulative percentage of diagnosed carriers by age was estimated also by Kaplan-Meier method for the same groups.

3.3: *TPM1* phenotype enrichment and functional domains

Regarding the phenotype enrichment of *TPM1* and its functional regions two different approaches were followed. The internal comparison separates cases and controls among all the NGS patients in which the gene was sequenced. Patients with variants located in each of the regions were grouped according to their phenotype and, simultaneously, the same was done for non- carrier patients. With these four values, fisher test is applied by regions resulting in an OR with a 95% confidence interval and its p- value.

The external comparison uses an external database (gnomAD) as control patients. Being the cases again the NGS sequenced phenotyped carriers and non-carriers of a variant for each region (HiC), different approaches were used to transform the allele count variant of gnomAD to group by carrier and non-carrier patients in the regions, since each variant in gnomAD has a different quantity of total alleles. Hence, using the average of total alleles among the variants located in each region was the most consistent approach after testing a sufficient amount of genes. Finally, the fourth value (control carriers) is obtained summing up alternative alleles for the variants located in each region attending to their zygosity.

3.4: Statistical analysis softwares

Statistical analyses were performed with the softwares R version 3.4.3 (R Foundation for Statistical Computing, Vienna, Austria) and HiC - mutations version 7.7.6 (Health in Code S.L., A Coruña, Spain).

Table 3.1: 213 genes related to inherited cardiovascular diseases and sudden death included in our custom probe library.
*** Priority genes for dilated/non-compaction cardiomyopathy.**
**** Priority genes for hypertrophic cardiomyopathy.**

AARS2	Alanine-tRNA ligase, mitochondrial
ABCC9	ATP-binding cassette, sub-family C (CFTR/MRP), member 9
ACAD9	Acyl-CoA dehydrogenase family member 9, mitochondrial
ACADM	Medium-chain specific acyl-CoA dehydrogenase, mitochondrial
ACADVL	Very long-chain specific acyl-CoA dehydrogenase, mitochondrial
ACTA1	Actin, alfa 1, skeletal muscle
ACTA2	Actin, aortic smooth muscle
ACTC1	**Actin, alpha cardiac muscle 1 **/***
ACTN2	Alpha-actinin-2
ACVRL1	Serine/threonine-protein kinase receptor R3
ADAMTSL4	ADAMTS-like protein 4
AGK	Acylglycerol kinase, mitochondrial
AGL	Glycogen debranching enzyme
AGPAT2	1-acyl-sn-glycerol-3-phosphate acyltransferase beta
AKAP9	A-kinase anchor protein 9
ALMS1	Alstrom syndrome protein 1
ANK2	Ankyrin 2
ANK3	Ankyrin-3
ANKRD1	Ankyrin repeat domain-containing protein 1
APOA5	Apolipoprotein A-V
APOB	Apolipoprotein B-100
APOC3	Apolipoprotein C-III
ATPAF2	ATP synthase mitochondrial F1 complex assembly factor 2
BAG3	**BAG family molecular chaperone regulator 3 ***
BMPR1B	Bone morphogenetic protein receptor type-1B
BMPR2	Bone morphogenetic protein receptor type II
BRAF	Serine/threonine-protein kinase B-raf
BSCL2	Seipin
CACNA1C	Voltage-dependent L-type calcium channel subunit alpha-1C
CACNA1D	Voltage-dependent L-type calcium channel subunit alpha-1D
CACNA2D1	Voltage-dependent calcium channel subunit alpha-2/delta-1
CACNB2	Voltage-dependent L-type calcium channel subunit beta-2
CALM1	Calmodulin
CALM2	Calmodulin
CALR3	Calreticulin 3
CAPN3	Calpain-3
CASQ2	Calsequestrin-2
CAV1	Caveolin-1
CAV3	Caveolin-3
CBL	E3 ubiquitin-protein ligase CBL
CBS	Cystathionine beta-synthase
CETP	Cholesteryl ester transfer protein
COL1A1	Collagen alpha-1(I) chain
COL1A2	Collagen alpha-2(I) chain
COL3A1	Collagen alpha-1(III) chain
COL5A1	Collagen alpha-1(V) chain
COL5A2	Collagen alpha-2(V) chain
COQ2	4-hydroxybenzoate polyprenyltransferase, mitochondrial
COX15	Cytochrome c oxidase assembly protein COX15 homolog
COX6B1	Cytochrome c oxidase subunit 6B1
CRELD1	Cysteine-rich with EGF-like domain protein 1
CRYAB	Alpha-crystallin B chain
CSRP3	Cysteine and glycine-rich protein 3
CTF1	Cardiotrophin 1
CTNNA3	Catenin alpha-3
DES	**Desmin **/***
DLD	Dihydrolipoyl dehydrogenase, mitochondrial
DMD	**Dystrophin ***
DNAJC19	Mitochondrial import inner membrane translocase subunit TIM14
DOLK	Dolichol kinase
DSC2	**Desmocollin 2 ***
DSG2	**Desmoglein 2 ***

DSP	**Desmoplakin ***
DTNA	Dystrobrevin alpha
ELN	Elastin
EMD	**Emerin ***
ENG	Endoglin
EYA4	Eyes absent homolog 4
FAH	Fumarylacetoacetase
FBN1	Fibrillin 1
FBN2	Fibrillin 2
FHL1	**Four and a half LIM domains protein 1 ****
FHL2	Four and a half LIM domains 2
FHOD3	**FH1/FH2 domain-containing protein 3 ****
FKRP	Fukutin-related protein
FKTN	Fukutin
FLNA	Filamin-A
FLNC	**Filamin-C ***
FOXD4	Forkhead box protein D4
GAA	Lysosomal alpha-glucosidase
GATA4	Transcription factor GATA-4
GATA6	Transcription factor GATA-6
GATAD1	GATA zinc finger domain-containing protein 1
GDF2	Growth/differentiation factor 2
GFM1	Elongation factor G, mitochondrial
GJA1	Gap junction alpha-1 protein
GJA5	Gap junction alpha-5 protein
GLA	**Alpha-galactosidase A ****
GLB1	Beta-galactosidase
GNPTAB	N-acetylglucosamine-1-phosphotransferase subunits alpha/beta
GPD1L	Glycerol-3-phospate dehydrogenase 1-like protein
GUSB	Beta-glucuronidase
HCN4	Potassium/sodium hyperpolarization-activated cyclic nucleotide-gated channel 4
HFE	Hereditary hemochromatosis protein
HRAS	GTPase HRas
JAG1	Jagged-1
JPH2	Junctophilin 2
JUP	**Junction plakoglobin ***
KCNA5	Potassium voltage-gated channel subfamily A member 5
KCND3	Potassium voltage-gated channel subfamily D member 3
KCNE1	Potassium voltage-gated channel subfamily E member 1
KCNE1L	Potassium voltage-gated channel subfamily E member 1-like protein
KCNE2	Potassium voltage-gated channel subfamily E member 2
KCNE3	Potassium voltage-gated channel subfamily E member 3
KCNH2	Potassium voltage-gated channel subfamily H member 2
KCNJ2	Inward rectifier potassium channel 2
KCNJ5	G protein-activated inward rectifier potassium channel 4
KCNJ8	ATP-sensitive inward rectifier potassium channel 8
KCNK3	Potassium channel subfamily K member 3
KCNQ1	Potassium voltage-gated channel subfamily KQT member 1
KLF10	Krueppel-like factor 10
KRAS	GTPase KRas
LAMA2	Laminin subunit alpha-2
LAMA4	Laminin subunit alpha-4
LAMP2	**Lysosome-associated membrane glycoprotein 2 **/***
LDB3	LIM domain-binding protein 3
LDLR	Low density lipoprotein receptor
LIAS	Lipoyl synthase, mitochondrial
LMNA	**Prelamin-A/C ***
LRP6	Low-density lipoprotein receptor-related protein 6
MAP2K1	Dual specificity mitogen-activated protein kinase kinase 1
MAP2K2	Dual specificity mitogen-activated protein kinase kinase 2
MIB1	E3 ubiquitin-protein ligase MIB1
MLYCD	Malonyl-CoA decarboxylase, mitochondrial
MRPL3	39S ribosomal protein L3, mitochondrial
MRPS22	28S ribosomal protein S22, mitochondrial
MTO1	Protein MTO1 homolog, mitochondrial
MURC	Muscle-related coiled-coil protein
MYBPC3	**Myosin-binding protein C, cardiac-type **/***

MYH11	Myosin-11
MYH6	Myosin-6
MYH7	**Myosin-7 **/***
MYL2	**Myosin regulatory light chain 2, ventricular/cardiac muscle isoform ****
MYL3	**Myosin light chain 3 ****
MYLK	Myosin light chain kinase, smooth muscle
MYLK2	Myosin light chain kinase 2, skeletal/cardiac muscle
MYOT	Myotilin
MYOZ2	Myozenin 2
MYPN	Myopalladin
NEBL	Nebulette
NEXN	Nexilin
NKX2-5	Homeobox protein Nkx-2.5
NOTCH1	Neurogenic locus notch homolog protein 1
NOTCH3	Neurogenic locus notch homolog protein 3
NPPA	Atrial natriuretic factor
NRAS	GTPase NRas
OBSL1	Obscurin-like protein 1
PCSK9	Proprotein convertase subtilisin/kexin type 9
PDHA1	Pyruvate dehydrogenase E1 component subunit alpha, somatic form, mitochondrial
PDLIM3	PDZ and LIM domain protein 3
PHKA1	Phosphorylase b kinase regulatory subunit alpha, skeletal muscle isoform
PITX2	Pituitary homeobox 2
PKP2	**Plakophilin 2 ***
PLN	Cardiac phospholamban
PLOD1	Procollagen-lysine,2-oxoglutarate 5-dioxygenase 1
PMM2	Phosphomannomutase 2
PRDM16	PR domain zinc finger protein 16
PRKAG2	**5'-AMP-activated protein kinase subunit gamma-2 ****
PRKG1	cGMP-dependent protein kinase 1
PSEN1	Presenilin-1
PSEN2	Presenilin 2
PTPN11	**Tyrosine-protein phosphatase non-receptor type 11 ****
RAF1	RAF proto-oncogene serine/threonine-protein kinase
RANGRF	Ran guanine nucleotide release factor
RBM20	**Probable RNA-binding protein 20 ***
RYR2	Ryanodine receptor 2
SCN10A	Sodium channel protein type 10 subunit alpha
SCN1B	Sodium channel subunit beta-1
SCN2B	Sodium channel subunit beta-2
SCN3B	Sodium channel subunit beta-3
SCN4B	Sodium channel subunit beta-4
SCN5A	**Sodium channel protein type 5 subunit alpha ***
SGCA	Alpha-sarcoglycan
SGCB	Beta-sarcoglycan
SGCD	Delta-sarcoglycan
SHOC2	Leucine-rich repeat protein SHOC-2
SKI	Ski oncogene
SLC22A5	Solute carrier family 22 member 5
SLC25A4	ADP/ATP translocase 1
SLC2A10	Solute carrier family 2, facilitated glucose transporter member 10
SLMAP	Sarcolemmal membrane-associated protein
SMAD1	Mothers against decapentaplegic homolog 1
SMAD3	Mothers against decapentaplegic homolog 3
SMAD4	Mothers against decapentaplegic homolog 4
SMAD9	Mothers against decapentaplegic homolog 9
SNTA1	Alpha-1-syntrophin
SOS1	Son of sevenless homolog 1
SPRED1	Sprouty-related, EVH1 domain-containing protein 1
SURF1	Surfeit locus protein 1
TAZ	Tafazzin
TBX1	T-box transcription factor TBX1
TBX20	T-box transcription factor TBX20
TBX5	T-box transcription factor TBX5
TCAP	Telethonin
TGFB2	Transforming growth factor beta-2
TGFB3	Transforming growth factor, beta 3

TGFBR1	TGF-beta receptor type-1
TGFBR2	TGF-beta receptor type-2
TMEM43	Transmembrane protein 43
TMEM70	Transmembrane protein 70, mitochondrial
TMPO	Thymopoietin
TNNC1	**Troponin C, slow skeletal and cardiac muscles **/***
TNNI3	**Troponin I, cardiac muscle **/***
TNNT2	**Troponin T, cardiac muscle **/***
TPM1	**Tropomyosin alpha-1 chain **/***
TRDN	Triadin
TRIM63	**E3 ubiquitin-protein ligase TRIM63 ****
TRPM4	Transient receptor potential cation channel subfamily M member 4
TSFM	Elongation factor Ts, mitochondria
TTN	**Titin ***
TTR	**Transthyretin ****
TXNRD2	Thioredoxin reductase 2, mitochondrial
VCL	Vinculin

4. *TPM1* p.Arg21Leu variant

4.1: The hypothesis for a founder variant: *TPM1* p.Arg21Leu

Hypertrophic cardiomyopathy is a common genetic disorder (prevalence >1/500), with wide phenotypic and *locus* heterogeneity (Gersh BJ et al, 2011; Elliott PM et al, 2014; Semsarian C et al, 2015). The *TPM1* gene (encoding α-tropomyosin) is considered one of the main causal hypertrophic cardiomyopathy-genes; nevertheless, it is a relatively rare etiology, accounting for 1-5% of the cases with this phenotype (Elliott et al, 2014).

Available clinical information on most of the patients carrying *TPM1* variants is restricted to a single pedigree or few index cases for each variant, which limits the assertive clinical interpretation of the genetic findings. The exception to this rule is the *TPM1* p.Asp175Asn variant identified in several Finnish hypertrophic cardiomyopathy-patients due to the fact that it is the single founder effect described in this gene to date. Clinical information about these variants are described in the first chapter of this thesis. Genotype-phenotype correlation studies of founder variants in hypertrophic cardiomyopathy-populations have contributed to a better understanding of the clinical course and prognosis associated with a same variant (Kubo T et al, 2005; Olive-Sandoval et al, 2010; van den Wijngaard A et al, 2011; Teirlinck CH et al, 2012; Calore C et al, 2015; Ross SB et al, 2017; Sabater-Molina M et al, 2017).

At our center, we have identified the variant *TPM1* p.Arg21Leu by next

generation sequencing in several individuals with hypertrophic cardiomyopathy, including homozygous carriers. Although we have undertaken genetic studies from several countries around the world, the p.Arg21Leu variant cases predominantly came from hospitals in northwest Spain and Portugal, suggesting that it could be a founder variant. This variant has been classified in public databases as of uncertain clinical significance (five independent submitters) and likely pathogenic (only one submitter) in ClinVar database; whereas the associated-phenotype is still unknown. Our first objective in this thesis was to describe the phenotypic features and associated prognosis of the *TPM1* p.Arg21Leu variant in a series of pedigrees.

4.2: *TPM1* p.Arg21Leu clinical description

TPM1 p.Arg21Leu was identified in 25/10,561 (0.23%) consecutive index cases with different inherited heart diseases sequenced in our center. It was present in 25/4,099 (0,61%) hypertrophic cardiomyopathy-probands, and absent in 6,462 index cases with other cardiac phenotypes (0/3,830 dilated, left ventricle non-compaction, and arrhythmogenic cardiomyopathies; 0/1,590 channelopathies and 0/1,042 other inherited heart diseases) ($p<0.0001$). This variant appears in simple heterozygosis in 10/62,784 (0.015%) individuals from TOPMed program population, and 5/120,158 (0.004%) individuals (age range 55-65 years) from gnomAD population (non-TOPMed samples).

Further, we have summed six index cases that were identified in other collaborating hospitals. In total, 83 carriers (50.6% male) were identified – 31 index cases (see population study; Table 4.1). Here we present the largest

HCM-population ever described carrying the same *TPM1* variant. In this chapter, we are showing that the *TPM1* p.Arg21Leu variant is significantly overrepresented in our hypertrophic cardiomyopathy cohort when compared with control populations, and familial cosegregation of the variant with the disease was documented with a significant LOD score.

Table 4.1: TPM1 p.Arg21Leu population study

Total individuals		***n* = 226**
1. Carriers		**83**
Male	42 (50.6%)	
Female	41 (49.4%)	
1.a: Carriers with clinical data		67
1.b: Carriers without clinical data		2
1.c: Obligated simple heterozygous carriers without clinical data		14
2. Patients reported as HCM without clinical and genetic data		**13**
3. Non-carriers with clinical data		**24**
4. First-degree relatives without clinical or genetic evaluation		**106**
5. Families		
5.a: Pedigrees		28
5.b: Index cases **		31
5.c: Nº carriers/pedigree		2.85
5.d: Pedigrees with affected carriers in ≥ 2 generations		12

** 3 index cases were reported without pedigree data.

4.2.1: *TPM1* p.Arg21Leu phenotypic features.

Clinical data obtained for 67 carriers (31 index cases and 36 relatives) are summarized at Table 4.2. 47/67 (70%) fulfilled the diagnostic criteria for HCM by left ventricle hypertrophy criteria (31 index cases and 16 relatives), 7/67 (10.4%) were relatives affected only with minor ECG changes. 13/67 (19%) were unaffected carriers. Most of the carriers were asymptomatic (79%), and syncope was reported in five (7.5%) cases.

In those cases with left ventricle hypertrophy (*n*=47), the predominant morphological pattern was asymmetric septal (37/47; 79%) and 15% (7/47) had apical hypertrophy. Figure 4.1 shows the distribution of left ventricle hypertrophy according to age of last follow up, sex and genotype. Carriers were concentrated between 15-25 mm of left ventricle hypertrophy. Mean maximum left ventricle wall thickness was 19.5 (±8.2) mm. Among the carriers diagnosed at younger ages, mean maximum left ventricle wall thickness was 25,8 (±11) mm. Left ventricle outflow tract obstruction was reported in 32% of cases (15/47) and abnormal blood pressure response to exercise testing in 10.6% (5/47). 14.9% (7/47) had a pseudonormalization pattern of the mitral valve inflow, and only one case had a restrictive pattern. No mid-ventricular obstruction was observed.

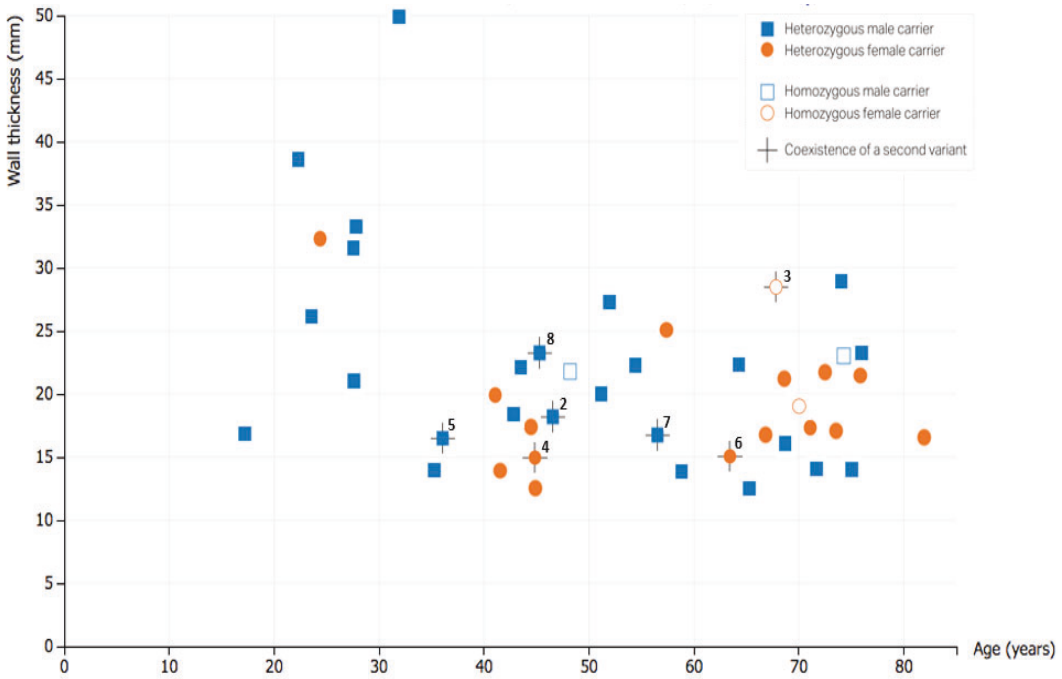


Figure 4.1: Maximum left ventricle wall thickness (in mm) by sex, genotype and age at the last follow up in *TPM1* p.Arg21Leu carriers diagnosed with LVH criteria (*n*=44). Male carriers are in blue and female carriers in orange. Numbers detail the genotype: 2. *TNNT2* p.Arg278Cys (+++); **3.** *MYBPC3* p.Asp75Asn (+?); **4.** *MYL3* p.Met173Val (+?); **5.** *MYH7* p.Lys351Asn (?); **6.** *MYH7* p.Leu1333Val (?); **7.** *MYH7* p.Tyr582Cys (?); **8.** *TPM1* p.Met281Val (+++). (+++) indicates a pathogenic variant, (+?) likely pathogenic variant, and (?) variant of unknown clinical significance.

Electrocardiographic abnormalities were reported in 84% of the carriers with clinical data; 9% (6/67) had atrial fibrillation, and the same number (9%; 6/67) was reported to have conduction system disorders. Only one patient underwent pacemaker implant due complete atrioventricular block after mitral valve surgery. 8/67 (12%) carriers had ventricular arrhythmias; five of them with non-sustained ventricular tachycardia recorded on 24-hour Holter. There were no reports of appropriate shocks in a mean follow-up of 3.8 years among the five patients who had implanted cardioverter-defibrillator for primary prevention. There were no arrhythmias induced by the exercise stress test. Only three clinical features showed significant statistical differences among index cases and relatives (Table 4.2). Index cases had more symptoms (dyspnea), atrial dilatation and higher ECG voltages than relatives.

Table 4.2: TPM1 p.Arg21Leu Clinical Features (*n*=67)

Carriers with data	Total *n* = 67	%	Average (SD)	Index cases *n* = 31	%	Average (SD)	Relatives *n* = 36	%	Average (SD)
Unaffected	**14/67**	**20.9**		-			**14/36**	**36.1**	
Affected	**53/67**	**79.1**		**31/53**	**58,5**		**22/53**	**41.5**	
Male:Female	1.01			1.38			0.53		
Only with minor ECG changes	9/53	**17**		0/31			9/22	40.9	
Age at diagnosis (years)			46.4 (±19.2) (range 11-73)			46.6 (±19.3)			46 (±19.5)
Mean follow-up time (years)	7.7 (±7.1)								
SYMPTOMS									
Dyspnea	**14/53**	**26.4**		**12/31**	**38.7**		**2/22**	**9.1**	
NYHA II	9/53	17		7/31	22.6		2/22	**9.1**	
NYHA III - IV	5/53	9.4		5/31	16.1		0/22		
Syncope	**5/53**	**9.4**		**5/31**	**16.1**		**0/36**		
Chest pain	**4/53**	**7.5**		**4/31**	**12.9**		**0/36**		
IMAGING									
LVH	**44/53**	**83**		**31/31**	**100**		**13/22**	**59.1**	
Apical	7/44	**15.9**		5/31	16.1		2/13	**15.4**	
Asymmetric septal	34/44	**77.3**		24/31	77.4		10/13	76.9	
Concentric	1/44	**2.3**		1/31	3.2		0/13		
Atypical	2/44	**4.5**		1/31	3.2		1/13	**7.7**	
Maximal LV wall thickness (mm)			21.4 (±7.65)			22.2 (±8.1)			15.1 (±6.6)
LV Mass (g)	**11/44**	**25**	**290.5 (±168.3)**	**5/31**	**16.1**	**338 (±173.9)**	**6/13**	**46.2**	**251 (±168.3)**
LVOTO	**15/44**	**34.1**		**12/31**	**38.7**		**3/13**	**23.1**	

Mean peak value (mmHg)			67.4 (±33.8)			65.1 (±35.6)			73.2 (±33.1)
Abnormal response to exercise testing	**5/44**	**11.4**		**4/31**	**12.9**		**1/13**	**7.7**	
Mid-ventricular obstruction	**0/44**			**0/31**			**0/13**		
LV apical aneurism	**0/44**			**0/31**			**0/13**		
Ejection fraction (%)			**68.3 (± 8.6)**			**68.2 (± 9.2)**			**68.5 (± 7.4)**
Systolic dysfunction	0/44			0/31			0/13		
LV diastolic dysfunction	**22/44**	**50**		**16/31**	**51.6**		**6/13**	**46.2**	
Grade I	14/44	**31.8**		10/31	32.2		4/13	**30.8**	
Grade II	7/44	**15.9**		5/31	16.1		2/13	**15.4**	
Restrictive pattern	1/44	**2.3**		1/31	3.2		0/13		
Left atrial dilatation (mm) ** [1]	**26/44**	**59.1**	**40.38 (±6.7)**	**21/31**	**67.7**	**41.64 (±6.5)**	**5/13**	**38.5**	**37.86 (±6.62)**
MRI	**18/53**	**33.9**		**15/31**	**48.4**		**3/22**	**13.6**	
No LGE	5/18	27.8		3/15	20		2/3	66.7	
LGE 1-2 segments	9/18	50		9/15	60		0/3		
LGE ≥ 3 segments	4/18	22.2		3/15	20		1/3	33.3	
ECG									
Minor ECG changes	**49/53**	**92.5**		**27/31**	**87.1**		**22/22**	**100**	
High voltages **	25/49	**51**		21/27	77.8		4/22	**18.2**	
T-wave inversion	16/49	**32.7**		10/27	37		6/22	**27.3**	
Pathologic Q waves	15/49	**30.6**		10/27	37		5/22	**22.7**	
Non-specific repolarization changes	16/49	**32.7**		8/27	29.6		8/22	**36.4**	
Atrial fibrillation	**6/53**	**11.3**		**5/31**	**16.1**		**1/22**	**4.5**	
Conduction disease	**6/53**	**11.3**		**5/31**	**16.1**		**1/22**	**4.5**	
Ventricular arrhythmias	**8/53**	**15.1**							
NSVT on 24-Holter	5/8	62.5		5/31	16.1		0/22		
Premature ventricular beats	4/8	50		2/31	6.5		2/22	**9.1**	
Arrhythmias induced by exercise	**0/53**			**0/31**			**0/22**		
TREATMENT									
Surgery [2]	**4/53**	**7.5**		**3/31**	**9.7**		**1/22**	**4.5**	
Pacemaker	**1/53**	**1.9**		**0/31**			**1/22**	**4.5**	
ICD [3]	**4/53**	**7.5**		**4/31**	**11.7**		**0/22**		
Appropriated shocks	0		Mean follow-up time 3.8 years						
LVH PREDISPOSING FACTORS									
Competitive sports	**6/53**	**11.3**		**3/31**	**9.7**		**3/22**	**13.6**	
Arterial hypertension	**7/53**	**13.2**		**6/31**	**19.4**		**1/22**	**4.5**	
Moderate-severe	2/7	28.6		2/31	6.5		0/22		

P-value Fisher exact test for the subpopulations index cases and relatives. Only shown [**] when p-value < 0.05 comparing index cases and relatives. [1] Statistical difference in Left atrial dilatation was observed only for *ratios (%),* not for *average (SD)* values. [2] Two myectomy; one valve mitral prosthesis surgery + myectomy; and one cardiac transplantation; [3] All cases for primary prevention.

ECG = electrocardiogram. ICD = implanted cardio defibrillator. NSVT = non-sustained ventricular tachycardia. LGE = Late gadolinium enhancement. LV = left ventricle. LVH = Left ventricular hypertrophy. LVOTO = Left ventricle outflow tract obstruction. MRI = Cardiac magnetic resonance. NYHA = New York Heart Association. SD = Standard deviation.

4.2.2: Age of diagnosis.

Cumulative percentage of diagnosed carriers by age and sex is shown in figure 4.2. At age of 30 years, 25% of male and 10% of female carriers had been clinically diagnosed with hypertrophic cardiomyopathy. The percentage rises to 50%

at the age of 50 years for both genders. At 70 years of age, approximately 12,5% of males and 20% of females were still unaffected (p = 0.24). Mean age at diagnosis was 46±19 years (range 11-73). Individuals diagnosed under the age of 35 years were 22.4% (15/67) of the carriers with detailed data (see Table 4.3).

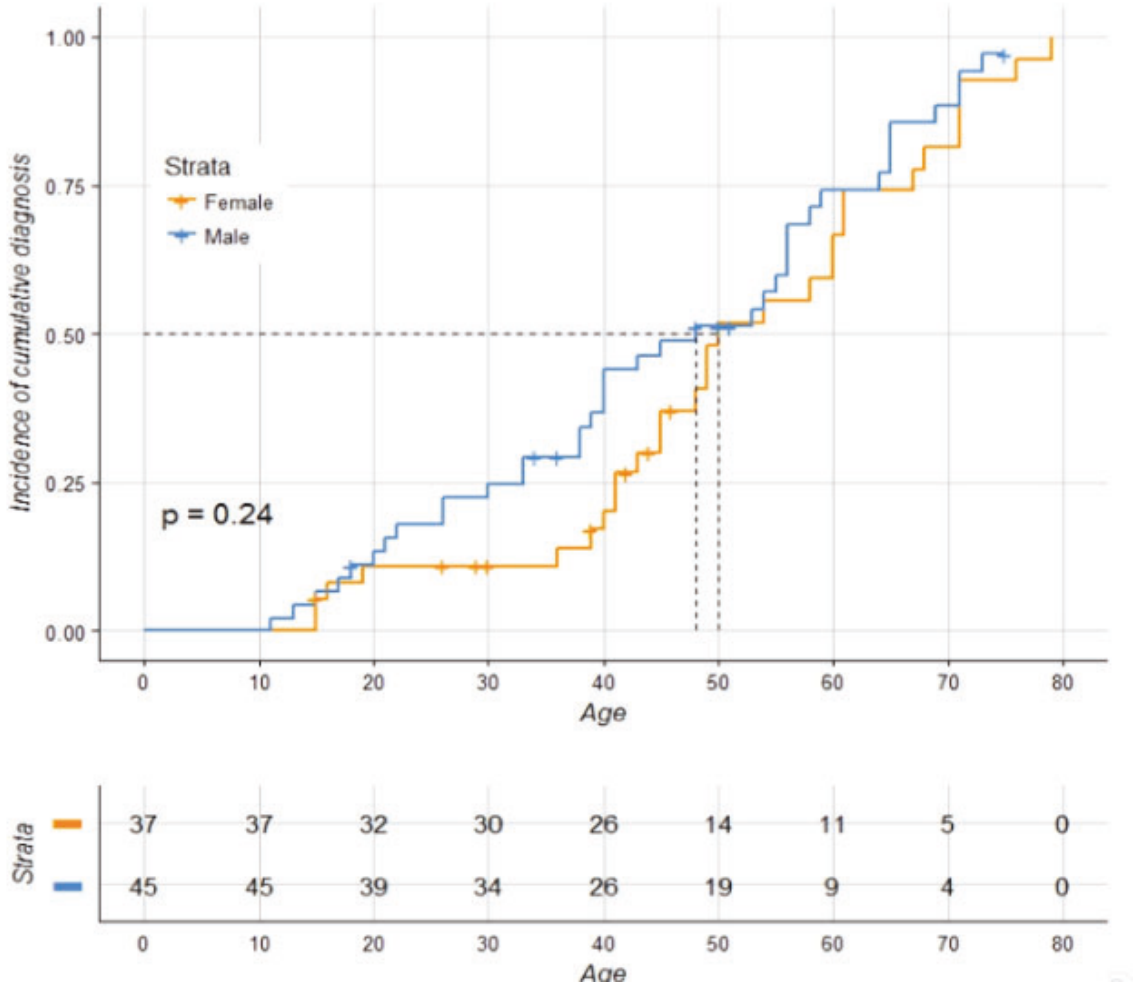


Figure 4.2: Cumulative percentage of TPM1 p.Arg21Leu carriers diagnosed with hypertrophic cardiomyopathy. Male carriers are in blue and female carriers in orange.

Table 4.3: Clinical features of the TPM1 p.Arg21Leu carriers diagnosed under the age of 35 years.

Pedigree	Id	Sex	Second variant	Index case	Phenotype	Age of Dx	Age	NYHA	AF	FHSD	Max LVH	TV/ FV	Syncope	Abn. Vasc Resp	LVOTO (Peak grad.)	LV disf. (EF)	Events	Additional features	Others	SCD Risk
#3	IV.1	M	No	No	HCM atypical	18	27	I	-	-	33	-	-	-	-	-		Echo: LA 40mm.	Competitive athlete – football	2.92
#6	IV.1	M		Yes	HCM septal	15	22	II	-	+	39	+	-	-	+(35 exerc.)	-	-	Holter: 1 episode NSVT (8 complex) MNR: LGE 3 segments	Competitive athlete - football ICD	6.37
#7	IV.2	M	No	No	HCM septal	26	35	I	-	+	14	-	-	-	-	-			Competitive athlete – football	2.48
#8	IV.2	F	No	No	HCM	15	19	I	-	-	11	-	-	-	-	-		ECO: apical hypertrabeculation (without criteria for NC) ECG: pathologic q wave inf and V3-6 Holter: Rare ectopic ventricular beats multifocal.		1.38
#11	V.1	M	No	No	HCM septal	17	28	I	-	-	22	-	-	-	-	-		ECO: LA 46mm ECG: negative T waves, high QRS voltages		3.29
	V.2	F	No	No	HCM	16	21	I	-	-	11	-	-	-	-	-		ECO: LA 46mm ECG: negative T waves, high		1.46
#13	II.3	M	No	Yes	HCM septal	30	44	I	-	-	18	-	-	+	+(60)	-		ECO: LA 40mm, diastolic dysfunction I. ECG: pathologic q wave, abnormal repolarization, high QRS voltages.		2.24
#17	II.1	M	MYH7 Lys351Asn (?)	Yes	HCM septal	33	36	I	-	-	16	-	-	-	-	-		ECO: LA 42mm ECG: normal		2.00
#18		M	No	Yes	HCM septal	13	32	III	-	-	51	-	+	+	+(140)	-		ECO: LA 47mm, RA enlarged, severe mitral regurgitation, SAM. Wave S <8 (tissue Doppler)	Myectomy. ICD	9.94

#19	II.1	M	No	Yes	HCM septal	33	47	I	-	-	23	+	-	-	-	-		ECG: First degree AVB, High QRS Voltage	Primary prevention – ICD implanted	4.59
#20	II.2	M	No	Yes	HCM septal	11	17	I	-	-	17	-	-	-	-	-				2.43
	II.1	M	No	No	HCM	22	23	I	-	-	10	-	-	-	-	-	-	ECG: High voltages		NR
#25	II3	M	No	Yes	HCM septal	26	28	I	-	-	32	+	-	-	-	-		Echo: LA 35mm	Treacher Collins syndrome	5.86
#26	II.1	M	No	Yes	HCM septal	20	23	I	-	-	26	-	-	-	-	-		MRI: LGE 2 segments, LA 41 mm	Competitive athlete – football	3.46
#29	II.3	F	No	Yes	HCM septal	15	24	II	-	+	33.5	-	-	-	+(90)	-	-	MRI: LGE in all segments, LA 32mm	Myectomy	5.35

4.2.3: Risk stratification and cardiovascular events.

Survival analysis (Figure 4.3) shows that less than 5% of our population had a cardiovascular death or heart transplant at the age of 30 years, and 10% at 50 years for both genders. 25% of females and 44% of males had suffered a major cardiovascular event at 70 years. No statistical difference between genders was observed ($p = 0.24$). Annual incidence of cardiovascular events was 0.25% in the survival analysis, which suggest a better prognosis for TPM1 p.Arg21Leu than expected for the overall hypertrophic cardiomyopathy population (≅0.5%/year).

Three cardiovascular deaths (one sudden cardiac death, one heart failure death, and one unspecified cardiac death) and one heart transplant (in total, 4/83; 5%) were reported in *TPM1* p.Arg21Leu carriers. During a mean follow-up time of 4.9 (±6.7) years, no sudden cardiac death or cardiac transplantation was additionally reported. Twelve other cardiovascular events were reported in first/second-degree relatives without genetic testing; five sudden cardiac deaths, two heart failure deaths, two stroke-related deaths, and three unspecified cardiac deaths. Five carriers were reported with minor cardiovascular events that were not included in the survival curves; two myectomies, one mitral valve replacement for left ventricle outflow tract obstruction due systolic anterior motion, and two non-lethal strokes. Each event, age of occurrence and sex of the patients are specified in Table 4.4.

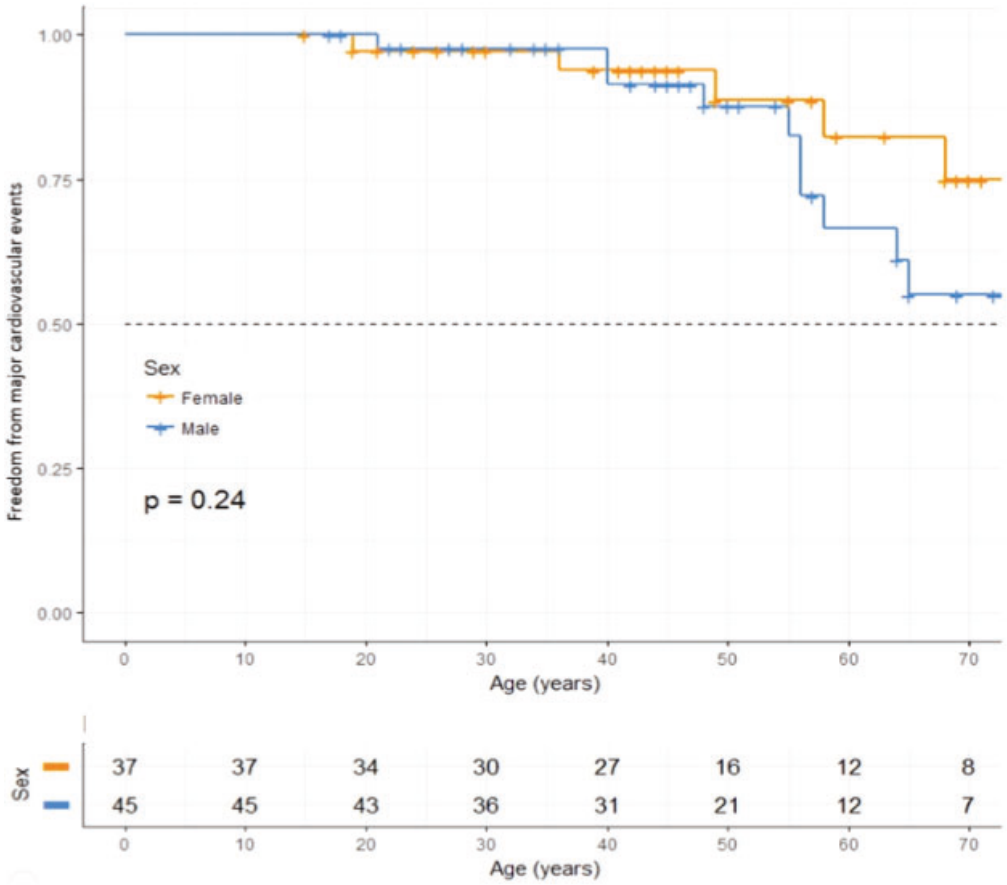


Figure 4.3: TPM1 p.Arg21Leu survival function by age and gender. Male carriers are in blue and female carriers in orange.

ESC sudden cardiac death risk was calculated for all carriers with left ventricle hypertrophy (*n*=47). Most of them (79%; 37/47) had a low risk score (<4% risk over 5 years), 17% (8/47) had intermediate values (4-6% risk over 5 years), and only 4% (2/47) had high risk (>6% risk over 5 years). Figure 4.4 shows carriers concentrated at ranges with low sudden cardiac death risk and advanced ages. Only 18/67 (26.8%) carriers with available clinical data had an cardiac magnetic resonance imaging; 5/18 (27.8%) were reported without late gadolinium enhancement, 9/18 (50%) had in one or two cardiac segments, and 4/17 (22.2%) had in ≥3 segments. No left ventricular apical aneurysm or end-stage hypertrophic cardiomyopathy was observed.

Table 4.4: Major and minor adverse cardiovascular events reported in the TPM1 p.Arg21Leu pedigrees.

Individual	Major CV event (age)	Observations
CARRIERS		
1. Male	Sudden death (55)	No left ventricle hypertrophy.
2. Male	Heart transplant (48)	
3. Female	Heart failure death (68)	Systemic sclerosis (Pulmonary fibrosis).
4. Obligated carrier, female	Unspecified cardiac death (79)	Unavailable clinical data.
FIRST-DEGREE RELATIVES		
5. Male	Sudden death (58)	Unknown genetic status.
6. Male	Sudden death (40)	Unknown genetic status. Additional familial genetic variant *TPM1* p.Met281Val – pathogenic.
7. Female	Sudden death (19)	Unknown genetic status. Additional familial genetic variant *MYH7* p.Gly741Arg – pathogenic.
8. Male	Stroke-related death (64)	Unknown genetic status.
9. Female	Heart failure death (58)	Unknown genetic status.
10. Female	Stroke-related death (36)	Unknown genetic status.
11. Female	Unspecified cardiac death (49)	Unknown genetic status. Valvular heart disease.
12. Male	Unspecified cardiac death (>65)	Unknown genetic status.
SECOND-DEGREE RELATIVES		
13. Male	Sudden death (21)	Unknown genetic status. SCD during military exercise.
14. Male	Sudden death (40)	Unknown genetic status.
15. Male	Heart failure death (40)	Unknown genetic status.
16. Male	Unspecified cardiac death (>56)	Unknown genetic status.
	Minor adverse CV events (age) **	
CARRIERS		
1. Female	Septal myectomy (23)	Echo 33,5 mm
2. Male	Septal myectomy (32)	Echo 51 mm
3. Female	Mitral v. replacement (41)	Systolic anterior motion of mitral valve
4. Male	Non-fatal stroke (65)	
5. Female	Non-fatal stroke (73)	

** Minor adverse cardiovascular events were not included in the survival curves.

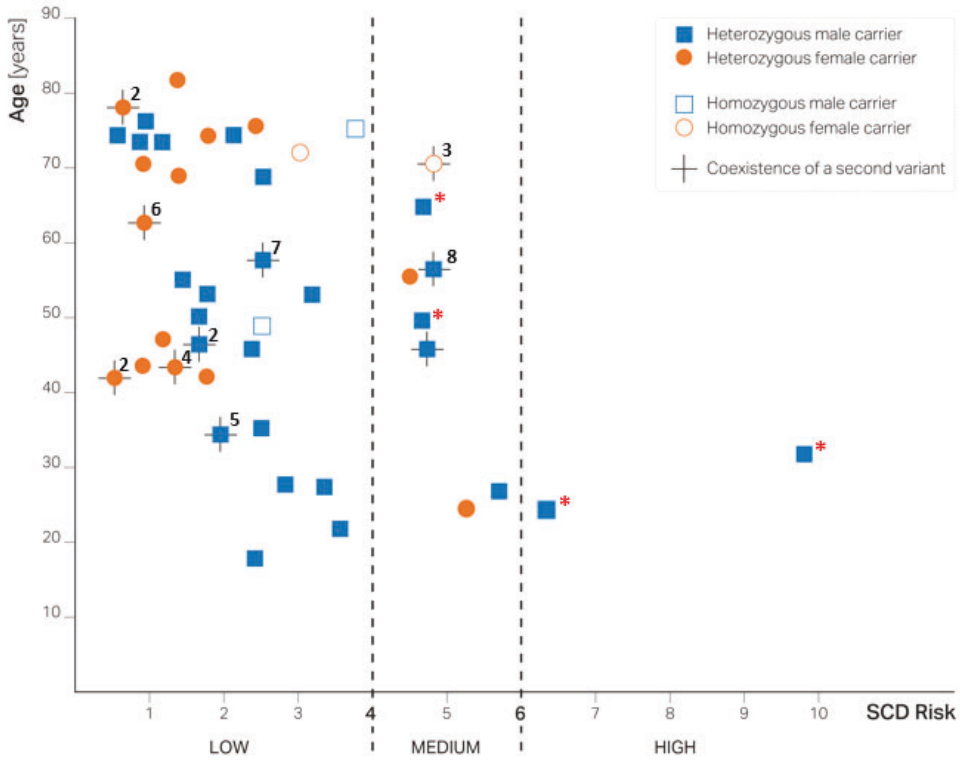


Figure 4.4: TPM1 p.Arg21Leu hypertrophic cardiomyopathy risk stratification (by ESC calculator) by age, risk score, gender, and genotype. Numbers detail the genotype: 2. *TNNT2* p.Arg278Cys (+++); **3.** *MYBPC3* p.Asp75Asn (+?); **4.** *MYL3* p.Met173Val (+?); **5.** *MYH7* p.Lys351Asn (?); **6.** *MYH7* p.Leu1333Val (?); **7.** *MYH7* p.Tyr582Cys (?); **8.** *TPM1* p.Met281Val (+++). (+++) indicates a pathogenic variant, (+?) likely pathogenic variant, and (?) variant of unknown clinical significance. (*) Red asterisks represent carriers with an ICD.

4.2.4: Homozygous carriers and additional genetic variants.

Fifteen individuals (15/67; 17.9%) from 10 pedigrees had complex genotypes. Four had *TPM1* p.Arg21Leu in homozygosity (4/67; 6%), and twelve had an additional genetic variant (12/67; 17.9%) – one of the homozygous carriers also had the pathogenic variant *MYBPC3* p.Asp75Asn. Clinical features of the homozygous carriers are described at table in the next page.

Table 4.5: Clinical features of homozygous TPM1 p.Arg21Leu carriers (*n*=4).

Pedigree	Id	Sex	Age of diagnosis	Age	Additional variant	Max wall thickness	Diastolic dysfunction	Left atrium	LVOTO	Ventricular arrhythmias	LGE	SCD risk
#1	II.3	Male	71	75	No	22 mm, asymmetric septal	II	48	120 mmHg exercise	NSVT	NR	3.85
#10	II.6	Female	67	70	No	17 mm, apical	II	46	118 mmHg in rest	Rare EVB	NR	3.04
#10	II.7	Female	61	68	*MYBPC3* p.Asp75Asn	28 mm, apical. Biventricular LVH	Restrictive	54	No	NSVT	Extensive	4.08
#13	II.2	Male	40	48	No	22 mm, asymmetric septal	II	45	40 mmHg in rest	No	NR	2.56

EVB: ectopic ventricular beats. LGE = Late gadolinium enhancement. LVH = Left ventricular hypertrophy. LVOTO = Left ventricle outflow tract obstruction. MRI = Cardiac magnetic resonance. NSVT = non-sustained ventricular tachycardia. NR = Not reported. SCD = Sudden cardiac death

Five genetic variants in the *MYH7* gene were identified (p.Gly741Arg and p.Thr1019Asn, respectively classified as pathogenic/likely pathogenic variants; p.Lys351Asn, p.Tyr582Cys and p.Leu1333Val as variants of unknown clinical significance). Other pathogenic sarcomeric variants were identified: *TPM1* p.Met281Thr, *TNNT2* p.Arg278Cys, and *MYL3* p.Met173Val. Each one of these variants was identified in different pedigrees. All these variants have been reported with very low allelic frequency (<0.01%) in the general population, except *MYH7* p.Lys351Asn.

No cardiovascular death or cardiac transplantation was described in confirmed carriers with an additional genetic variant. Two sudden cardiac deaths described in first-degree relatives without genetic testing were reported in pedigrees with a second pathogenic genetic variant (*MYH7* p.Gly741Arg, and *TPM1* p.Met281Val, respectively).

Specific clinical features of the carriers with an additional genetic variant is detailed in the following table.

Table 4.6: Clinical features of TPM1 p.Arg21Leu carriers with an additional genetic variant (*n*=12).

Pedigree	Id	Sex	Second variant	Phenotype	Age of Dx	Age	NYHA	AF	FHSD	Max LVH	TV/ FV	Syncope	Abn. Vasc Resp	LVOTO (Peak grad.)	LV disf. (EF)	Events	Additional features	Others	SCD Risk
#3	III.3	M	MYH7 p.Thr1019Asn	Not affected		51	I	-	-	11	-	-	-	-	-	-	ECG normal		-
#9	II.1	M	TNNT2 p.Arg278Cys	HCM Apical	43	45	I	-	-	17	-	-	-	-	-	-	Echo: LA 40mm. MNR: LGE 4 segments		1.67
	II.3	F	TNNT2 p.Arg 278Cys	SAM Mitral	41	41	I	-	-	-	-	-	-	-	-	-	ECG normal		0.87
	I.2	F	TNNT2 p.Arg 278Cys	HCM apical	76	76	I	-	-	+	-	-	-	-	-	-	MNR: no LGE		0.61
#10	III.6	F	MYBPC3 p.Asn75Asp	Not affected	-	42	I	-	-	-	-	-	-	-	-	-	-		-
	II.7	F	MYBPC3 p.Asp75Asn	HCM Apical	<61	68	III	-	-	28	+	-	-	-	-		Holter: NSVT. MNR: LA 54 mm, restrictive pattern, biventricular hypertrophy and LGE (subepicardical, medium mural, RV and apical). Apical hypokinesis.	Homozygous p.Arg21Leu	4.8
#15	III.5	F	MYL3 p.Met173Val	HCM septal	43	45	II	-	-	15	-	-	-	+(30)	-	-	MNR: Mild LGE in 2 segments	SAH.	1.48
	II.3	M	MYL3 p.Met173Val	HCM informed	?	75	?	?	?	+	?	?	?	?	?	-	?	Patients were evaluated in other centre.	-
#17	II.1	M	MYH7 p.Lys351Asn	HCM septal	33	36	I	-	-	16	-	-	-	-	-	-	ECO: LA 42mm	Mild SAH	2.0
#23	II.2	F	MYH7 p.Leu1333Val	HCM apical	61	63	I	-	-	15	-	-	-	-	-	-			0.87
#28		M	MYH7 p.Tyr582Cys	HCM septal	56	57	II-III	-	-	17	-	-	-	+(110)	-	-	MRI: No LGE, LA 45mm		2.39
#30	II.2	M	TPM1 p.Met281Val	HCM septal	39	56	I	-	+	23	+	-	-	-	-	-	MRI: LGE >3 segments Holter: NSVT 3 beats		4.71

We have identified twelve *TPM1* p.Arg21Leu carriers with an additional genetic variant (12/67; 17.9%). The presence of an additional genetic variant has been classically associated with a more severe disease expression; however, we consider that the clinical interpretation of genetic findings must be done always considering the clinical features and penetrance associated specifically to each genetic variant. Note that some of our carriers with a second genetic variant have mild phenotypes or even unaffected. It could be explained by the presence of two genetic variants of late/incomplete penetrance, or by the uncertainties on the pathogenic role of the additional variant (three cases with *MYH7* unknown clinical significance variants). Comments regarding disease expression of each patient and the pathogenicity of the additional genetic variant are briefly described below:

- Pedigree #3: The single p.Arg21Leu carrier (III.3) with the additional variant (*MYH7* p.Thr1019Asn) in this family was unaffected at age 51 years. Both variants have been described as having late/incomplete penetrance.

  We consider that *MYH7* p.Thr1019Asn is a likely pathogenic rare variant (present in four heterozygous carriers in gnomAD database). It has been reported in the literature in an African descendent French family that four carriers had dilated cardiomyopathy and five carriers were unaffected. In our centre, we have identified it in some DCM cases, but mainly in multiple HCM patients. In some cases, the variant was identified in association with another sarcomere variant as in this family.

- Pedigree #9: Individuals II.1, II.3 and I.2 were carriers of a pathogenic *TNNT2* variant, and expressed relatively mild phenotypes

We consider *TNNT2* p.Arg278Cys as a pathogenic HCM-associated variant. It has been identified in more than 220 carriers from >130 families. The variant appears to be associated with late/incomplete penetrance and mild-moderate hypertrophies. This incomplete penetrance explains the presence of this variant in a relevant number of individuals in control populations (98 heterozygous carriers in the gnomAD database). Other three missense variants affecting the same amino acid p.Arg278Pro/Leu/His have been also identified in multiple HCM-patients with a similar clinical profile. Among the families carrying variants in Arg278, we have reported: 9 sudden deaths in carriers and 17 sudden deaths in first/second-degrees relatives without genetic testing.

- Pedigree #10: *TPM1* p.Arg21Leu homozygous sisters (II.7 and II.6) showed severe phenotypes, although diagnosed at advanced ages (see: *Supplementary A - Pedigree #10*). The homozygous carrier II.7 had a more marked phenotype that may be related to the presence of an additional likely pathogenic variant in *MYBPC3*. On other hand, his 42-years-old daughter (III.6), clinically unaffected, was a double heterozygous carrier of *TPM1* p.Arg21Leu and *MYBPC3* p.Asp75Asn variants.

We consider *MYBPC3* p.Asp75Asn as a likely pathogenic rare variant (four carriers in gnomAD database). This variant has been identified in several HCM index cases, and familial studies showed the presence of both affected and unaffected carriers, suggesting late/incomplete penetrance.

- Pedigree #15: There are two carriers with complex genotype in this pedigree, but the individual II.3 was studied in another centre and no clinical details could be obtained.

We consider *MYL3* p.Met173Val as a likely pathogenic variant that has been associated with the development of hypertrophic cardiomyopathy (28 carriers from 14 families –half of them either unaffected of with unknown phenotype; so, familial cosegregation has not been clearly documented). It is present in a single individual from gnomAD database (control). A functional study suggesting a damaging effect has been published. Data suggests that this variant could be associated with a late disease onset and incomplete penetrance.

- Pedigree #17: The young age at diagnosis of this carrier (II.1) could be related with the presence of the *MYH7* variant; however, he did not present a severe phenotype.

We consider *MYH7* p.Lys351Asn as a variant of unknown clinical significance. It has been described to date in this single HCM-patient, and also in a single carrier from gnomAD population (control). Two missense variants located at the same amino acid (p.Lys351Glu/Thr) have been identified in HCM cases (six unrelated carriers).

- Pedigree #23: Patient II.2, with a mild phenotype, carried an additional *MYH7* variant of uncertain significance.

We consider *MYH7* p.Leu1333Val is a variant of unknown clinical significance. This is the third HCM index case that we have identified with the variant, and we have identified it in another index with DCM phenotype. It has been reported in four heterozygous individuals from gnomAD database. These data could suggest a late/incomplete penetrance for the variant, but its pathogenicity is still uncertain.

- Pedigree #28: Carrier of an additional variant of uncertain significance in *MYH7* with mild phenotype and low SCD risk. No myocardial fibrosis in MRI.

We consider *MYH7* p.Tyr582Cys is a variant of unknown clinical significance. It was identified only in this single patient to date. The variant is absent in control populations. It is located within a protein domain in which many other variants have been associated with HCM.

- Pedigree #30: Two pathogenic variants in *TPM1* were identified in this family. Only the individual II.2 carried both variants. Compared to his brother (II.1), who carried only the *TPM1* p.Met281Val, the clinical expression of the index case was more severe and could suggest a synergic effect of both variants. The paternal uncle (I.1) had a sudden death at 40 years of age; we cannot determine if he had only one of the family's variants or both.

We consider *TPM1* p.Met281Val as a pathogenic rare variant (8 individuals in gnomAD). This variant has been identified in at least 25 HCM-pedigrees (30 affected carriers and 8 unaffected relatives). Familial cosegregation has been documented in at least one large family. Clinical data of the carriers suggest late/incomplete

penetrance (only five carriers had been diagnosed under the age of 45 years). Another rare variant affecting the same amino acid (p.Met281Thr) has been identified in five HCM-patients (from four pedigrees).

Almost 18% of the carriers had a second genetic variant, a percentage higher than that described in the literature (5%) for hypertrophic cardiomyopathy (Gersh BJ et al, 2011). Previous studies have demonstrated that the number of genetic variants may be a determinant of disease severity (Ingles J et al, 2005; Girolami F et al, 2010). However, our data demonstrate that the presence of a second variant is not necessarily associated with a more severe phenotype. Some of our carriers with an additional genetic variant had mild phenotypes or were even unaffected, which could be explained by the presence of two variants with late/incomplete penetrance. The clinical interpretation of genetic findings must be done always considering the clinical features and penetrance described specifically for each variant.

Different studies have associated sarcomere variants in homozygosis to more severe presentations in hypertrophic cardiomyopathy than when these variants are identified in simple heterozigozity (Wessels MW et al 2015; Kissopoulou A et al, 2018). *TPM1* p.Arg21Leu homozygous carriers in our study showed in general a more pronounced phenotype than the simple heterozygous carriers (especially considering the existence of moderate-severe diastolic dysfunction, and ventricular arrhythmias); however, these homozygous carriers showed late onset manifestations, supporting that this variant would be associated with late onset and favourable prognosis in the absence of additional factors.

TPM1 p.Arg21Leu carriers diagnosed at younger ages were predominantly male individuals and competitive sport practice was the only left ventricle hypertrophy predisposing factor reported among them. No additional pathogenic genetic variant (except one who had a *MYH7* variant of unknown clinical significance) was reported in this group. Their sudden cardiac death risk scores were higher than the overall average, and they had more prominent ventricular hypertrophy (both myectomies described in our study were in individuals from this group), but no cardiovascular death or heart transplant was documented among the younger carriers. Some groups have described similar marked intrafamilial clinical heterogeneity in other *TPM1* hypertrophic-variants (Thierfelder L et al, 1993; Nakajima-Taniguchi C et al, 1995; Watkins H et al, 1995, Yamauchi-Takihar et al, 1996; Karibe A et al, 2001; Van Driest SL et al 2002; Jongbloed et al, 2003; Vieira E et al, 2016); nevertheless, sudden cardiac death episodes were frequently reported among young carriers, which did not occur in our population.

TPM1 p.Arg21Leu was identified exclusively in patients with hypertrophic cardiomyopathy phenotype. Most of the carriers in our population were simple heterozygous carriers with late onset disease, mild to moderate phenotype, asymptomatic clinical course, and a low number of cardiovascular deaths or heart transplant was reported. Survival analysis shows a better prognosis for *TPM1* p.Arg21Leu than expected for the overall HCM population (≅0.5%/year) (Maron BJ et al, 2018; Lorenzini M et al, 2019), and the sudden cardiac death risk scores were predominantly in the low risk range, followed by approximately 20% of the cases with intermediate scores. The identification of unaffected carriers at advanced ages, both in our pedigrees and in the general population, suggests incomplete penetrance.

In comparison, another *TPM1* variant, p.Asp175Asn, has been described in the literature as associated to mild-moderate hypertrophic cardiomyopathy phenotype and favourable prognosis; nonetheless, a higher penetrance in the adulthood (91-95%) was reported, based on two unrelated smaller cohorts (Coviello DA et al, 1997; Jääskeläinen P et al, 2004). Other variants in the *MYBPC3* and *MYH7* genes previously reported as founder effects in hypertrophic-populations have shown a worse prognosis than *TPM1* p.Arg21Leu (Kubo T et al, 2005; Olive-Sandoval et al, 2010; Teirlinck CH et al, 2012; Calore C et al, 2015; Ross SB et al, 2017; Sabater-Molina M et al, 2017). Mean age of diagnosis in some of these variants was also lower, approximately one decade less.

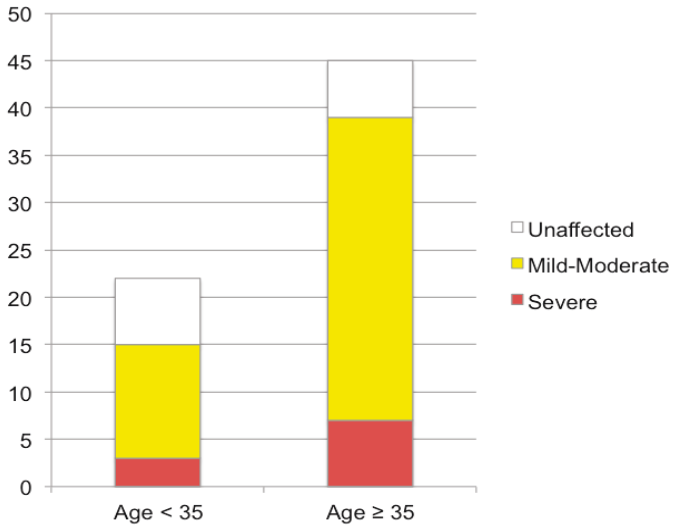


Figure 4.5: Severe phenotype criteria: *TPM1* p.Arg21Leu carriers that suffered a major CV event or those at high SCD risk.

4.2.5: Founder Variant

Index cases had been evaluated from 13 hospitals (four Portuguese and nine Spanish). The reported origin of the families was concentrated in the western part of the

Iberian Peninsula (the region of Braga in northern Portugal, as well as the Spanish regions of Galicia and Extremadura) (Figure 4.6). We have not identified the *TPM1* p.Arg21Leu variant in hospitals from other parts of the world. These territories share common historical and geopolitical factors dating back to the 11th century, or even earlier (Trillo-Santamaría JM and Paül V, 2014). Four index cases were identified in collaborating hospitals located outside of these zones, but families' origin was reported in the founder region. Furthermore, the identification of homozygous carriers with non-consanguineous progenitors would also reflect the greater prevalence of the variant in specific regions.

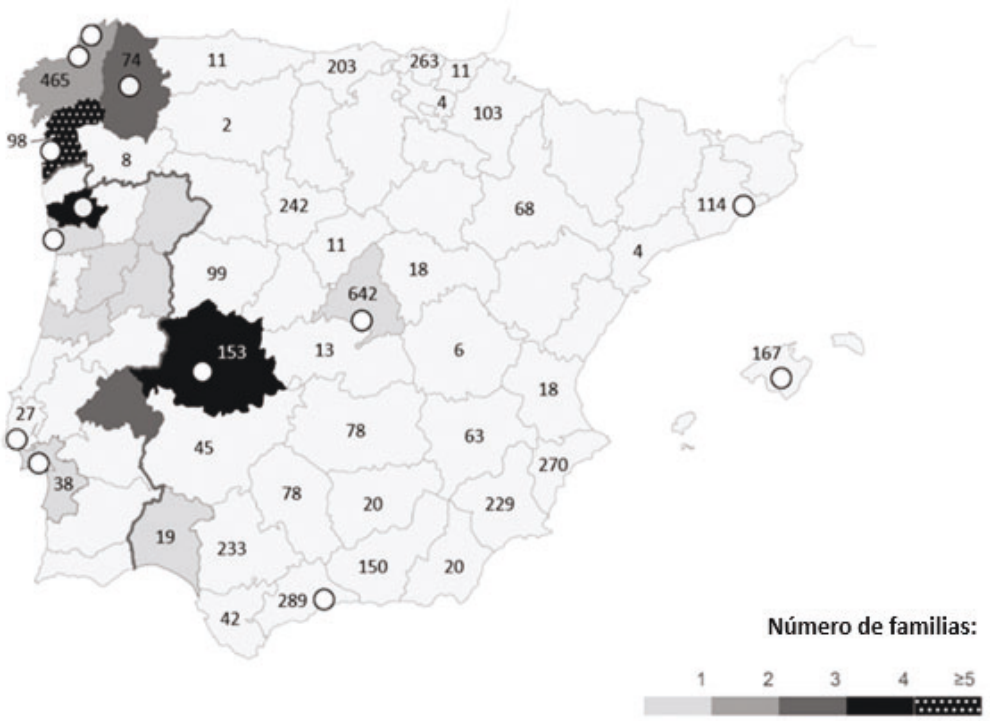


Figure 4.6: Geographic distribution (by origin) of the families carrying the variant *TPM1* p.Arg21Leu. Portugal and Spain maps divided by region. Concentration of families (origin) is represented in grey scale. White circles mean the reference hospitals where the index cases were identified. Numbers (*n*) represent the HCM-studies requested by each region.

A recent study had already described a founder variant for the *GLA* gene in the same northern Portuguese region (Guimarães, Braga), where we also identified a high number of families carrying the *TPM1* p.Arg21Leu variant (Azevedo et al, 2019). The authors demonstrated that it was related to cultural and socioeconomic particularities (i.e. high level of endogamy due the marriages occurring within the same social stratum) existing since the 17th until 19th century in this region. They considered that social factors and the late penetrance of the variant perpetuated the disease transmission in that region until the contemporary era. These characteristics could be hypothesized to explain the distribution observed for the *TPM1* p.Arg21Leu variant.

The presence of our variant exclusively in Latin individuals from gnomAD and TOPMed populations could reflect a possible common ancestrally from Spain and Portugal, once that these countries had a central role in Latin America's colonization. In comparison, another *TPM1* variant, p.Asp175Asn, described as a founder effect in Finland, is reported predominantly in Finnish European individuals in gnomAD database.

4.2.6: *TPM1* p.Arg21Leu as a pathogenic variant

Even though previously reported in ClinVar database as a variant of unknown clinical significance by 3 submitters, and likely pathogenic by another submitter, the availability of a large number of carriers with the same *TPM1* variant from a defined geographic area in Galicia/Extremadura/Northern Portugal has enabled genotype-phenotype evaluation which reveals the variant to be pathogenetic and associated with late onset disease, incomplete penetrance, and a generally favorable clinical course. Based on these findings, we now have sufficient criteria to describe *TPM1* p.Arg21Leu as pathogenic (see Table 4.7).

Table 4.7: Criteria for classifying TPM1 p.Arg21Leu pathogenicity.

Criteria*	Description	References **
(PS4) The prevalence of the variant in affected individuals is significantly increased compared with the prevalence in controls.	● *TPM1* p.Arg21Leu present in 23 / 4099 (0.56%) HCM-probands, including four homozygous carriers. It is not present in 6462 patients (controls) sequenced with other inherited cardiac disorders in the same period (p < 0,0001). ● The variant is listed in simple heterozygosity in 10/62784 (0.015%) individuals from TOPMed program, and 5/120,158 individuals (0.004%) (age range 55-65 years) from gnomAD database (non-TOPMed samples).	This study.
(PP1) Cosegregation with disease in multiple affected family members in a gene definitively known to cause the disease.	● Combined LOD score = 3.95	This study.
(PP2) Missense variant in a gene that has a low rate of benign missense variation and in which missense variants are common mechanisms of disease.	● *TPM1* gene Z = 2.87	gnomAD database, 13
(PP3) Multiple lines of computational evidence supporting a deleterious effect.	Polyphen-2, MutationTaster, FATHMM, DANN	*In silico* predictors
(PM5) Novel missense change at an amino acid residue where a different missense change determined to be pathogenic has been seen before.	● p.Arg21His was identified in a single hypertrophic cardiomyopathy index case. ● *In vitro* functional evidence supporting a damaging effect caused by p.Arg21His.	1, 2
(PM1) Located in a critical and well-established functional domain (N-terminal amino acids 1-25).	● *TPM1 v*ariants: p.Met8Arg, p.Gln9Lys/His/Leu, p.Lys15Asn/Glu/Arg, p.Glu16Gln, p.Asp20Asn, p.Ala22Thr, p.Glu23Gln, and p.Ala25Thr have been reported in multiple affected carriers (16 HCM and 8 DCM-patients) versus only p.Asn17Lys and p.Arg21Leu, p.Ala22Thr in the general population (gnomAD). [see figure S4, next page]. ● *In vitro* functional assays: 1) Disruption of the coiled coil structure in residues 15–22 N-terminal. Lys15, Ala18 and Ala22 and those of their coiled-coil mates were critical for stability of N:C-terminal junction. Salt bridges and H-bonds, including Asp-20, Arg-21, Gln-24, and Glu-26, reinforce the close interhelix packing. N-terminal region of tropomyosin is necessary for stable binding to actin filaments. 2) p.Met8Arg and p.Lys15Asn cause changes to various properties of *TPM1* molecules, disrupting the interaction with F-actin. *In vitro* data obtained point out that the N-terminal variants are more crucial for the head-to-tail interaction than those in the C-terminus. 3) 1-21 N-terminal residues of tropomyosin are involved in interactions with leiomodin protein (actin-binding capsular protein). p.Lys15Asn reduces binding affinity for both leiomodin and tropomodulin, which are responsible for correct lengths of thin filaments. 4) An intact coiled-coil at the N-terminus of the *TPM1* is essential for tropomodulin binding.	2-12

[*] ACMG Criteria for interpretation of sequenced variants. [**] See specific references for this section below. HCM: hypetrophic cardiomyopathy DCM: Dilated cardiomyopathy.

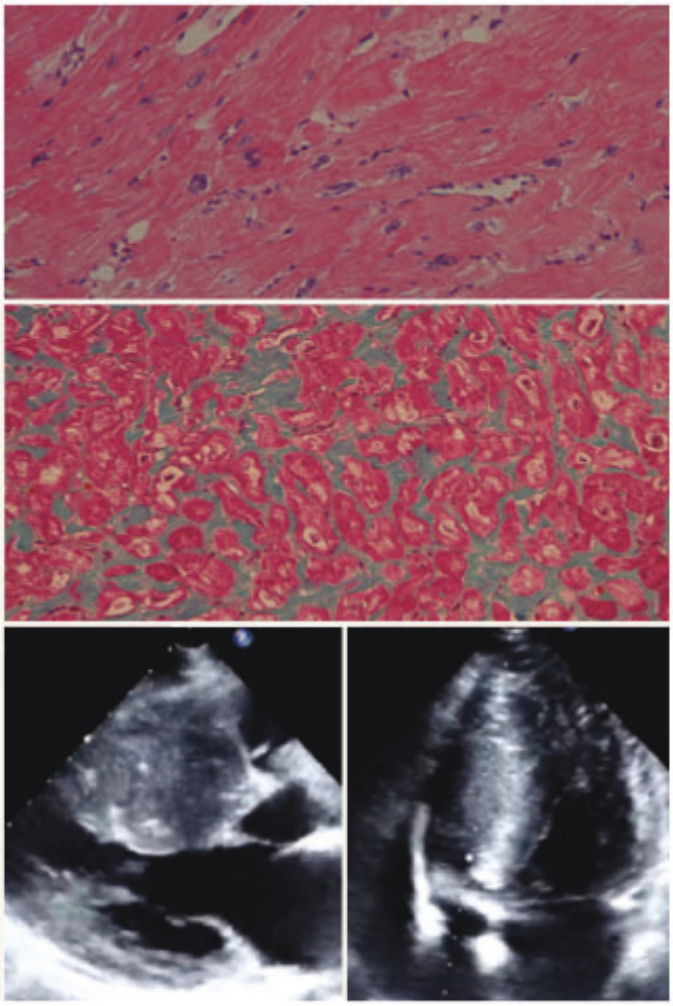


Fig. 4.7: Clinical features of TPM1 p.Arg21Leu carriers. **At the top:** Histopathology findings (myectomy sample) in a female simple heterozygous carrier at age 23. There is hypertrophy (fibres with a large diameter and large nuclei). The disarray is minimal and limited to the right and central region of the figure, next to a blood vessel. **Image at the middle:** Masson's Trichrome staining: collagen – green; myocardium – red. Fibres have a compressed aspect due to the widening of the interstice as a result of collagen fibrosis. **Image below:** Echocardiogram parasternal long axis and apical 4 chamber view; LVH = 51 mm in a male simple heterozygous carrier at age 32.

4.7: *TPM1* p.Arg21Leu Pedigrees

Twenty-eight pedigrees were constructed (familial data was not reported in three probands), and familial cosegregation of the variant was documented with an autosomal dominant inheritance pattern (combined LOD score = 3.95). Specific LOD scores values suggesting familial cosegregation were described in each pedigree. All pedigrees, individual clinical features, and sudden cardiac death risk scores are detailed in the following pages.

Legends:

Narrow indicates the **P**roband or index-case

Normal male individual / female - clinically evaluated.

Male individual is square / female is circle

Deceased male individual / female individual

HCM affected male carrier / female carrier

*** Obligate carrier** is described below of the individual.

HCM affected male carrier only with minor ECG changes / female carrier

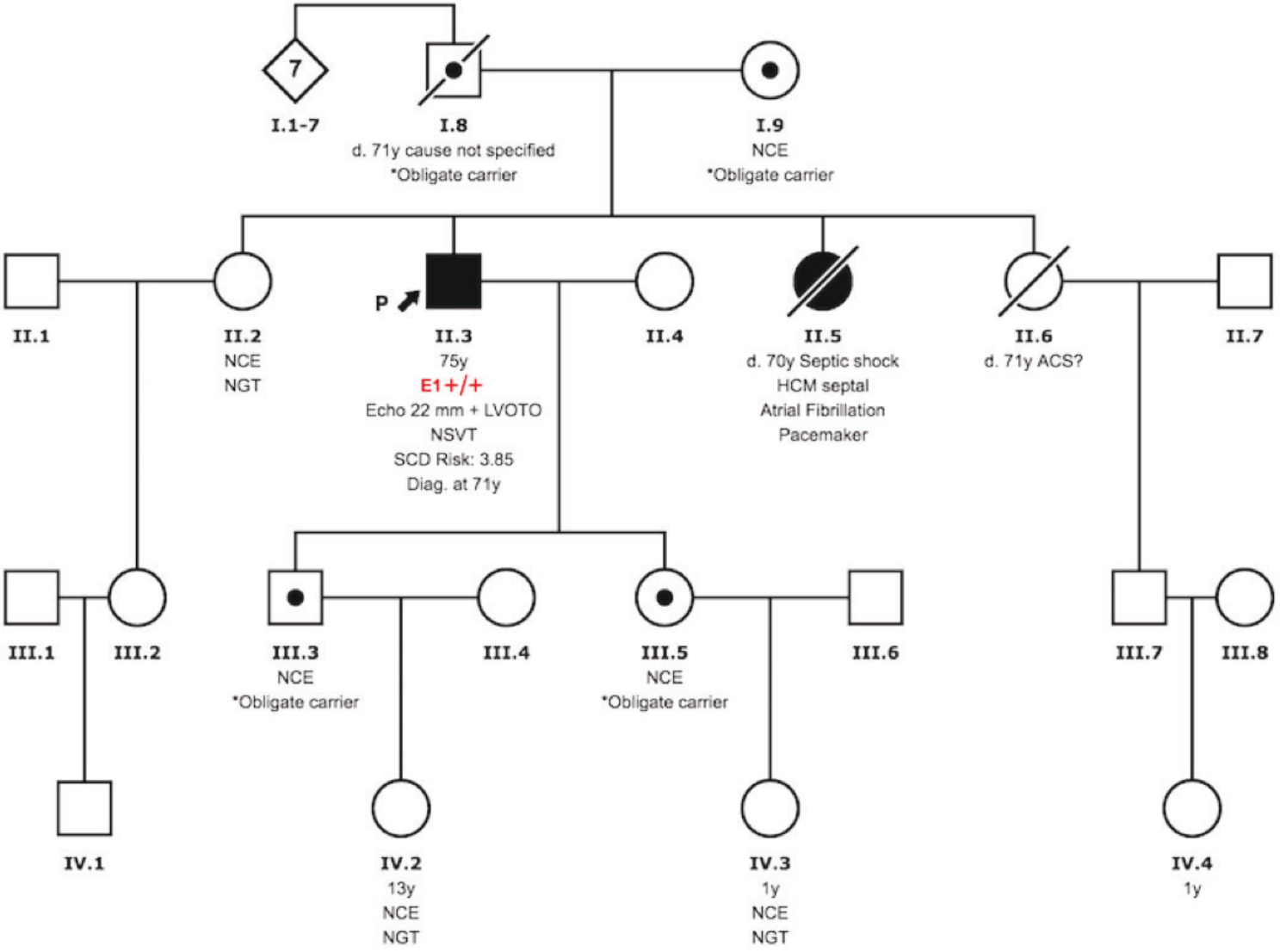
NCE No clinical evaluation
NGT No genetic testing

Unaffected male carrier / female carrier

Presence of a second genetic disorder in a male / female individual

Male individual with strong suspicious of HCM / female individual or clinical data not reported

Pedigree #1

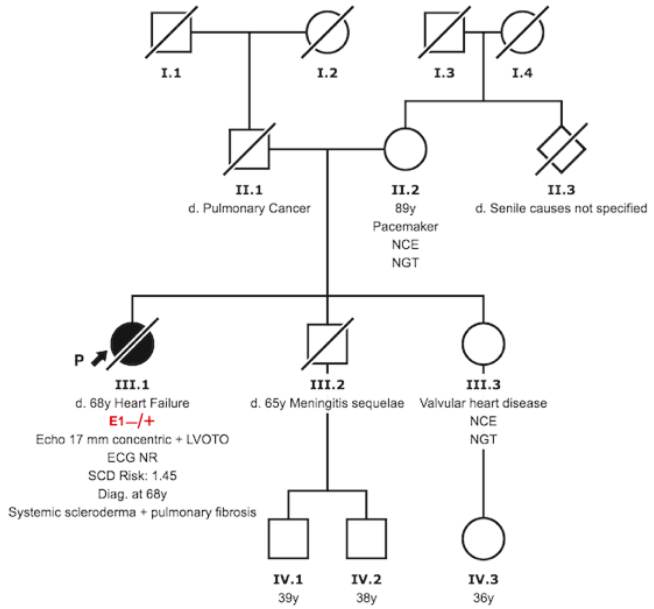


E1 TPM1 (g.63335090G>T, c.62G>T, p.Arg21Leu)

"+/+" = Homozygous, "-/+" = Heterozygous, "+" = Hemizygous, "-/-" = Not found, "-" = Not found

	Id	Sex	Kinship	TPM1 Arg21Leu	Other mutat.	Phenotype	Age of Dx	Age	NYHA	AF	FHSD	Max LVH	TV/FV	Syncope	Abn. Vasc Resp	LVOTO (Peak grad.)	LV disf. (EF)	Events
#1	**II.3**	M	Index case	Yes (Homoz.)	No	HCM septal	71	75	I	-	-	22	NSTV	-	-	+ (zero in rest; 120 exercise)	-	
	III.5	F	Daughter	Obligate carrier		NCE												
	III.3	M	Son	Obligate carrier		NCE												
	I.8	M	Father	Obligate carrier		NCE												
	I.9	F	Mother	Obligate carrier		NCE												
	II.5	F	Sister	NGT		HCM Septal	?	70	?	+	-	?	?	?	?		?	

Pedigree #2

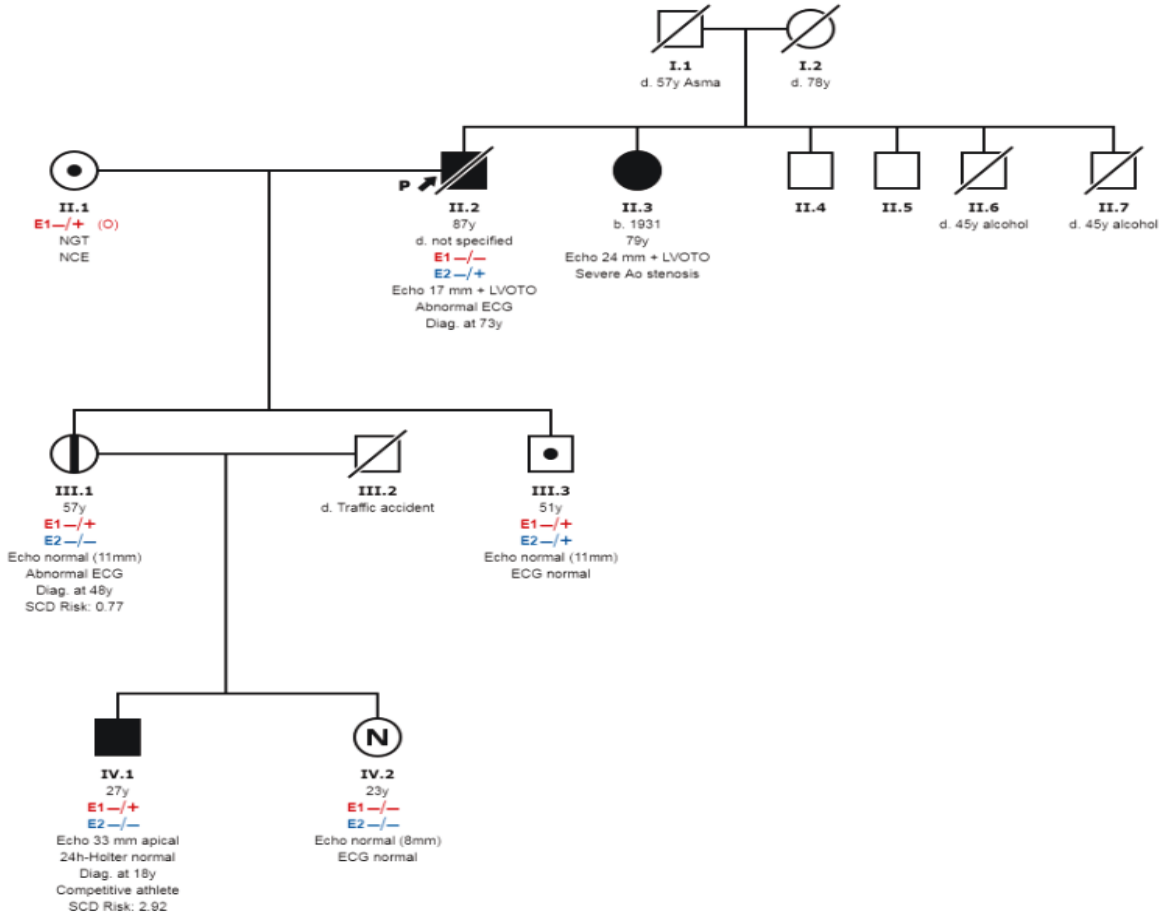


E1 TPM1 (g.63335090G>T, c.62G>T, p.Arg21Leu)

"+/+" = Homozygous, "-/+" = Heterozygous, "+" = Hemizygous, "-/-" = Not found, "-" = Not found

	Id	Sex	Kinship	TPM1 Arg21Leu	Other mutat.	Phenotype	Age of Dx	Age	NYHA	AF	FHSD	Max LVH	TV/FV	Syncope	Abn. Vasc Resp	LVOTO (peak grad.)	LV Disf. (EF)	Events
#2	**III.1**	F	Index case	Yes	No	HCM concentric	68	68	IV	-	-	17	-	-	-	+ (53)	-(75)	Heart failure death 68y

Pedigree #3



LOD SCORE 0.16

E1 TPM1 (g.63335090G>T, c.62G>T, p.Arg21Leu)

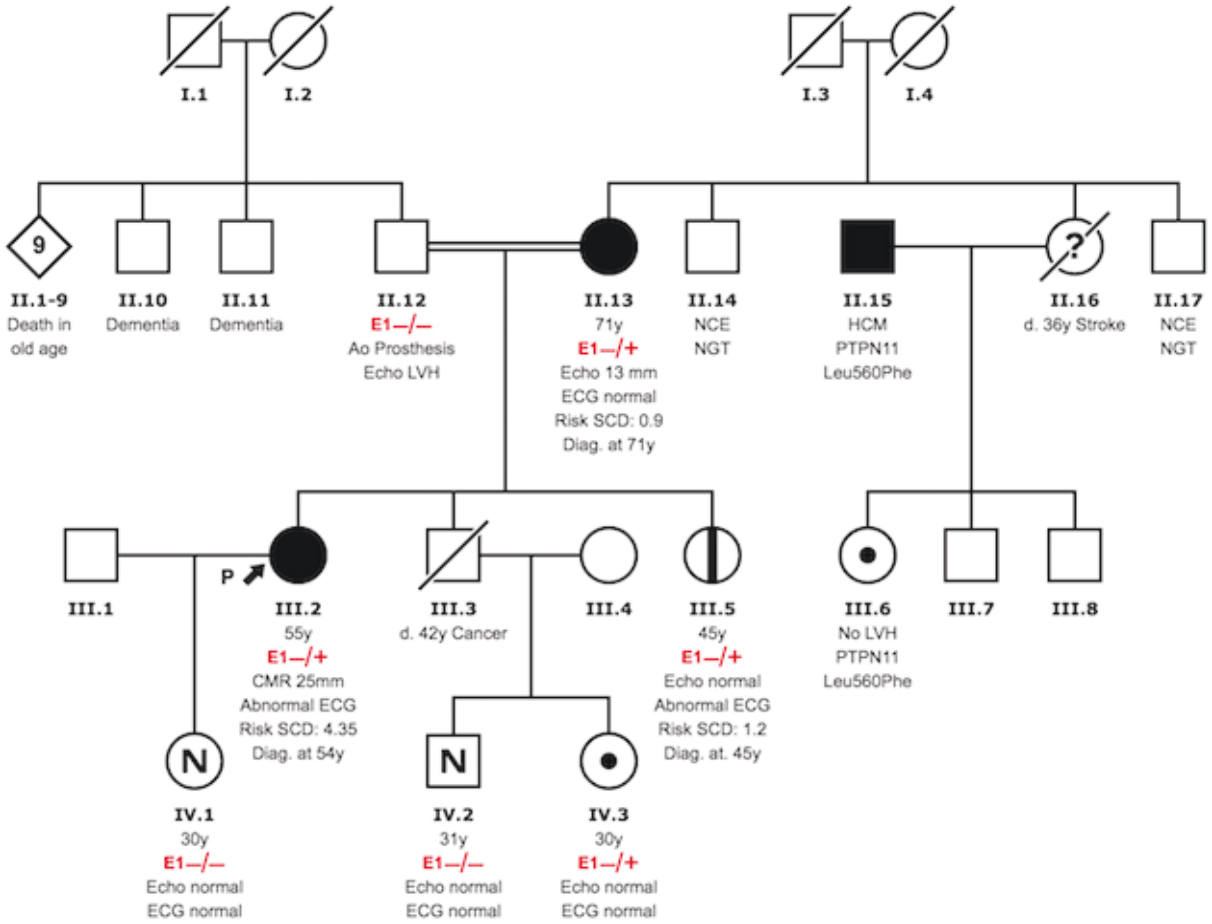
"+/+" = Homozygous, "-/+" = Heterozygous, "+" = Hemizygous, "-/-" = Not found, "-" = Not found

E2 MYH7 (g.23892799G>T, c.3056C>A, p.Thr1019Asn)

"+/+" = Homozygous, "-/+" = Heterozygous, "+" = Hemizygous, "-/-" = Not found, "-" = Not found

	Id	Sex	Kinship	TPM1 Arg21Leu	Other mutat.	Phenotype	Age of Dx	Age	NY HA	A F	FH SD	Max LVH	TV/ FV	Sync ope	Abn. Vasc Resp	LVOTO (Peak grad.)	LV disf. (EF)	Events
#3	**II.2**	M	Index case	**No**	MYH7 Thr1019Asn (+?)	HCM septal	73	83	II	+	-	16	-	-	-	+ (30-55 in rest, >250 Valsalva).	-(>55)	
	III.1	F	Daughter	Yes	No	Not affected (?)		57	I	-	-	11	-	-	-	-	-(83)	
	III.3	M	Son	Yes	MYH7 Thr1019Asn (+?)	Not affected		51	I	-	-	11	-	-	-	-	-(61)	
	IV.1	M	Grandson	Yes	No	HCM atypical	18	27	I	-	-	33	-	-	-	-	-(67)	
	II.1	F		Obligate carrier	?	NCE												

Pedigree #4



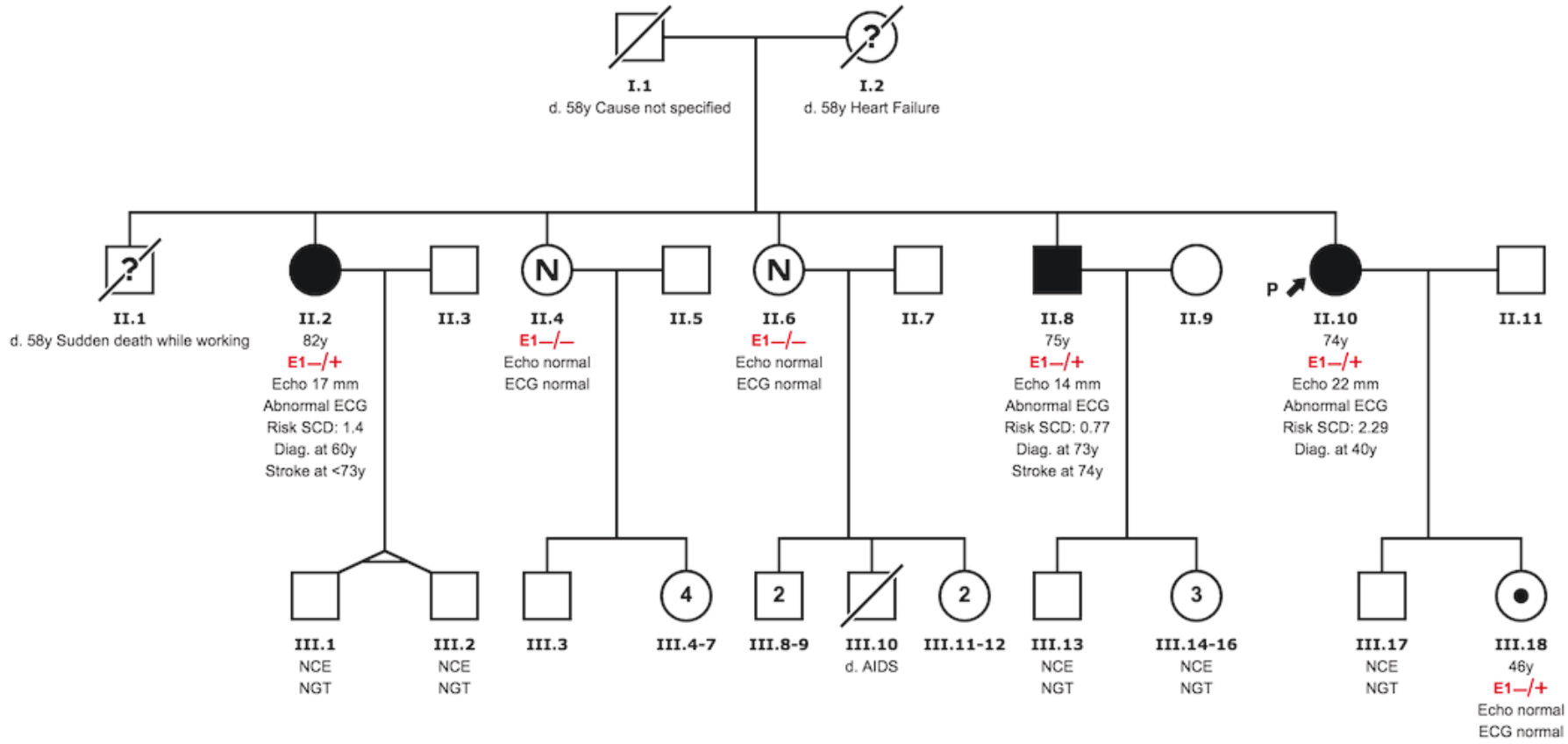
LOD SCORE : 0.68

E1 TPM1 (g.63335090G>T, c.62G>T, p.Arg21Leu)

"+/+" = Homozygous, "-/+" = Heterozygous, "+" = Hemizygous, "-/-" = Not found, "-" = Not found

	Id	Sex	Kinship	TPM1 Arg21Leu	Other mutat.	Phenotype	Age of Dx	Age	NY HA	AF	FHS D	Max LVH	TV/ FV	Synco pe	Abn. Vasc Resp	LVOTO (Peak grad.)	LV disf. (EF)	Events
#4	**III.2**	F	Index case	Yes	No	HCM septal	54	58	II	+	-	25	-	+	-	-	-(60)	
	III.5	F	Sister	Yes		Not affected (?)	45	45	I	-	-	13	-	-	-	-	-(>55)	
	II.13	F	Mother	Yes		HCM	71	71	I	-	-	13	-	-	-	-	-(>55)	
	IV.3	F	Niece	Yes		Not affected		30	I	-	-	-	-	-	-	-	-	
	II.16	F	Aunt	NGT		?		36										Stroke-related death 36-y

Pedigree #5



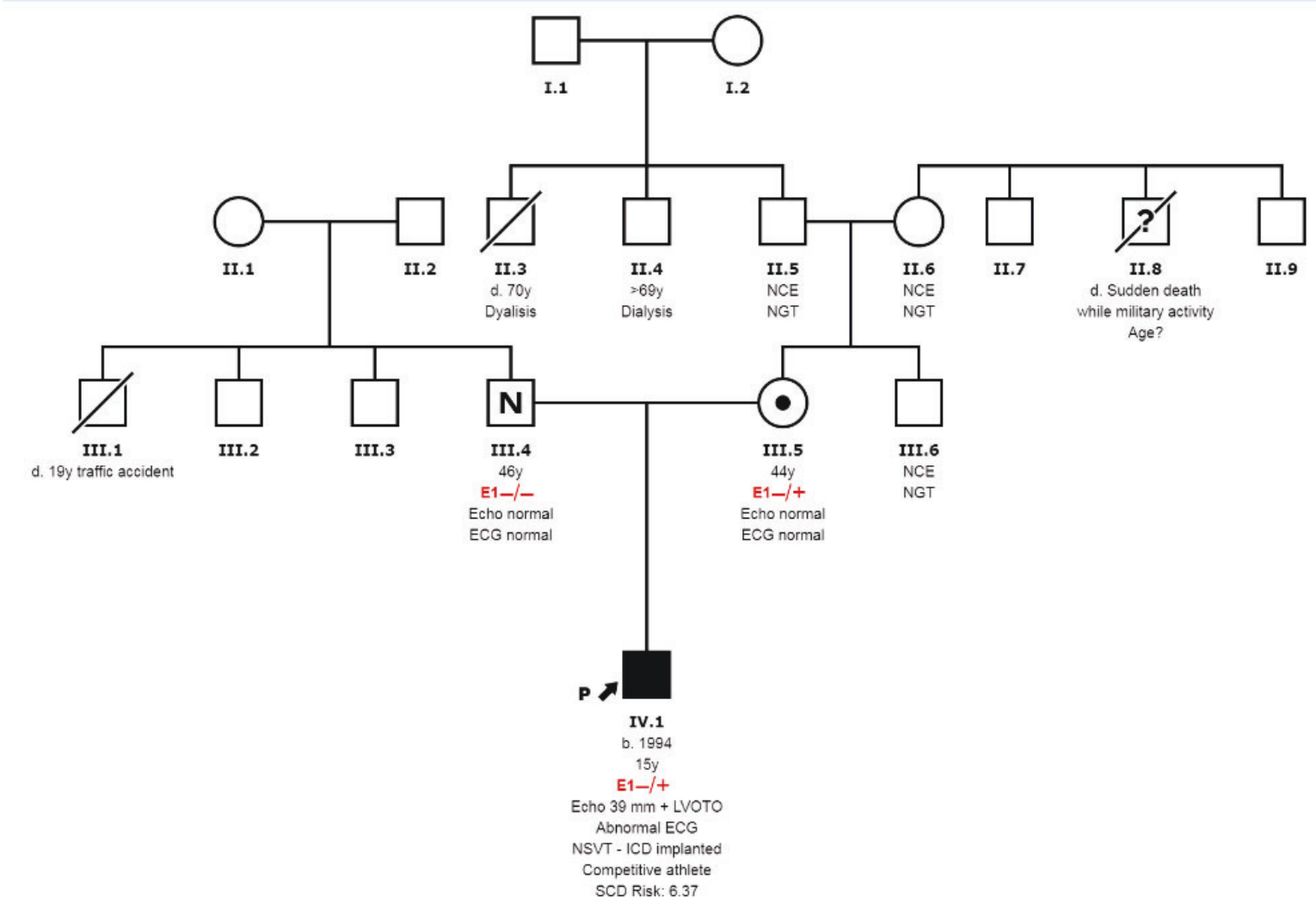
E1 TPM1 (g.63335090G>T, c.62G>T, p.Arg21Leu)

"+/+" = Homozygous, "-/+" = Heterozygous, "+" = Hemizygous, "-/-" = Not found, "-" = Not found

LOD SCORE: 0.17

	Id	Sex	Kinship	TPM1 Arg21Leu	Other mutat	Phenotype	Age of Dx	Age	NY HA	AF	FHS D	Max LVH	TV/ FV	Syncope	Abn. Vasc Resp	LVOTO (Peak grad.)	LV disf. (EF)	Events
#5	**II.10**	F	Index case	Yes	No	HCM septal	40	74	II	+	+	22	-	-	-	+ (50)	-(78)	
	II.2	F	Sister	Yes		HCM septal	60	82	I	-	+	17	-	-	-	-	-(70)	Stroke at <73y
	II.8	M	Brother	Yes		HCM septal	73	75	I	-	+	14	-	-	-	-	-(79)	Stroke at 74y
	I.2	F	Mother	NGT		?		58									+	Heart failure death 58y
	II.1	M	Brother	NGT		?		58										Sudden death 58-y while working.
	III.18	F	Daughter	Yes		Not affected		46										

Pedigree #6

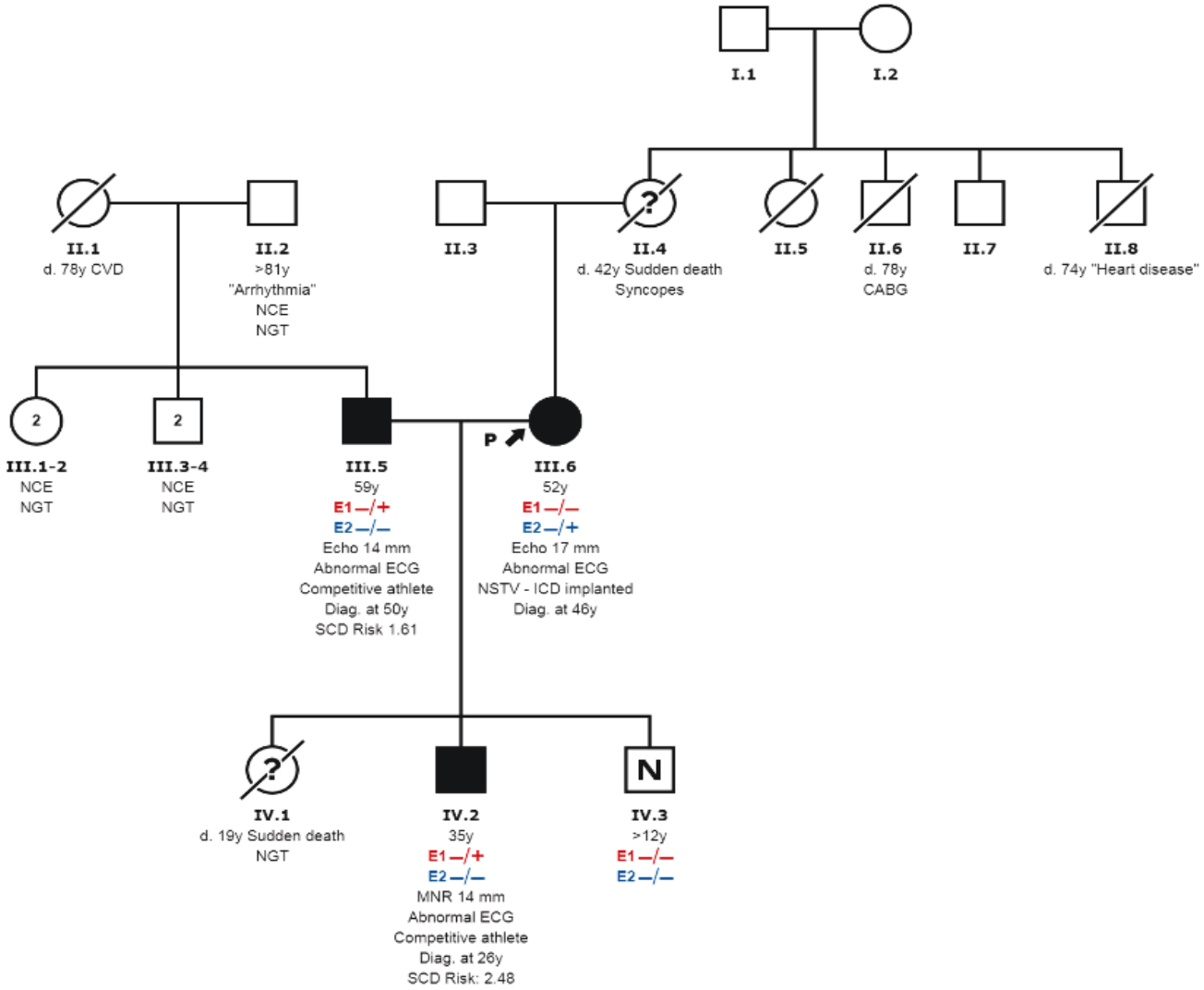


E1 TPM1 (g.63335090G>T, c.62G>T, p.Arg21Leu)

"+/+" = Homozygous, "-/+" = Heterozygous, "+" = Hemizygous, "-/-" = Not found, "-" = Not found

	Id	Sex	Kinship	TPM1 Arg21Leu	Other mutat.	Phenotype	Age of Dx	Age	NYHA	AF	FHSD	Max LVH	TV/FV	Syncope	Abn. Vasc Resp	LVOTO (Peak grad.)	LV disf. (EF)	Events
#6	**IV.1**	M	Index case	Yes		HCM septal	15	22	II	-	+	39	+	-	-	+ (35 exerc.)	-(78)	
	III.5	F	Mother	Yes	No	Not affected		44	-	-	+	-	-	-		-	-	
	III.4	M	Father	No		Not affected		46	-	-	-	-	-	-		-	-	
	II.8	M		?		?		21										Sudden death - age 21y

Pedigree #7



LOD SCORE: 0.17

E1 TPM1 (g.63335090G>T, c.62G>T, p.Arg21Leu)

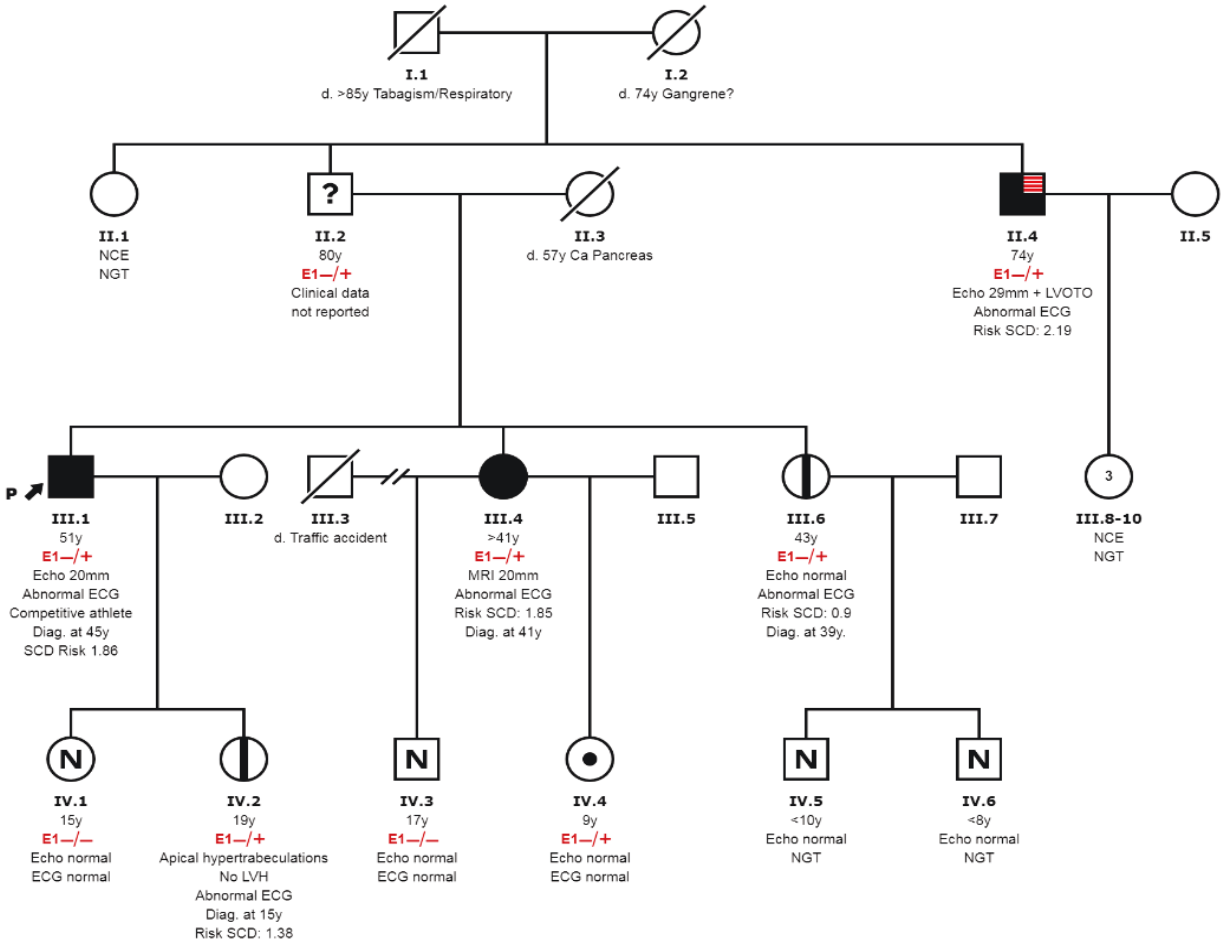
"+/+" = Homozygous, "-/+" = Heterozygous, "+" = Hemizygous, "-/-" = Not found, "-" = Not found

E2 MYH7 (g.23894969C>T, c.2221G>A, p.Gly741Arg)

"+/+" = Homozygous, "-/+" = Heterozygous, "+" = Hemizygous, "-/-" = Not found, "-" = Not found

	Id	Sex	Kinship	TPM1 Arg21Leu	Other mutat.	Phenotype	Age of Dx	Age	NYHA	AF	FHSD	Max LVH	TV/FV	Syncope	Abn. Vasc Resp	LVOTO (Peak grad.)	LV disf. (EF)	Events
#7	**III.6**	F	Index-case	**No**	MYH7 Gly741Arg (+++)	HCM septal	46	52	I	-	+	17	+	-	-	-	-(61)	
	III.5	M	Husband	Yes		HCM septal	50	59	II	-	-	14	-	-	-	-	-(58)	
	IV.2	M	Son	Yes	No	HCM septal	26	35	I	-	+	14	-	-	-	-	-(75)	
	IV.1	F	Daughter	NGT		SCD		19			+		+					Sudden death - 19y

Pedigree #8



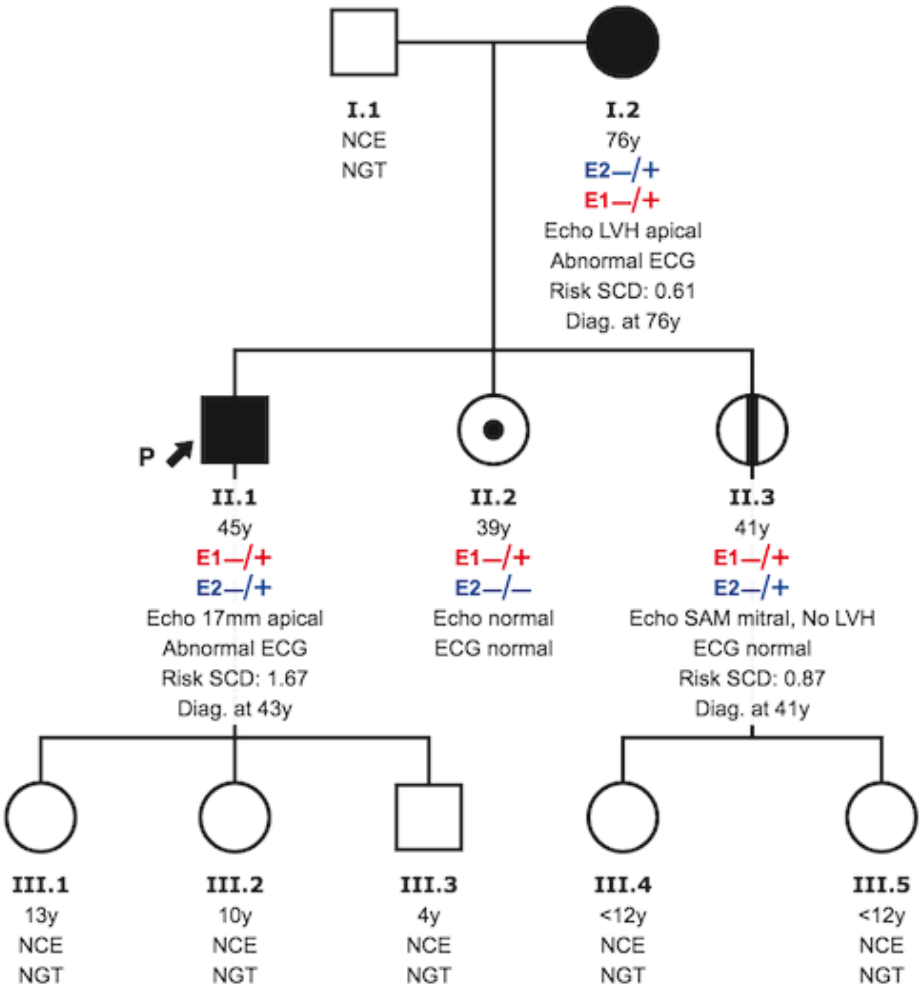
LOD SCORE: 1.12

E1 TPM1 (g.63335090G>T, c.62G>T, p.Arg21Leu)

"+/+" = Homozygous, "-/+" = Heterozygous, "+" = Hemizygous, "-/-" = Not found, "-" = Not found

	Id	Sex	Kinship	TPM1 Arg21Leu	Other mutat.	Phenotype	Age of Dx	Age	NYHA	AF	FHSD	Max LVH	TV/FV	Syncope	Abn. Vasc Resp	LVOTO (Peak grad.)	LV disf (EF)	Events
#8	**III.1**	M	Index case	Yes	No	HCM septal	45	51	I	-	-	20	-	-	-	-	-(78)	
	III.4	F	Sister	Yes		HCM septal	41	>41	I	-	-	20	-	-	-	-	-(78)	
	III.6	F	Sister	Yes		Not affected (?)		43	I	-	-	-	-	-	-	-	-	
	II.4	M	Uncle	Yes		HCM septal	48	74	I	+	-	29	-	-	-	+(60)	-(55)	
	IV.2	F	Daughter	Yes		Not affected (?)		19	I	-	-	-	-	-	-	-	-(62)	
	IV.4	F	Niece	Yes		Not affected		9	-	-	-	7	-	-	-	-	-	
	II.2	M	Father	Yes		?												

Pedigree #9



LOD SCORE: 0.34

E1 TPM1 (g.63335090G>T, c.62G>T, p.Arg21Leu)

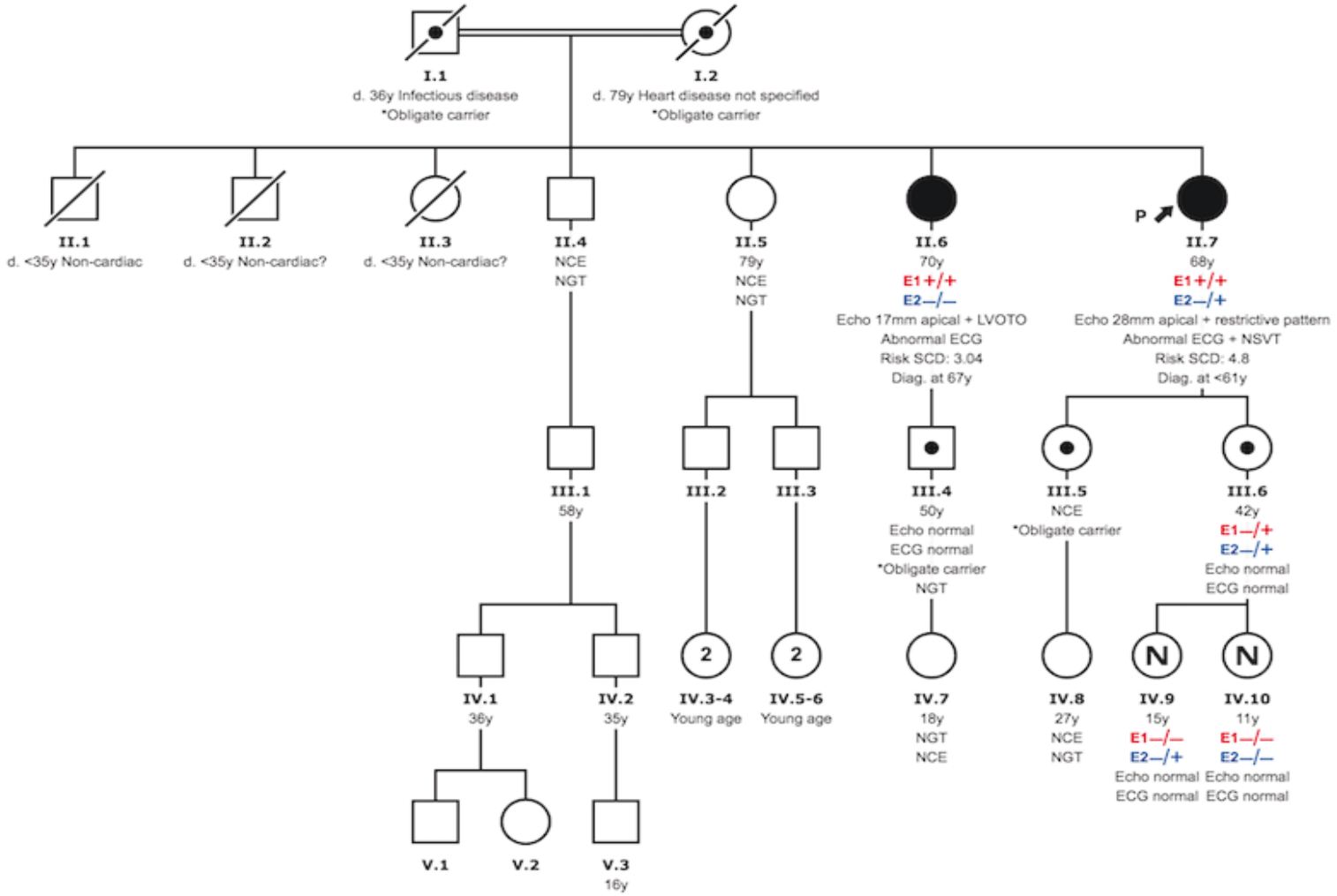
"+/+" = Homozygous, "-/+" = Heterozygous, "+" = Hemizygous, "-/-" = Not found, "-" = Not found

E2 TNNT2 (g.201328373G>A, c.832C>T, p.Arg278Cys)

"+/+" = Homozygous, "-/+" = Heterozygous, "+" = Hemizygous, "-/-" = Not found, "-" = Not found

	Id	Sex	Kinship	TPM1 Arg21Leu	Other mutat.	Phenotype	Age of Dx	Age	NY HA	AF	FH SD	Max LVH	TV/ FV	Syncope	Abn. Vasc Resp	LVOTO (Peak grad.)	LV disf. (EF)	Events
#9	**II.1**	M	Index case	Yes	TNNT2 Arg278Cys (+++)	HCM Apical	43	45	I	-	-	17	-	-	-	-	-(62)	
	II.2	F	Sister	Yes	No	Not affected		39	-	-	-	-	-	-	-	-	-	
	II.3	F	Sister	Yes	TNNT2 Arg278Cys (+++)	Not affected (?)		41	I	-	-	-	-	-	-	-	-	
	I.2	F	Mother	Yes	TNNT2 Arg278Cys (+++)	HCM apical	76	76	I	-	-	+	-	-	-	-	-	

Pedigree #10



E1 TPM1 (g.63335090G>T, c.62G>T, p.Arg21Leu)

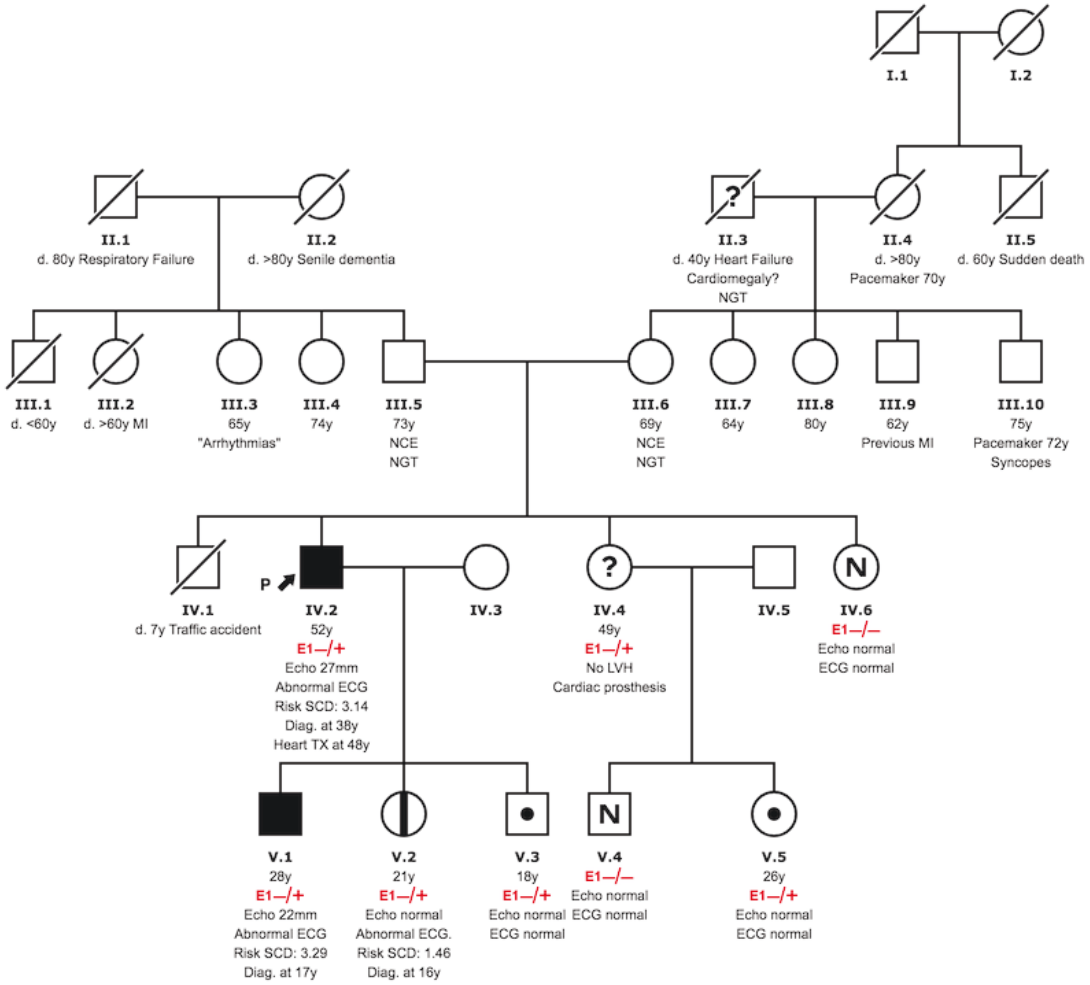
"+/+" = Homozygous, "-/+" = Heterozygous, "+" = Hemizygous, "-/-" = Not found, "-" = Not found

E2 MYBPC3 (g.47372859C>T, c.223G>A, p.Asp75Asn)

"+/+" = Homozygous, "-/+" = Heterozygous, "+" = Hemizygous, "-/-" = Not found, "-" = Not found

	Id	Sex	Kinship	TPM1 Arg21Leu	Other mutat.	Phenotype	Age of Dx	Age	NYHA	AF	FHSD	Max LVH	TV/FV	Syncope	Abn. Vasc Resp	LVOTO (Peak grad.)	LV disf. (EF)	Events
#10	**II.7**	F	Index case	Yes (Homoz.)	MYBPC3 Asp75Asn (+?)	HCM Apical	<61	68	III	-	-	28	+	-	-	-	-(63)	
	II.6	F	Sister	Yes (Homoz.)	No	HCM Apical	67	70	II	-	-	17	-	-	-	+ (118 in rest)	-(64)	
	III.6	F	Daughter	Yes	MYBPC3 Asp75Asn (+?)	Not affected		42	I	-	-	-	-	-	-	-	-	
	I.2	F	Mother	Obligate carrier	?	Cardiac disease not specified		79										Death at 79y – heart disease.
	I.1	M	Father	Obligate carrier	?	NCE		36										
	III.4	M	Nephew	Obligate carrier		Not affected		50	I	-	-	-	-	-	-	-	-	
	III.5	F	Daughter	Obligate carrier		NCE												

Pedigree #11



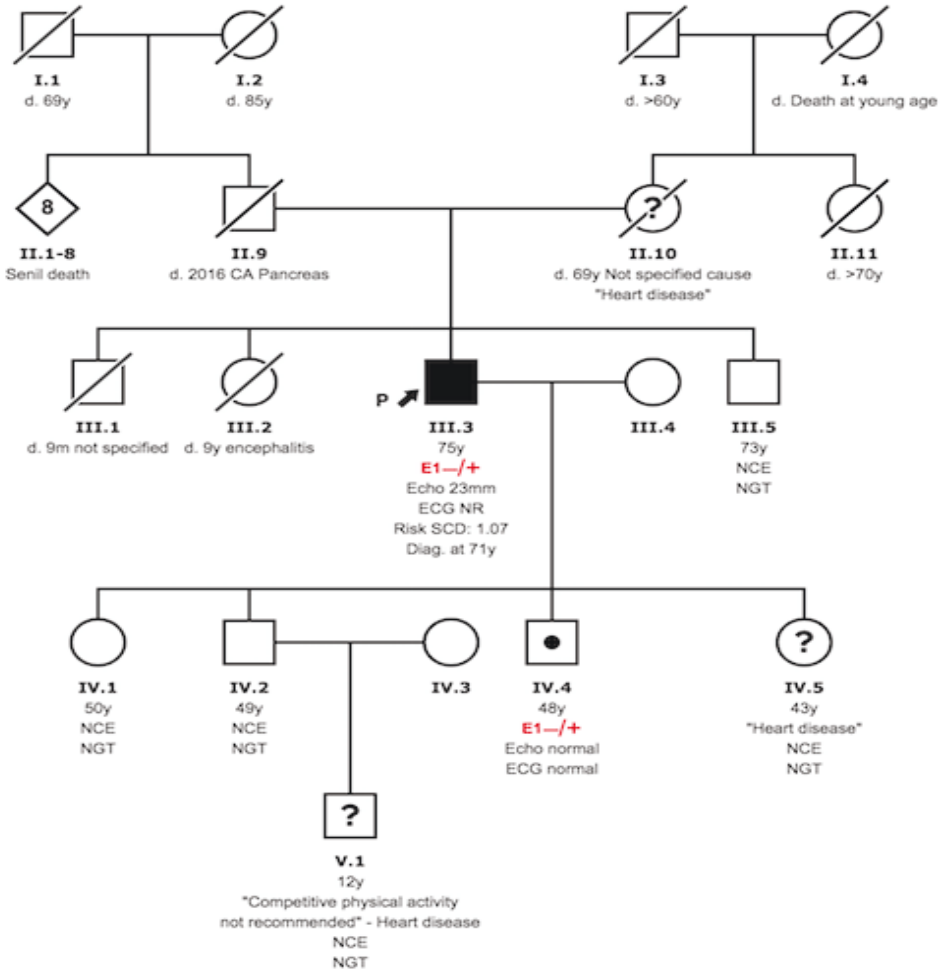
LOD SCORE : 0.17

E1 TPM1 (g.63335090G>T, c.62G>T, p.Arg21Leu)

"+/+" = Homozygous, "-/+" = Heterozygous, "+" = Hemizygous, "-/-" = Not found, "-" = Not found

	Id	Sex	Kinship	TPM1 Arg21Leu	Other mutat.	Phenotype	Age of Dx	Age	NYHA	AF	FH SD	Max LVH	TV/ FV	Syncope	Abn. Vasc Resp	LVOTO (Peak grad.)	LV disf. (EF)	Events
#11	**IV.2**	M	Index case	Yes	No	HCM septal	38	52	III-IV	+	-	27	-	-	-	-	-(65)	Heart transplant at 48y
	IV.4	F	Sister	Yes		?		49										
	V.1	M	Son	Yes		HCM septal	17	28	I	-	-	22	-	-	-	-	-(72)	
	V.2	F	Daughter	Yes		Not affected (?)		21	I	-	-	11	-	-	-	-	-	
	V.3	M	Son	Yes		Not affected		18										
	V.5	F	Niece	Yes		Not affected		26										
	II.3	M	Grandfather	NGT		?		40										Heart failure death - 40y

Pedigree #12

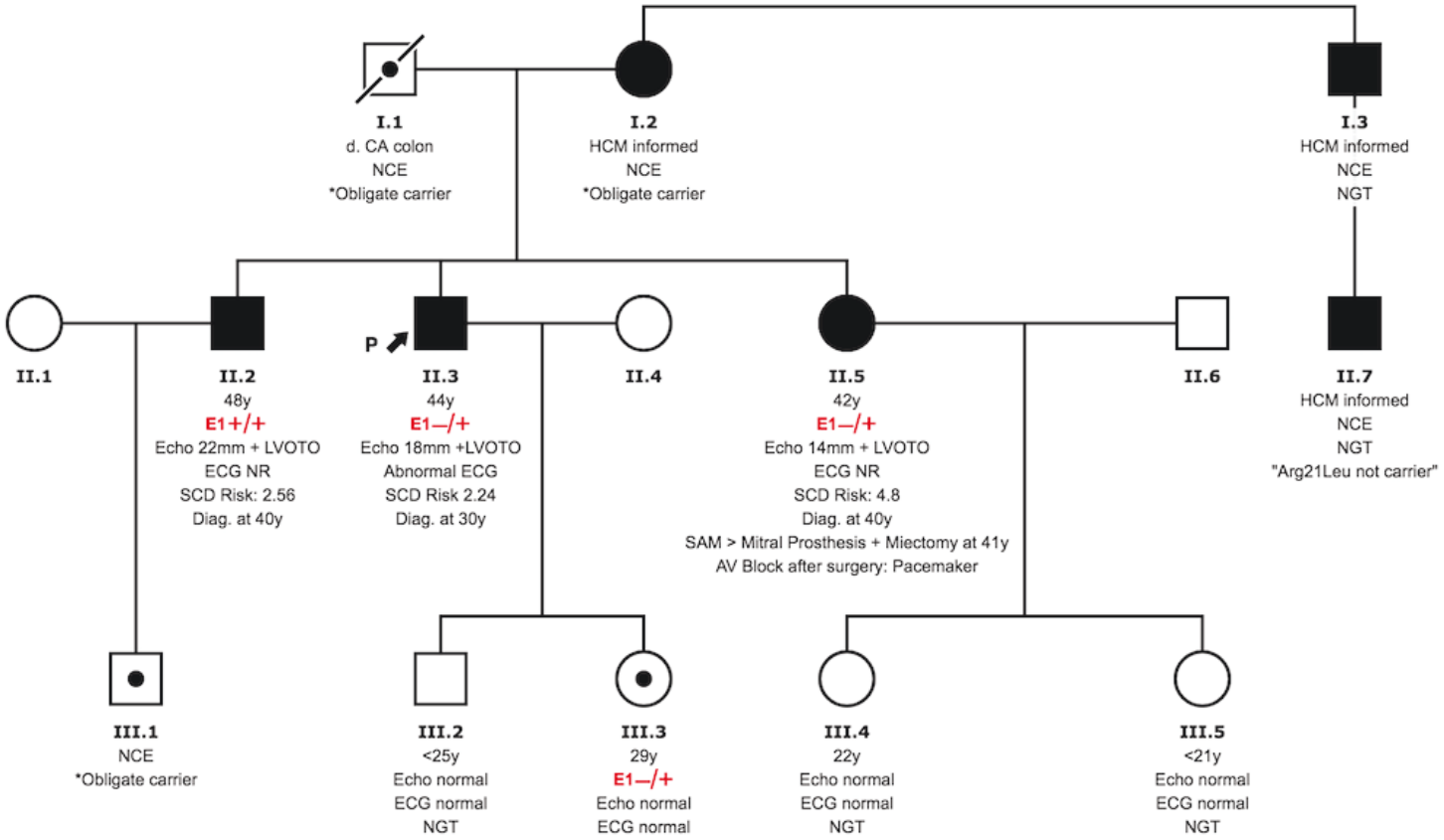


E1 TPM1 (g.63335090G>T, c.62G>T, p.Arg21Leu)

"+/+" = Homozygous, "-/+" = Heterozygous, "+" = Hemizygous, "-/-" = Not found, "-" = Not found

	Id	Sex	Kinship	TPM1 Arg21Leu	Other mutat.	Phenotype	Age of Dx	Age	NYHA	AF	FHSD	Max LVH	TV/FV	Syncope	Abn. Vasc Resp	LVOTO (Peak grad.)	LV disf. (EF)	Events
#12	**III.1**	M	Index case	Yes	No	HCM septal	71	75	I	-	-	23	-	-	+	-	-(67)	
	IV.4	M	Son	Yes		Not affected		48										
	II.10	F	Mother	NGT		Heart disease not specified		69										
	IV.5	F	Daughter	NGT		Heart disease not specified		43										
	V.1	M	Grandson	NGT		Heart disease not specified		12										

Pedigree #13



LOD SCORE 0.34

E1 TPM1 (g.63335090G>T, c.62G>T, p.Arg21Leu)

"+/+" = Homozygous, "-/+" = Heterozygous, "+" = Hemizygous, "-/-" = Not found, "-" = Not found

	Id	Sex	Kinship	TPM1 Arg21Leu	Other mutat.	Phenotype	Age of Dx	Age	NYHA	AF	FHSD	Max LVH	TV/FV	Syncope	Abn. Vasc Resp	LVOTO (Peak grad.)	LV disf. (EF)	Events
#13	**II.3**	M	Index case	Yes	No	HCM septal	30	44	I	-	-	18	-	-	+	+(60)	-(68)	
	II.2	M	Brother	Yes (Homozy.)		HCM septal	38	48	I	-	-	22	-	-	-	+(40)	-(70)	
	II.5	M	Sister	Yes		HCM septal	40	42	III	+	-	14	-	+	-	+(75)	-(68)	
	III.3	F	Daughter	Yes		Not affected		29										
	I.1	M	Father	Obligate carrier		NCE												
	I.2	F	Mother	Obligate carrier		HCM informed – NCE		71										
	III.1	M	Nephew	Obligate carrier		NCE												

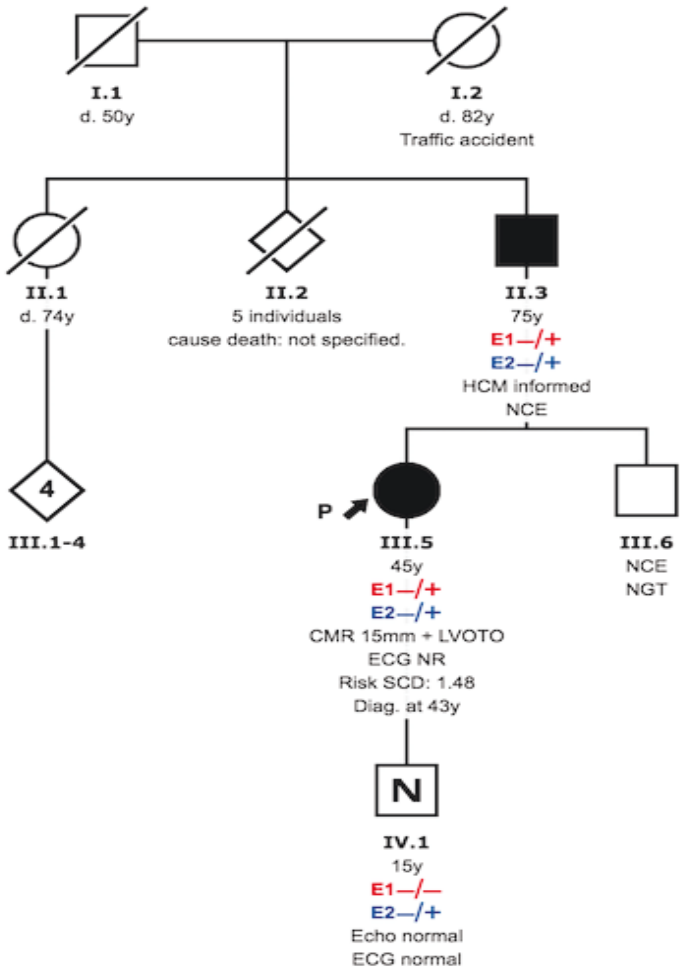
Pedigree #14

	Id	Sex	Kinship	TPM1 Arg21Leu	Other mutat.	Phenotype	Age of Dx	Age	NYHA	AF	FH SD	Max LVH	TV/ FV VE	Syncope	Abn. Vasc Resp	LVOTO (Peak grad.)	LV disf. (EF)	Events
#14		M	Index case	Yes	No	HCM apical	69	72	I	-	-	14	-	-	-	-	-(67)	

SCD Risk: 1.04

No pedigree was reported.

Pedigree #15



LOD SCORE 0.23

E1 TPM1 (g.63335090G>T, c.62G>T, p.Arg21Leu)

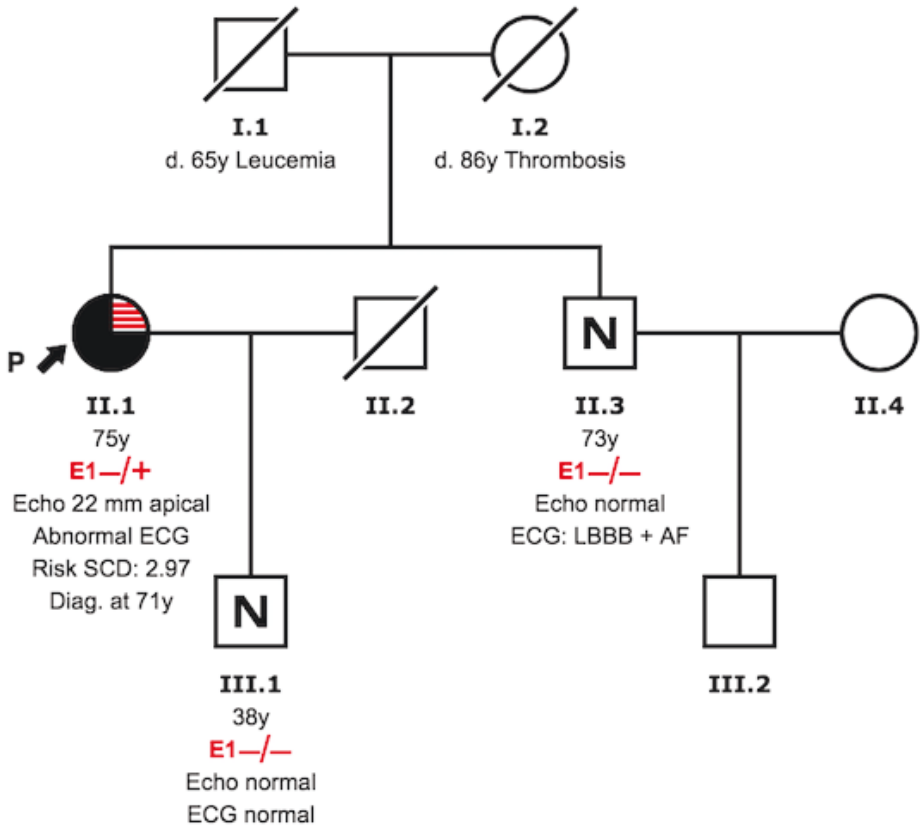
"+/+" = Homozygous, "-/+" = Heterozygous, "+" = Hemizygous, "-/-" = Not found, "-" = Not found

E2 MYL3 (g.46899916T>C, c.517A>G, p.Met173Val)

"+/+" = Homozygous, "-/+" = Heterozygous, "+" = Hemizygous, "-/-" = Not found, "-" = Not found

	Id	Sex	Kinship	TPM1 Arg21Leu	Other mutat.	Phenotype	Age of Dx	Age	NYHA	AF	FH SD	Max LVH	TV/ FV	Syncope	Abn. Vasc Resp	LVOTO (Peak grad.)	LV disf. (EF)	Events
#15	**II.1**	F	Index case	Yes	MYL3 Met173Val (+?)	HCM septal	43	45	II	-	-	15	-	-	-	+(>30)	-(66)	
	II.3	M	Father	Yes	MYL3 Met173Val (+?)	HCM informed		75				+						

Pedigree #16

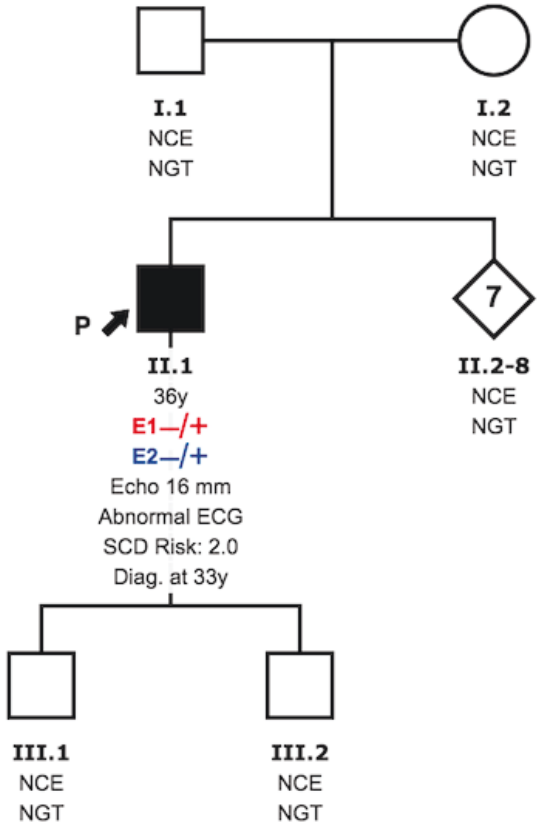


E1 TPM1 (g.63335090G>T, c.62G>T, p.Arg21Leu)

"+/+" = Homozygous, "-/+" = Heterozygous, "+" = Hemizygous, "-/-" = Not found, "-" = Not found

	Id.	Sex	Kinship	TPM1 Arg21Leu	Other mutat.	Phenotype	Age of Dx	Age	NYHA	AF	FHSD	Max LVH	TV/ FV VE	Syncope	Abn. Vasc Resp	LVOTO (Peak grad.)	LV disf. (EF)	Events
#16	**II.1**	F	Index case	Yes	No	HCM apical	71	75	II	+	-	22	-	+	-	-	-(82)	

Pedigree #17



E1 TPM1 (g.63335090G>T, c.62G>T, p.Arg21Leu)

"+/+" = Homozygous, "-/+" = Heterozygous, "+" = Hemizygous, "-/-" = Not found, "-" = Not found

E2 MYH7 (g.23899069C>A, c.1053G>T, p.Lys351Asn)

"+/+" = Homozygous, "-/+" = Heterozygous, "+" = Hemizygous, "-/-" = Not found, "-" = Not found

	Id	Sex	Kinship	TPM1 Arg21Leu	Other mutat.	Phenotype	Age of Dx	Age	NYHA	AF	FHSD	Max LVH	TV/FV	Syncope	Abn. Vasc Resp	LVOTO (Peak grad.)	LV disf. (EF)	Events
#17	**II.1**	M	Index case	Yes	MYH7 Lys351Asn (?)	HCM septal	33	36	I	-	-	16	-	-	-	-(17)	-(84)	

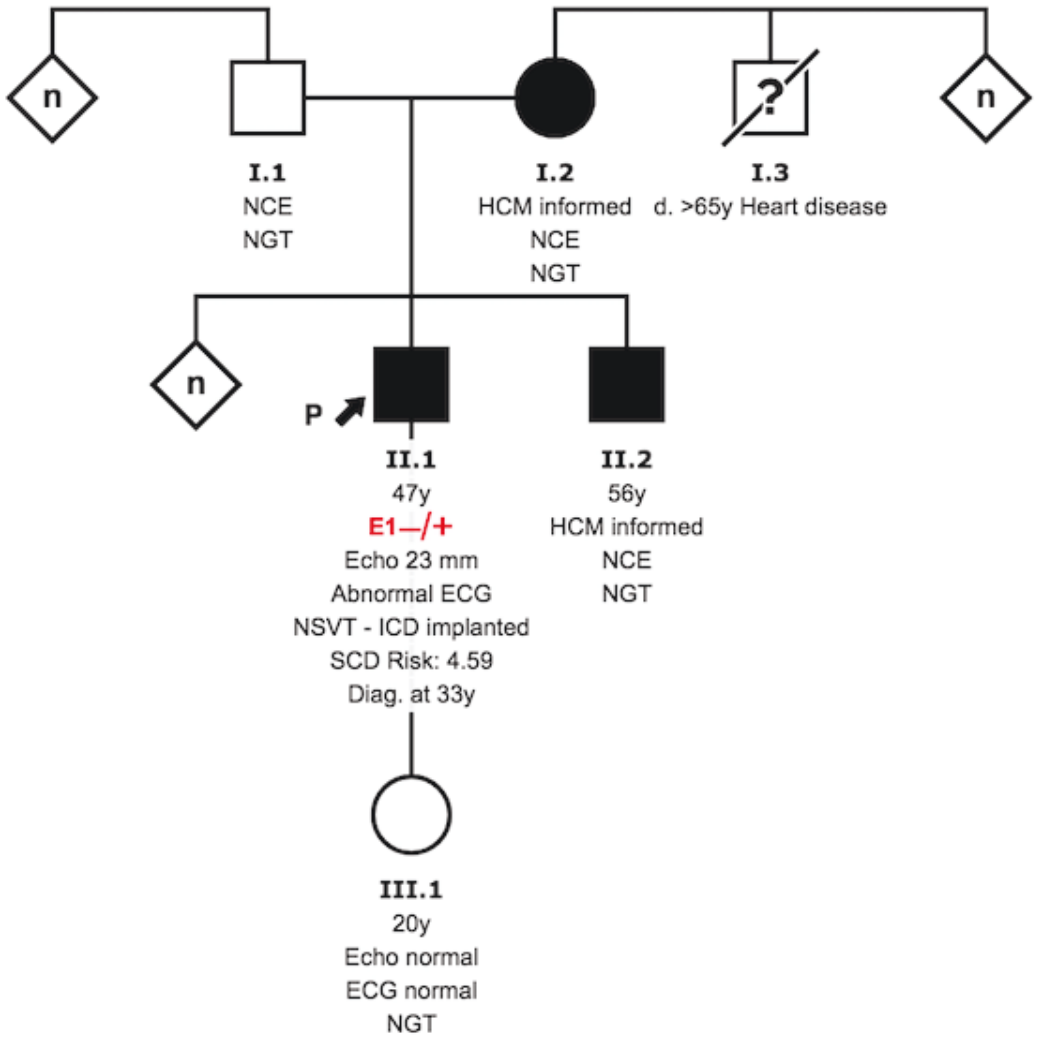
Pedigree #18

	Id	Sex	Kinship	TPM1 Arg21Leu	Other mutat.	Phenotype	Age of Dx	Age	NYHA	AF	FHSD	Max LVH	TV/FV	Syncope	Abn. Vasc Resp	LVOTO (Peak grad.)	LV disf. (EF)	Events
#18		M	Index case	Yes		HCM septal	13	32	III	-	-	51	-	+	+	+(140)	-(55)	

SCD Risk: 9.94

No pedigree was reported.

Pedigree #19

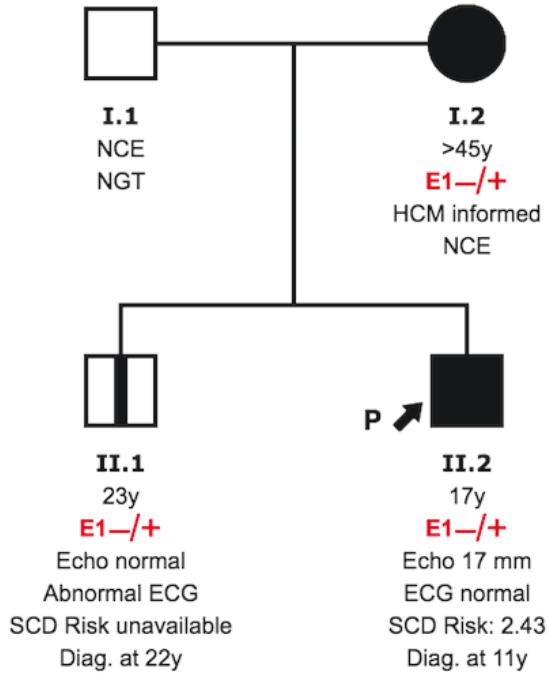


E1 TPM1 (g.63335090G>T, c.62G>T, p.Arg21Leu)

"+/+" = Homozygous, "-/+" = Heterozygous, "+" = Hemizygous, "-/-" = Not found, "-" = Not found

	Id	Sex	Kinship	TPM1 Arg21Leu	Other mutat.	Phenotype	Age of Dx	Age	NYHA	AF	FH SD	Max LVH	TV/ FV	Syncope	Abn. Vasc Resp	LVOTO (Peak grad.)	LV disf. (EF)	Events
#19	**II.1**	M	Index case	Yes	No	HCM septal	33	47	I	-	-	23	+	No	No	-	-(>55)	
	II.2	M	Brother	NGT		HCM informed		56										
	I.2	F	Mother	NGT		HCM informed		?										
	I.3	M	Uncle			Heart disease not specified		>65										Heart disease death >65y

Pedigree #20



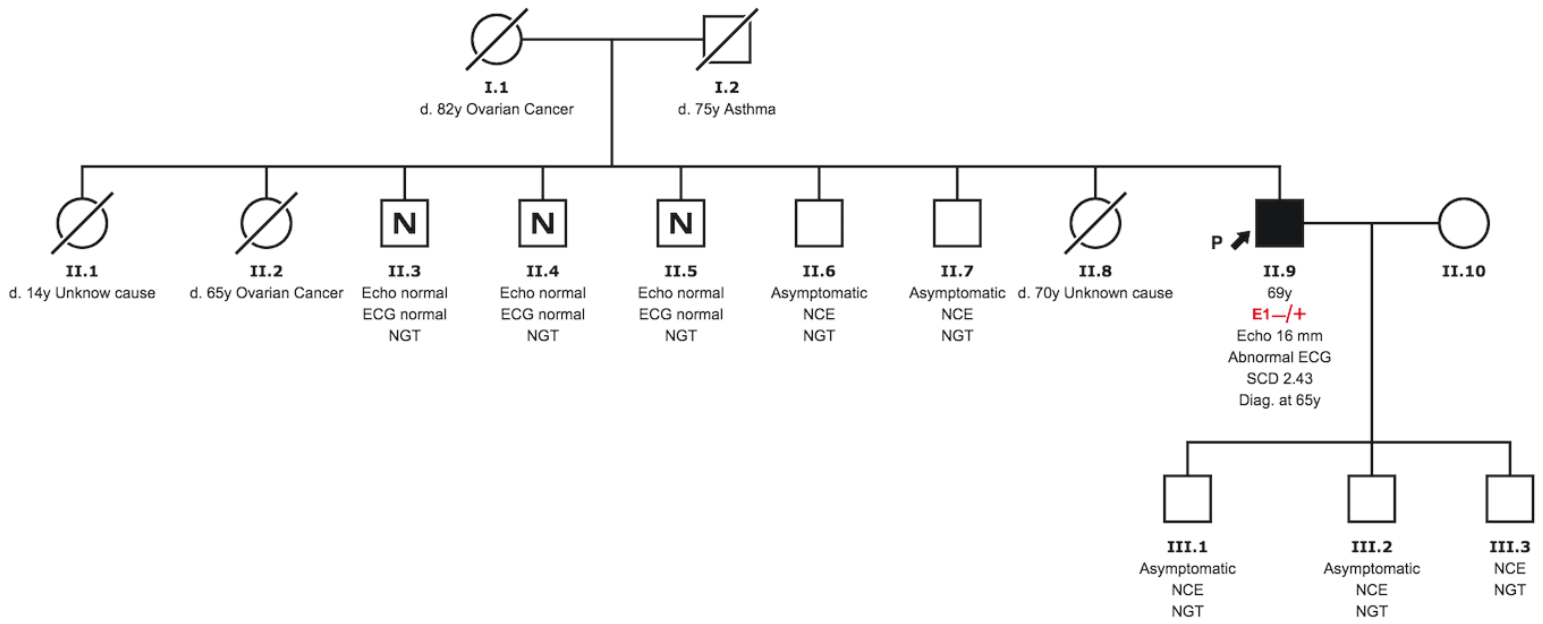
LOD SCORE 0.17

E1 TPM1 (g.63335090G>T, c.62G>T, p.Arg21Leu)

"+/+" = Homozygous, "-/+" = Heterozygous, "+" = Hemizygous, "-/-" = Not found, "-" = Not found

	Id	Sex	Kinship	TPM1 Arg21Leu	Other mutat.	Phenotype	Age of Dx	Age	NYHA	AF	FHSD	Max LVH	TV/FV	Syncope	Abn. Vasc Resp	LVOTO (Peak grad.)	LV disf. (EF)	Events
#20	**II.2**	M	Index case	Yes	No	HCM septal	11	17	I	-	-	17	-	No	No	-	-(77)	
	I.2	F	Mother	Yes		HCM informed	<45	>45										
	II.1	M	Brother	Yes		HCM	22	23	I	-	-	-	-	No	-	-	-	

Pedigree #21

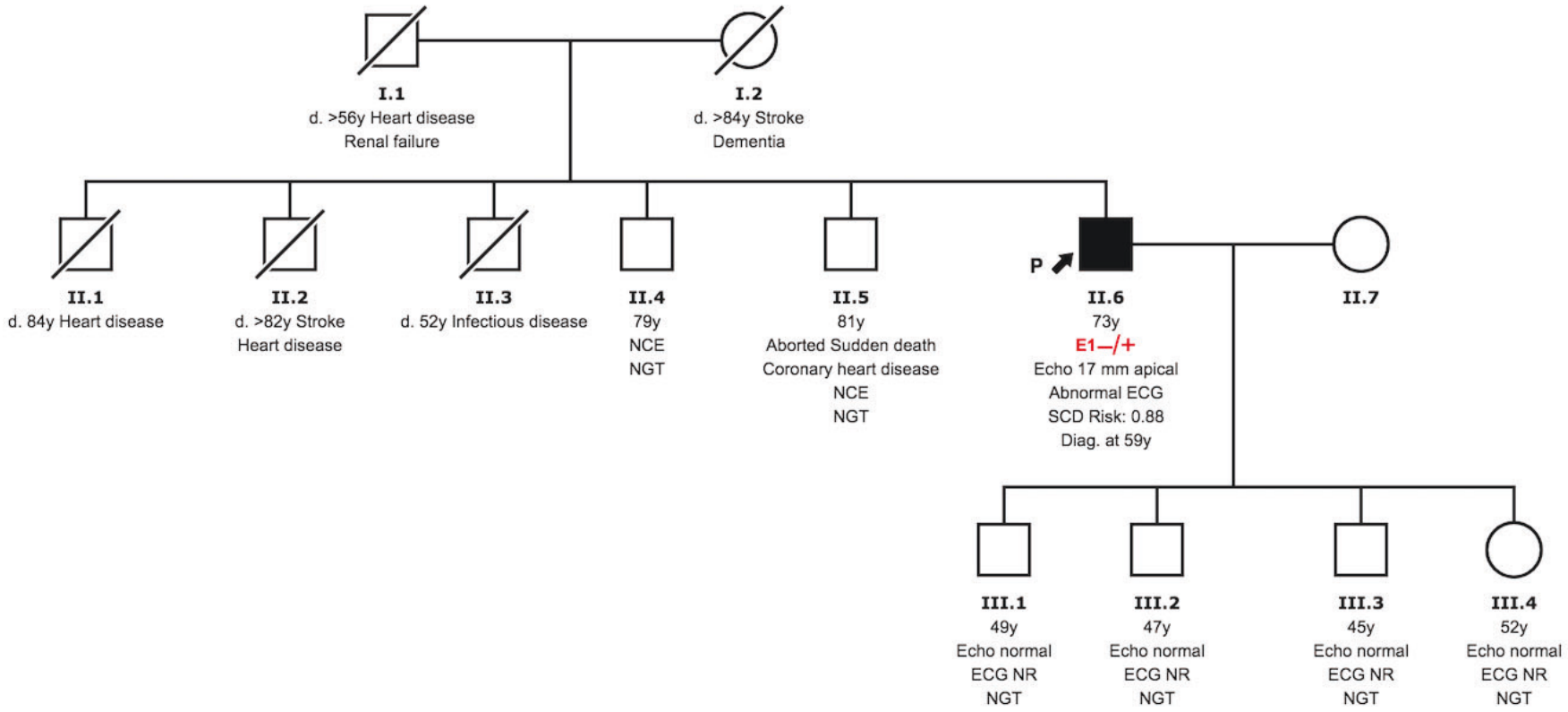


E1 TPM1 (g.63335090G>T, c.62G>T, p.Arg21Leu)

"+/+" = Homozygous, "-/+" = Heterozygous, "+" = Hemizygous, "-/-" = Not found, "-" = Not found

	Id	Sex	Kinship	TPM1 Arg21Leu	Other mutat.	Phenotype	Age of Dx	Age	NYHA	AF	FH SD	Max LVH	TV/ FV	Syncope	Abn. Vasc Resp	LVOTO (Peak grad.)	LV disf. (EF)	Events
#21	**II.9**	M	Index case	Yes	No	HCM septal	65	69	I	-	-	16	-	No	No	-	-(72)	

Pedigree #22

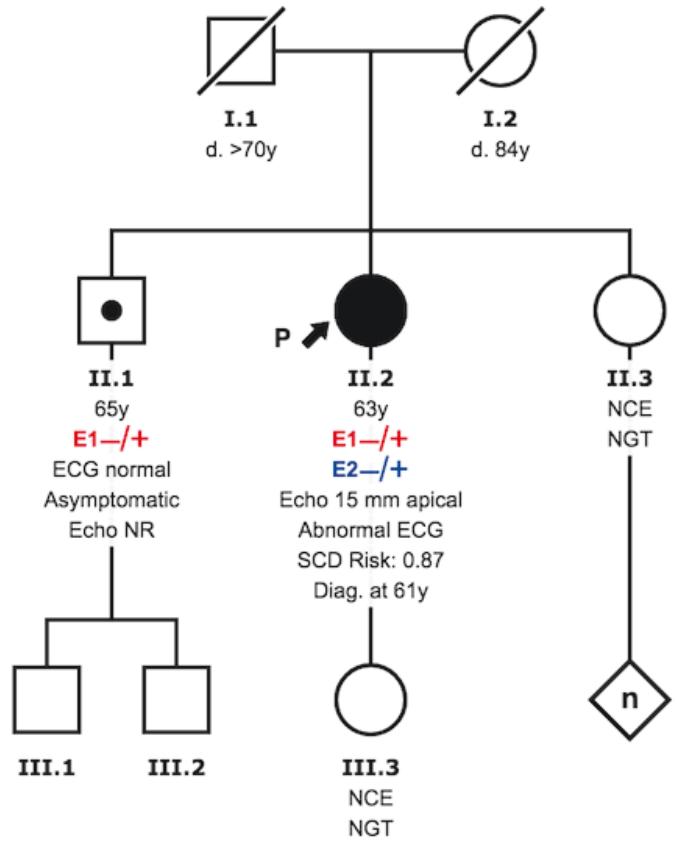


E1 TPM1 (g.63335090G>T, c.62G>T, p.Arg21Leu)

"+/+" = Homozygous, "-/+" = Heterozygous, "+" = Hemizygous, "-/-" = Not found, "-" = Not found

	Id	Sex	Kinship	TPM1 Arg21Leu	Other mutat.	Phenotype	Age of Dx	Age	NYHA	AF	FH SD	Max LVH	TV/ FV	Syncope	Abn. Vasc Resp	LVOTO (Peak grad.)	LV disf. (EF)	Events
#22	**II6**	F	Index case	Yes	No	HCM apical	59	73	I	-	-	17	-	-	-	-	-(77)	
	I.1	M	Father				56											Heart disease death - at 56y

Pedigree #23



E1 TPM1 (g.63335090G>T, c.62G>T, p.Arg21Leu)

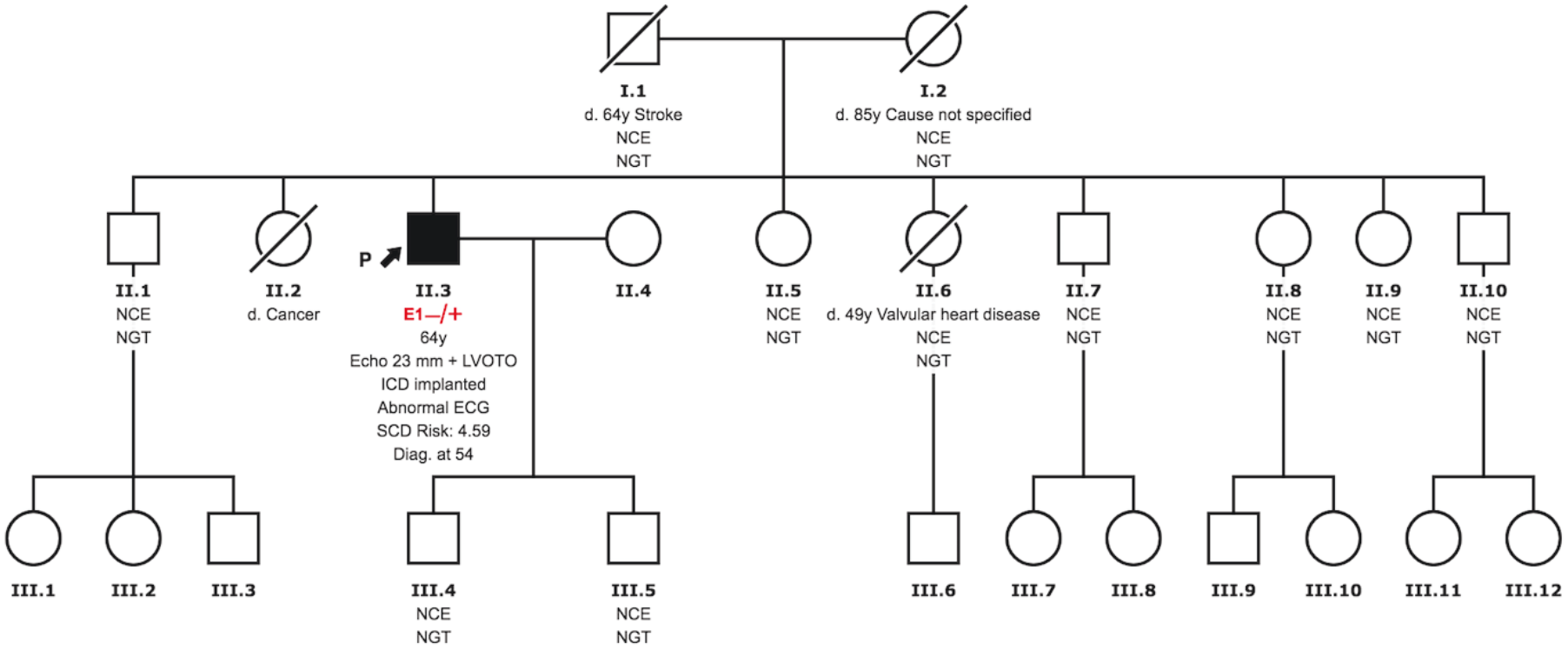
"+/+" = Homozygous, "-/+" = Heterozygous, "+" = Hemizygous, "-/-" = Not found, "-" = Not found

E2 MYH7 (g.23887591G>C, c.3997C>G, p.Leu1333Val)

"+/+" = Homozygous, "-/+" = Heterozygous, "+" = Hemizygous, "-/-" = Not found, "-" = Not found

	Id	Sex	Kinship	TPM1 Arg21Leu	Other mutat.	Phenotype	Age of Dx	Age	NYHA	AF	FH SD	Max LVH	TV/ FV	Syncope	Abn. Vasc Resp	LVOTO (Peak grad.)	LV disf. (EF)	Events
#23	**II2**	F	Index case	Yes	MYH7 Leu1333Val (?)	HCM apical	61	63	I	-	-	15	-	-	-	-	-(58)	

Pedigree #24

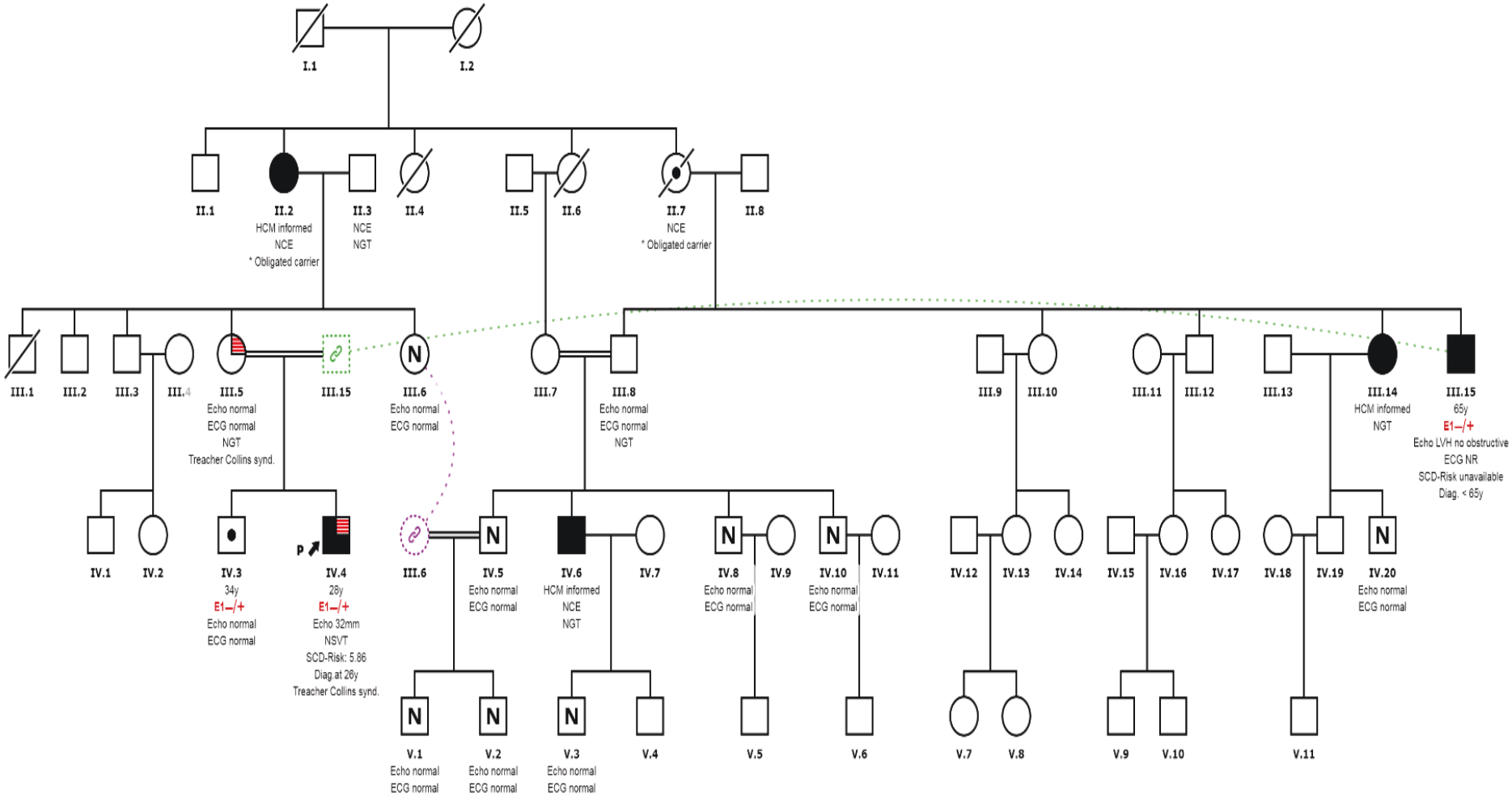


E1 TPM1 (g.63335090G>T, c.62G>T, p.Arg21Leu)

"+/+" = Homozygous, "-/+" = Heterozygous, "+" = Hemizygous, "-/-" = Not found, "-" = Not found

	Id	Sex	Kinship	TPM1 Arg21Leu	Other mutat.	Phenotype	Age of Dx	Age	NYHA	AF	FH SD	Max LVH	TV/FV	Syncope	Abn. Vasc Resp	LVOTO (Peak grad.)	LV disf. (EF)	Events
#24	**II.3**	M	Index case	Yes	No	HCM septal	54	64	I	-	-	23	-	+	+	+(88)	-(60)	
	I.1	M	Father	NGT		NCE		64										Stroke-related death – at 64y
	II.6	F	Sister	NGT		Heart disease		49										Valvular heart disease – death 49y

Pedigree #25



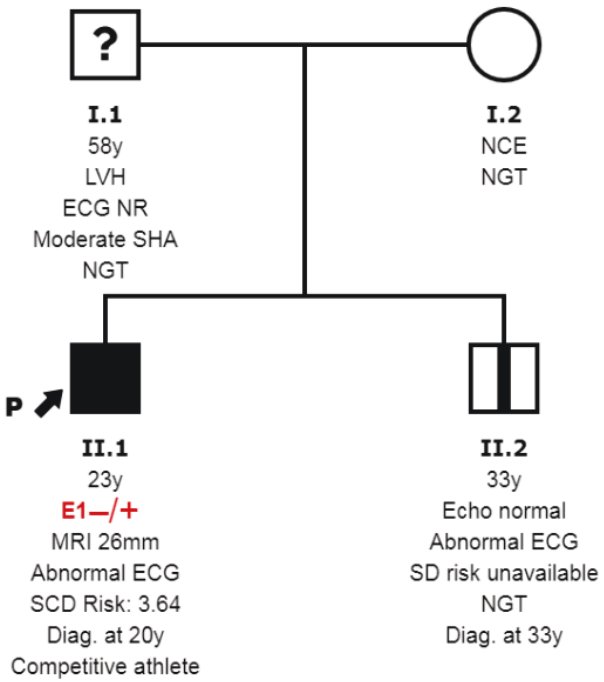
E1 TPM1 (g.63335090G>T, c.62G>T, p.Arg21Leu)

"+/+" = Homozygous, "-/+" = Heterozygous, "+" = Hemizygous, "-/-" = Not found, "-" = Not found

LOD SCORE 0.17

	Id	Sex	Kinship	TPM1 Arg21Leu	Other mutat.	Phenotype	Age of Dx	Age	NYHA	AF	FH SD	Max LVH	TV/ FV	Syncope	Abn. Vasc Resp	LVOTO (Peak grad.)	LV disf. (EF)	Events
#25	**IV.4**	M	Index case	Yes	No	HCM septal	26	28	I	-	-	32	+	-	-	-	-(70)	
	III.15	M	Father	Yes		HCM	<65	65	?	?	-	+	?	?	?	-	-	
	IV.3	M	Brother	Yes		Not affected		34	I	-	-	-	-	-	-	-	-	
	II.2	F	Grandmother	Obligate carrier		HCM informed												
	II.7	F		Obligate carrier		NCE												

Pedigree #26



E1 TPM1 (g.63335090G>T, c.62G>T, p.Arg21Leu)

"+/+" = Homozygous, "-/+" = Heterozygous, "+" = Hemizygous, "-/-" = Not found, "-" = Not found

	Id	Sex	Kinship	TPM1 Arg21Leu	Other mutat.	Phenotype	Age of Dx	Age	NYHA	AF	FHSD	Max LVH	TV/FV	Syncope	Abn. Vasc Resp	LVOTO (Peak grad.)	LV disf. (EF)	Events
#26	**II.1**	M	Index case	Yes	No	HCM septal	20	23	I	-	-	26	-	-	-	-	-	
	II.2	M	Brother	NGT		Not affected (?)		33	I	-	-	-	-	-	-	-	-	
	I.1	M	Father	NGT		?		58	I	-	-	+	-	-	-	-	-	

Pedigree #27

	Id	Sex	Kinship	TPM1 Arg21Leu	Other mutat.	Phenotype	Age of Dx	Age	NYHA	AF	FH SD	Max LVH	TV/ FV	Syncope	Abn. Vasc Resp	LVOTO (Peak grad.)	LV disf. (EF)	Events
#27		F	Index case	Yes	No	HCM septal	60	69	II	-	-	21	-	-	-	-	-	

SCD Risk: 1.73

No pedigree was reported.

Pedigree #28

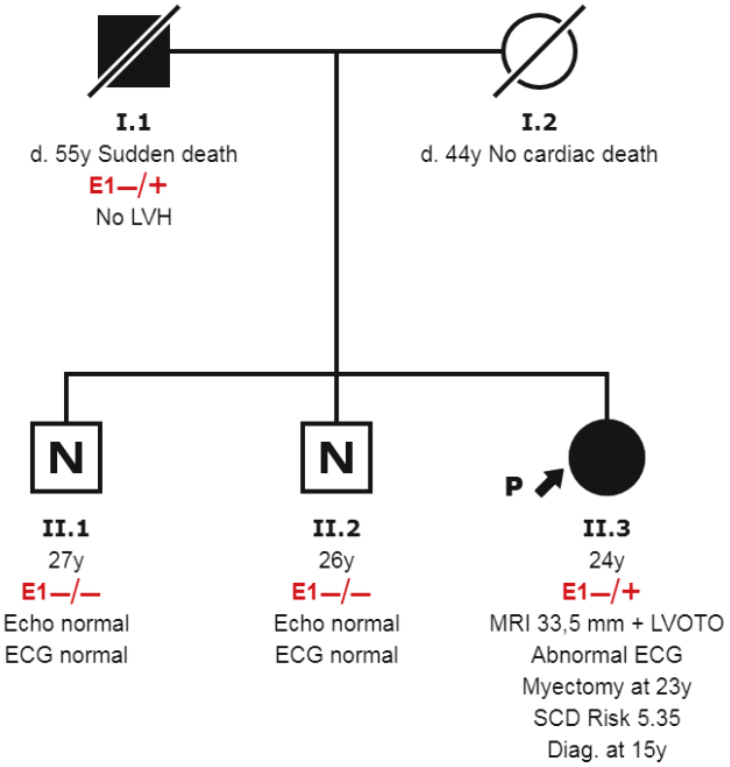
	Id	Sex	Kinship	TPM1 Arg21Leu	Other mutat.	Phenotype	Age of Dx	Age	NYHA	AF	FH SD	Max LVH	TV/ FV	Syncope	Abn. Vasc Resp	LVOTO (Peak grad.)	LV disf. (EF)	Events
#28		M	Index case	Yes	MYH7 Tyr582Cys (?)	HCM septal	56	57	II-III	-	-	17	-	-	-	+ (110)	-(61)	

SCD Risk: 2.39

No pedigree was reported.

.

Pedigree #29



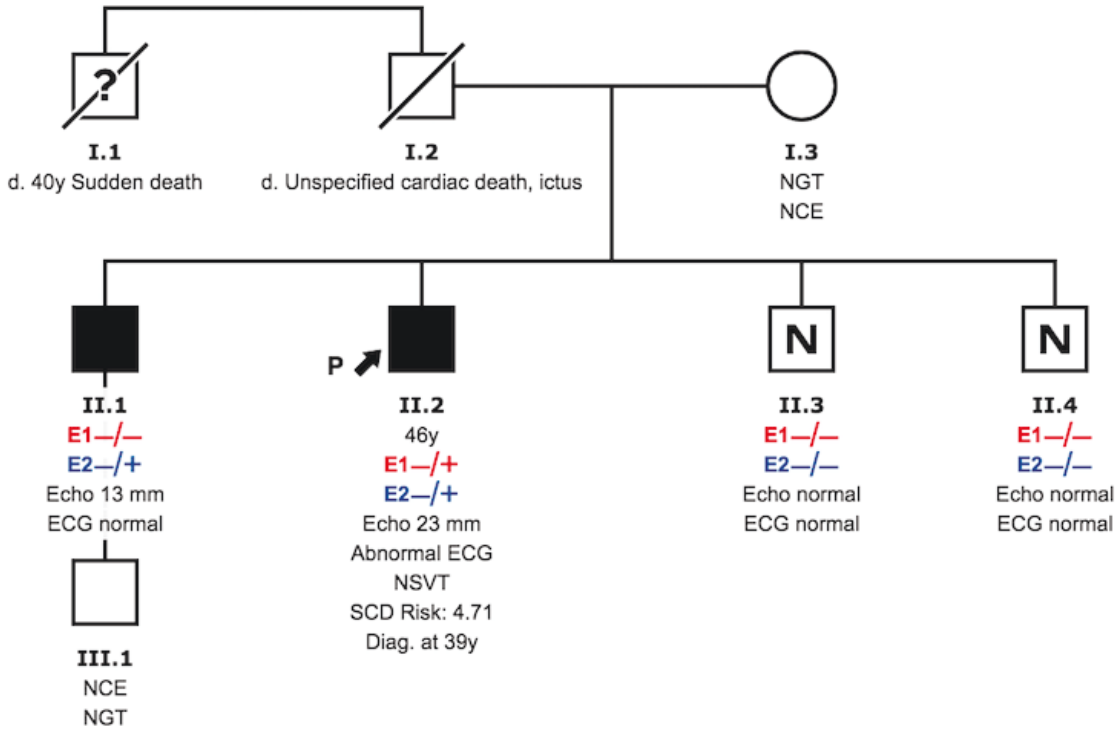
LOD SCORE 0.23

E1 TPM1 (g.63335090G>T, c.62G>T, p.Arg21Leu)

"+/+" = Homozygous, "-/+" = Heterozygous, "+" = Hemizygous, "-/-" = Not found, "-" = Not found

	Id	Sex	Kinship	TPM1 Arg21Leu	Other mutat.	Phenotype	Age of Dx	Age	NYHA	AF	FH SD	Max LVH	TV/ FV	Syncope	Abn. Vasc Resp	LVOTO (Peak grad.)	LV disf. (EF)	Events
#29	**II.3**	F	Index case	Yes	No	HCM septal	15	24	II	-	+	33.5	-	-	-	+ (90)	-(72)	
	I.1	M	Father	Yes	No	Sudden Death		55				No						Sudden death at 55y

Pedigree #30



E1 TPM1 (g.63335090G>T, c.62G>T, p.Arg21Leu)

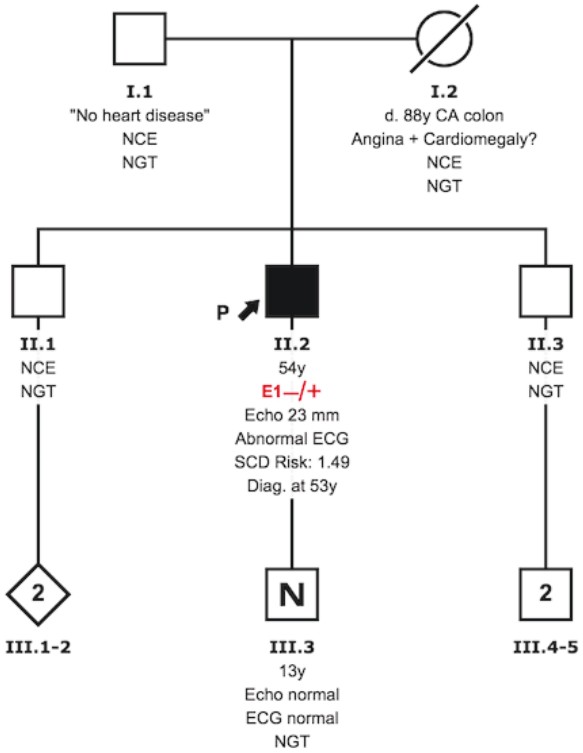
"+/+" = Homozygous, "-/+" = Heterozygous, "+" = Hemizygous, "-/-" = Not found, "-" = Not found

E2 TPM1 (g.63356332T>C, c.842T>C, p.Met281Thr)

"+/+" = Homozygous, "-/+" = Heterozygous, "+" = Hemizygous, "-/-" = Not found, "-" = Not found

	Id	Sex	Kinship	TPM1 Arg21Leu	Other mutat.	Phenotype	Age of Dx	Age	NYHA	AF	FH SD	Max LVH	TV / FV	Syncope	Abn. Vasc Resp	LVOTO (Peak grad.)	LV disf. (EF)	Events
#30	**II.2**	M	Index case	Yes	TPM1 Met281Val (+++)	HCM septal	39	46	I	-	+	23	+	-	-	- (20)	- (77)	
	I.1	M	Uncle	?	?			40										Sudden death at 40y

Pedigree #31



E1 TPM1 (g.63335090G>T, c.62G>T, p.Arg21Leu)

"+/+" = Homozygous, "-/+" = Heterozygous, "+" = Hemizygous, "-/-" = Not found, "-" = Not found

	Id	Sex	Kinship	TPM1 Arg21Leu	Other mutat.	Phenotype	Age of Dx	Age	NYHA	AF	FH SD	Max LVH	TV/ FV	Syncope	Abn. Vasc Resp	LVOTO (Peak grad.)	LV disf. (EF)	Events
#31	**II.2**	M	Index case	Yes	No	HCM septal	53	54	I	-	-	23	-	-	-	- (23)	- (80)	

5. *TPM1* variants

This chapter was described considering the database carried out in this study which we are presenting in the Supplementary Material - Excel table Chapter 5. In this database, we have listed all *TPM1* variants identified in our collaborator hospitals and different databases, including; nomenclature, heptad position, functional domain, prevalence of the variant in cases vs controls, ClinVar phenotype, and pathogenicity, number of references in the literature (clinical reports and functional studies) and total number of carriers, families and associated phenotype.

The Supplementary Material - Excel table Chapter 5 allows an overview on all *TPM1* variants, and the data used to support our pathogenicity classification for each one of the variants. The analysis described in this chapter must be accompanied by the read of this supplementary material.

Sarcomeric proteins such as alpha-tropomyosin are in general codified by extense genes, in which it is possible to house hundreds of variants. By consequence, it leads to difficulties in the classification of the variants' pathogenicity. Only pathogenic and likely pathogenic variants have been considered able to determine the clinical decision making process. More recently, variants of "uncertain significance - potentially relevant" have emerged as a classification category, which could put more clinical emphasis (specially for its familial cosegregation study) in this context of several sarcomeric variants (Arbustini E et al, 2022). In some of the following analysis, we do

not consider variants of uncertain significance “VUS”, because their causality is still low to define an association with a specific phenotype.

5.1: *TPM1* variants by pathogenicity criteria.

We have identified 281 *TPM1* variants in total. Missense-type variants were predominant (256 variants) in this study. Only one single-amino acid deletion variant was identified, and 24 truncating variants additionally. Analysis about intronic variants was carried out separately in the section 5.5 of this chapter. The other variants were classified and divided in four groups according to their pathogenicity: (1.) pathogenic, (2.) likely pathogenic, (3.) variant of uncertain significance - potentially relevant (VUS+), and (4.) variant of uncertain significance (VUS) (see Table 5.1).

Forty three variants (19 pathogenic and 24 likely pathogenic) were considered disease causing. Fifty eight variants were classified as of uncertain significance; however, potentially relevant. Available information on other 156 variants was insufficient to establish their pathogenicity, therefore, these variants were classified as of uncertain significance (VUS).

Table 5.1: TPM1 variants divided by pathogenicity criteria.

Pathogenicity	*n*	Variants
Pathogenic (+++)	19	p.Arg21Leu; p.Glu40Lys; p.Glu54Lys; p.Glu62Gln; p.Glu62Lys; p.Ala63Val; p.Leu71Val; p.Asp84Asn; p.Val95Ala; p.Leu113Val; p.Asp175Asn; p.Glu180Gly; p.Glu192Lys; p.Thr201Met; p.Ser215Leu; p.Asp219Val; p.Asp230Asn; p.Lys248Glu; p.Met281Val.
Likely pathogenic (++)	24	p.Gly3Arg; p.Lys5del; Met8Arg; p.Lys15Asn; p.Lys15Asn; p.Arg21Gly; p.Arg21Gln; p.Lys70Thr; p.Ala83Asp; p.Ile92Thr; p.Ala109Val; p.Ala120Val; p.Ile130Leu; p.Ile130Val; p.Ile172Thr; p.Glu180Val; p.Ala183Val; p.Leu185Arg; p.Lys198Glu; p.Asp219Asn; p.Gln263Glu; p.Ala277Val; p.Met281Thr; p.Ile284Val.
Variants of uncertain significance, potentially relevant (+?)	58	p.Met8Lys; p.Gln9Lys; p.Gln9Arg; p.Gln9Leu; p.Gln9His; p.Lys15Glu; p.Lys15Arg; p.Asp20Asn; p.Ala22Thr; p.Ala22Gly; p.Ser36Gly; p.Ser36Asn; p.Lys37Glu; p.Lys37Thr; p.Glu40Gln; p.Asp55Asn; p.Asp55Tyr; p.Asp55Gly; p.Asp58His; p.Glu62Gly; p.Ala63Pro; p.Ala63Thr; p.Lys70Arg; p.Asp84Glu;

		p.Asp84Ala; p.Ile92Met; p.Ala102Asp; p.Glu104Trp; p.Ser123Thr; p.Gly126Val; p.Met127Lys; p.Lys128Gln; p.Ile130Thr; p.Arg133Gln; p.Asp159Asn; p.Arg160His; p.Ile172Phe; p.Ile172Leu; p.Ile172Met; p.Asp175Gly; p.Glu180Gln; p.Leu185Phe; p.Gln210Arg; p.Gln210His; p.Ala211Gly; p.Ala211Val; p.Tyr221Cys; p.Arg238Trp; p.Arg238Gln; p.Ala242Val; p.Ser245Leu; p.Leu249Trp; p.Asp275His; p.Asp275Tyr; p.Ala277Thr; p.Ala277Gly; p.Asn279His; p.Ile284Thr.
Variants of uncertain significance (?)	156	p.Asp2His; p.Ile4Val; p.Lys6Arg; p.Asp14Tyr; p.Asp14Gly; p.Glu16Gln; p.Asn17Lys; p.Ala18Val; p.Glu23Gln; p.Glu23Asp; p.Gln24His; p.Ala25Thr; p.Glu26Gly; p.Asp28His; p.Asp28Asn; p.Lys30Ala; p.Ala31Thr; p.Glu33Lys; p.Glu33Ala; p.Arg35Thr; p.Arg35Lys; p.Arg35Ser; p.Gln38Glu; p.Asp42Tyr; p.Leu43Pro; p.Gln47Lys; p.Gln47Glu; p.Gln47Arg; p.Leu50Phe; p.Leu50Ile; p.Lys51Arg; p.Lys51Thr; p.Gly52Ser; p.Tyr60Cys; p.Ser61Cys; p.Asp66Asn; p.Asp66His; p.Gln68Lys; p.Glu72Lys; p.Glu72Gly; p.Lys76Glu; p.Thr79Ile; p.Ala81Val; p.Val85Ile; p.Val85Leu; p.Ala86Thr; p.Ala86Val; p.Ala86Gly; p.Leu88Val; p.Asn89Lys; p.Arg90Lys; p.Arg91Cys; p.Arg91His; p.Glu97Gln; p.Arg101Gly; p.Arg101His; p.Arg101Pro; p.Glu104Gln; p.Arg105His; p.Ala107Thr; p.Gln111Glu; p.Gln111His; p.Glu114Gln; p.Glu114Gly; p.Glu115Lys; p.Glu122Lys; p.Glu124Gly; p.Met127Thr; p.Met127Ile; p.Glu131Gln; p.Gln135Lys; p.Lys136Asn; p.Asp137Glu; p.Asp137Val; p.Glu138Lys; p.Glu139Val; p.Glu139Gly; p.Lys140Gln; p.Met141Val; p.Met141Leu; p.Met141Ile; p.Met141Lys p.Ile143Ser; p.Ile143Asn; p.Gln144Arg; p.Ile146Ser; p.Ala151Thr; p.Lys152Glu; p.His153Asp; p.His153Arg; p.Ile154Val; p.Ile154Thr; p.Lys161Glu; p.Ala166Thr; p.Val170Ile; p.Ile171Val; p.Ile171Leu; p.Ile171Met; p.Glu173Asp; p.Arg178His; p.Glu181Lys; p.Glu181Met; p.Glu187Gln; p.Glu187Val; p.Gly188Ser; p.Cys190Arg; p.Cys190Tyr; p.Ala191Val; p.Glu195Lys; p.Asn202Ser; p.Asn203Asp; p.Asn203Lys; p.Ser206Ala; p.Ala209Ser; p.Ala209Thr; p.Glu212Val; p.Lys213Gln; p.Ile225Val; p.Lys226Gln; p.Lys226Arg; p.Lys226Asn; p.Ser229Phe; p.Lys231Arg; p.Leu232Arg; p.Lys233Asn; p.Ala235Thr; p.Thr237Ser; p.Arg238Leu; p.Ala239Thr; p.Arg244Lys; p.Val246Ile; p.Lys248Thr; p.Glu250Val; p.Ser252Thr; p.Ile253Thr; p.Asp254Gly; p.Asp254Glu; p.Asp254Glu; p.Asp258Glu; p.Glu259Lys; p.Glu259Asp; p.Ala262Thr; p.Lys264Glu; p.Lys266Arg; p.Lys268Gln; p.Lys268Glu; p.Lys268Arg; p.Ala269Val; p.Ile270Val; p.Ser271Asn; p.Glu272Lys; p.Glu272Ala; p.Glu272Gly; p.Asp280Ala; p.Met281Arg.
Total	**257**	

5.2: Changes in *TPM1* variant's pathogenicity from ClinVar

We have evaluated changes in the pathogenicity of missense-type *TPM1* variants reported in ClinVar, taking into account the clinical data input from this study. The changes are summarized in Table 5.2.

Several *TPM1* variants are reported in ClinVar without clinical and/or functional evidence supporting the pathogenicity of these variants. In this sense, we have

reclassified the variants based on the available data from this study and present in the literature. In total, we have changed the pathogenicity of 87 ClinVar variants.

From 140 ClinVar variants classified as VUS; no change in the pathogenicity of 99 ClinVar VUS was observed in our study. Other 41 variants were reclassified as 36 VUS+ and 5 as likely pathogenic.

Thirty likely pathogenic variants are listed in ClinVar database. Twenty-five variants had their pathogenicity altered in this study: 17 of them were relegated as VUS, four as VUS+ and three pathogenic. Other six variants classified as likely pathogenic in ClinVar had the same pathogenicity classification here.

Seven pathogenic variants are listed in ClinVar. Only two pathogenic variants from Clinvar were classified as VUS (1 variant) and VUS+ (1 variant).

Finally, 19 variants having conflicting interpretation in ClinVar were defined in this study: 6 VUS, 4 VUS+, 2 likely pathogenic and 7 pathogenic variants.

Grouping variants from different databases, literature and our collaborators centers, we have classified the pathogenicity of 280 genetic variants (255 missense-type), in a gene composed of 284 amino acid-positions. We have not considered those likely benign/benign variants in this work. Thus, it is a number that should be underlined in our results. Our analysis on our *TPM1* variants database allowed reclassification of the pathogenicity of 87 previously described in ClinVar database, including 22 variants which now can be considered clearly actionable

(pathogenic/likely pathogenic). These data were essential to become a more achievable individual medical approach to each one of these carriers.

Table 5.2: Changes in TPM1 variant's pathogenicity from ClinVar

ClinVar database	This study
140 VUS	99 VUS 36 VUS+ 5 Likely Pathogenic
30 Likely pathogenic	17 VUS 4 VUS+ 6 Likely Pathogenic 3 Pathogenic
7 Pathogenic	1 VUS 1 VUS+ 5 Pathogenic
19 Conflicting interpretations	6 VUS 4 VUS+ 2 Likely pathogenic 7 Pathogenic

VUS: Variants of uncertain significance, VUS+: Variants of uncertain significance, potentially relevant.

5.3: *TPM1* variants by heptad position and phenotype

Alpha-tropomyosin is a helical coiled-coil molecule composed of subsequents heptad repeats. Each position in the heptad can be represented by a letter *a* to *g* (Gupte et al, 2015). Some authors have suggested that these positions could have different relevance for the protein structure and stability due to interaction characteristics, polarity and position (Van Driest, et al 2002; Gupte et al, 2015; Kuruba et al 2021).

No genotype-phenotype correlation between *TPM1* heptad position and cardiomyopathy-type was described in the literature to date. Taking into consideration this information, we have analyzed the *TPM1* variants-associated phenotype and their

respective heptad position. We have included in this analysis only pathogenic, likely pathogenic and VUS+ variants in *TPM1*, since only these variants could be really informative about their association with the phenotype. The number of variants identified in each residue is different, in descending order in the following positions: *a, d, g, f, b, e* and *c*, with respectively, 22, 22, 18, 13, 13, 10 and 5 variants in each position.

Some particularities can be observed in this analysis, such as only one pathogenic variant was identified in the position *b* (with 13 variants), and two likely pathogenic variants were reported in this same position. Further, the positions *g* and *f* had a higher number of hypertrophic cardiomyopathy-variants than dilated cardiomyopathy/left ventricle non-compaction; however, there was no significant statistical difference (p value >0,05). Only the heptad position *e* seems to have predominantly dilated cardiomyopathy-variants than hypertrophic cardiomyopathy-variants (p=0,009). Nonetheless, we need to consider that the number of variants with available information on phenotype is still low to really determine a heptad position-phenotype correlation, and it could cause inconsistencies in this result.

Genotype-phenotype correlations on *TPM1* heptad positions suggested that this way to look for these variants is probably not relevant to clinical practice at this point, although our data could be still limited to definitive conclusions. It could reflect the complexity of the tropomyosin functional mechanisms, and its molecular relationships across cardiac contractility movement. Table 5.3 shows TPM1 variants by each heptat position and respectives pathogenicity (see next page).

Table 5.3: TPM1 variants by heptad position and pathogenicity

Heptad position	Pathogenic (+++)	Likely pathogenic (++)	Variants of uncertain significance, potentially relevant (+?)	Total genotype/ phenotype analysis
a	*n*= 3 (2 HCM, 1 DCM)	*n*= 7 (4 DCM, 2 HCM)	*n*= 12 (5 HCM, 4 DCM)	*n*= 22 (9 HCM, 9 DCM)
	p.Leu71Val (HCM); p.Leu113Val (DCM); p.Met281Val (HCM)	p.Met8Arg (DCM); p.Lys15Asn (DCM); p.Lys15Asn; p.Ile92Thr (DCM); p.Ala120Val; (DCM); p.Ala183Val (HCM); p.Met281Thr (HCM)	p.Met8Lys (HCM); p.Lys15Glu (HCM); p.Lys15Arg; p.Ala22Thr (HCM); p.Ala22Gly; p.Ser36Gly (DCM); p.Ser36Asn (DCM); p.Ile92Met; p.Met127Lys (HCM); p.Arg133Gln (DCM) p.Ala211Gly (DCM); p.Ala211Val (HCM);	
b	*n*= 1 (1 HCM)	*n*=2 (2 HCM)	*n*= 10 (4 HCM, 4 DCM)	*n*= 13 (7 HCM, 4 DCM)
	p.Asp219Val	p.Lys198Glu p.Asp219Asn	p.Gln9Lys (HCM); p.Gln9Arg; p.Gln9Leu; p.Gln9His (HCM); p.Lys37Glu (DCM/LVNC); p.Lys37Thr (HCM); p.Asp58His (HCM); p.Lys128Gln (DCM); p.Asp275His (DCM); p.Asp275Tyr (DCM)	
c	*n*=2 (1 HCM, 1 DCM)	*n*=2 (2 HCM)	*n*=1 (1 DCM)	*n*= 5 (3 HCM, 2 DCM)
	p.Glu192Lys (HCM); p.Lys248Glu (DCM)	p.Gly3Arg (HCM); p.Leu185Arg (HCM);	p.Leu185Phe (DCM)	
d	*n*= 1 (1 HCM)	*n*= 7 (4 DCM, 2 HCM)	*n*= 12 (5 HCM, 4 DCM)	*n*= 20 (10 HCM, 6 DCM)
	p.Val95Ala (HCM)	p.Ala109Val (DCM); p.Ile130Leu (HCM); p.Ile130Val; p.Ile172Thr (HCM); p.Gln263Glu (HCM); p.Ala277Val (DCM); p.Ile284Val (HCM)	p.Ala102Asp (HCM); p.Ser123Thr (DCM); p.Ile130Thr (DCM); p.Ile172Phe ; p.Ile172Leu; p.Ile172Met; p.Tyr221Cys (HCM); p.Ala242Val (DCM); p.Leu249Trp (HCM); p.Ala277Thr (HCM); p.Ala277Gly (DCM); p.Ile284Thr (HCM)	
e	*n*= 5 (4 DCM, 1 HCM)	*n*= 2	*n*= 3 (1 DCM)	*n*= 10 (1 HCM, 6 DCM) *p=0,009*
	p.Glu40Lys (DCM); p.Glu54Lys (DCM); p.Glu180Gly (DCM); p.Thr201Met (DCM); p.Ser215Leu (HCM)	p.Glu180Val; p.Lys5del	p.Glu40Gln p.Asp159Asn (DCM) p.Glu180Gln	
f	*n*= 3 (2 HCM, 1 DCM)	*n*= 1 (1 HCM)	*n*= 9 (4 HCM, 2 DCM)	*n*= 13 (7 HCM, 3 DCM)
	p.Glu62Gln (HCM); p.Glu62Lys (HCM); p.Asp230Asn (DCM)	p.Ala83Asp (HCM)	p.Asp20Asn (HCM); p.Asp55Asn (DCM); p.Asp55Tyr; p.Asp55Gly; p.Glu62Gly ; p.Glu104Trp (HCM); p.Arg160His (DCM); p.Asp175Gly (HCM); p.Asn279His (HCM)	
g	*n*= 4 (3 HCM, 1 DCM)	*n*= 3 (3 HCM)	*n*= 11 (3 HCM, 4 DCM)	*n*= 18 (9 HCM, 5 DCM)
	p.Arg21Leu (HCM); p.Ala63Val (HCM); p.Asp84Asn (DCM/LVNC); p.Asp175Asn (HCM)	p.Arg21Gly (HCM); p.Arg21Gln (HCM); p.Lys70Thr (HCM)	p.Ala63Pro (DCM); p.Ala63Thr (HCM); p.Lys70Arg; p.Asp84Glu (HCM); p.Asp84Ala; p.Gly126Val; p.Gln210Arg (HCM); p.Gln210His; p.Arg238Trp (DCM); p.Arg238Gln (DCM); p.Ser245Leu (DCM)	
Total	*n*= 18	*n*= 23	*n*= 59	*n*= 100

HCM: Hypertrophic cardiomyopathy. DCM: Dilated cardiomyopathy/left ventricle non-compaction.

5.4: Phenotype enrichment of *TPM1* variants by functional domain

Missense-type variants in some sarcomere proteins have not been frequently identified in the general population. Along *TPM1*, gnomAD database has proposed that other two sarcomeric genes (*MYH7* and *ACTN1*) would also have a relevant intolerance of variations caused by missense alterations (with minor allele frequency <0,01%). This fact has been considered as criteria to define variant's pathogenicity, when a missense variant in a gene that has a low rate of benign missense variation, and where this type of variants are a common mechanism of disease (supporting evidence by ACMG).

More recently, Kelly et al (2018) validated an adapted ACMG pathogenicity criteria for *MYH7* variants. They have considered that the interpretation of this supporting evidence could be modified by statistically significant clustering of pathogenic variants in specific functional domains. They ruled out the supporting evidence that missense variants identified in a gene with a low rate of benign variations could be a pathogenicity criteria, and their conclusion was that the unique criteria which should be applied for classification of the variants in *MYH7* would be where variants located in a hotspot and/or critical domain without benign variation (moderate evidence).

Taking into account this premise, we have carried out an overrepresentation analysis for missense variants clustered in *TPM1* domains, considering the associated-phenotype. The results are presented in the following tables 5.4 and 5.5.

Table 5.4: Hypertrophic cardiomyopathy phenotype enrichment by regions

Domain name	Internal controls (non-HCM)		External controls (gnomAD)	
	Odds ratio	p value	Odds ratio	p value
Overlapping Head-Tail regions	**9,86**	5.90e-24	**8,34**	8.32e-45
N-terminal	**6,79**	1.85e-11	**7,13**	2.19e-19
C-terminal	**20,73**	4.04e-14	**15,66**	8.41e-34
Leiomodin 2-binding region	**9,88**	9.85e-13	**20,31**	2.49e-34
Tropomodulin 1-binding region	1,83	4.65e-01 **	2,7	8.54e-02 **
Actin binding site repeat 1	**5,35**	8.65e-11	**9,35**	1.03e-28
Actin binding site repeat 2	2,34	3.22e-02 **	**7,48**	1.93e-08
Actin binding site repeat 3	0,37	1.75e-01 **	1,32	5.05e-01 **
Actin binding site repeat 4	1,03	1.00e+00 **	**15,16**	6.35e-03
Actin binding site repeat 5	2,89	5.49e-03	**6,25**	4.37e-09
Actin binding site repeat 6	**9,63**	4.02e-04	1,65	1.12e-01 **
Actin binding site repeat 7	**13**	3.70e-13	**3,94**	1.21e-12
Middle region	0,63	4.87e-01 **	3,79	1.12e-02 **
Flexible central domain	1,97	3.01e-03	2,31	2.35e-06
Troponin T-binding regions	**12,46**	1.51e-14	**7,31**	2.67e-23

In bold: Stronger odds ratio values with statistically significant difference. (**) means p value not significantly different. HCM: hypertrophic cardiomyopathy.

There is an overlapping among the different *TPM1* functional domains. It can be related with the azimuthal switch of tropomyosin during cardiac contraction, and its complex relation with different sarcomere proteins. Phenotype enrichment by regions observed for variants associated with hypertrophic cardiomyopathy patients showed several relevant domains; however, some of them correspond to the same protein structure. Hypertrophic cardiomyopathy enrichment by regions versus internal controls (with other inherited cardiac phenotypes) pointed out the regions N-terminal portion of the head-tail overlapping junction, leiomodin-binding site, and actin-binding site repeat 1 as overrepresented. Three domains corresponding virtually to the same

N-terminal protein region. Here the number of hypertrophic cardiomyopathy patients came mainly from the *TPM1* p.Arg21Leu variant.

Table 5.5: Dilated/non-compaction cardiomyopathies phenotype enrichment by regions

Domain name	Internal controls (non-DCM/LVNC)		External controls (gnomAD)	
	Odds ratio	p value	Odds ratio	p value
Overlapping Head-Tail regions	0,19	1.13e-07	0,95	1.00e+00 **
N-terminal	0,24	3.15e-04	2,34	7.69e-02 **
C-terminal	0,12	1.81e-04	0,51	5.91e-01 **
Leiomodin 2-binding region	0,1	1.25e-05	1,32	6.66e-01 **
Tropomodulin 1-binding region	0,48	6.85e-01 **	1,1	6.11e-01 **
Actin binding site repeat 1	0,3	7.25e-04	1,83	1.22e-01 **
Actin binding site repeat 2	0,82	8.27e-01 **	**4,29**	5.35e-03
Actin binding site repeat 3	**5,16**	2.79e-03	**6,44**	5.55e-05
Actin binding site repeat 4	**3,82**	7.91e-02 **	**32,92**	1.47e-04
Actin binding site repeat 5	1	1.00e+00 **	**3,87**	2.37e-03
Actin binding site repeat 6	0,19	8.76e-02 **	0,19	6.41e-02 **
Actin binding site repeat 7	0,24	1.35e-03	0,44	3.31e-01 **
Middle region	**6,21**	1,22e-04	**13,39**	2.02e-09
Flexible central domain	1,38	2.00e-01 **	**2,08**	1.02e-03
Troponin T-binding regions	0,2	2.90e-04	0,88	1.00e+00 **

In bold: Stronger odds ratio values with statistically significant difference. (**) means p value not significantly different. DCM/LVNC:dilated/left ventricle non-compaction cardiomyopathy.

In the opposite extremity of the molecule the same occurs with the C-terminal portion of the head-tail overlapping junction and the actin-binding site repeat 7. *TPM1* p.Met281Leu is the main variant in this region. Over both protein extremities is one of the troponin T-binding regions, so these same hypertrophic cardiomyopathy variants also collaborate with the relevant enrichment observed in this domain. Finally, the actin-binding site repeat 6 is also overrepresented due to the presence of several

hypertrophic cardiomyopathy variants in this region, but no highly prevalent variant was identified in this protein segment.

The results for hypertrophic cardiomyopathy enrichment versus external controls (from gnomAD) have similar distribution; nonetheless, we observe differences in the actin-binding repeats. The actin-binding repeat 6 is not highlighted here because an elevated number of control individuals is identified in this region. The actin-binding site repeats 2, 4 and 5 appear overrepresented in the analysis with external controls. In the repeat 2, there are important hypertrophic cardiomyopathy variants, such as p.Ala63Thr, p.Leu71Val and p.Glu54Lys. The repeat 5 is detached due to an elevated number of patients carrying the founder Finnish variant, p.Asp175Asn, and the p.Glu192Lys variant. The repeat 4 overrepresentation is likely related to the very low number of control individuals in the gnomAD database. This is a very conservate protein region with the lower number of variants in gnomAD database among all actin-binding repeats. Enrichment analysis for dilated cardiomyopathy variant also showed the repeat 4 overrepresented with an odds ratio higher than in hypertrophic cardiomyopathy (32,92 vs 15,16, respectively), so this region should be probably associated with dilated/left ventricle non-compaction cardiomyopathy.

Phenotype enrichment for dilated cardiomyopathy variants vs internal controls shown that the middle region is overrepresented. Here, we have included the dilated cardiomyopathy and left ventricle non-compaction cardiomyopathy as a single group in comparison with other inherited cardiac phenotypes. The middle region corresponds

to the actin-binding sites repeats 3 and 4, so these periods are also highlighted in this analysis.

When we have compared these variants versus those observed in external controls (gnomAD), the same periods are overrepresented; however, with higher odds ratio values. The actin-binding site repeat 4 has a very high odds ratio value probably due to the low number of control individuals in gnomAD in this database, as commented in the anterior paragraph. This fact also elevated the value observed for the middle region. Further, we also have identified an enrichment in the actin-binding site repeats 2 and 5 in the analysis versus external control.

Our analysis about phenotype enrichment of *TPM1* variants by functional domain pointed out relevant results for variants' pathogenicity classification. We have identified differences for each tropomyosin region according to the cardiomyopathy-type. This finding can be useful to clinical interpretation of the genetic findings in this gene, as well as have been proposed for *MYH7* variants in this same context (Kelly et al, 2018). For enrichment analysis, we have used only the patients sequenced in Health in Code center, due to greater agility in the method. Nonetheless, these results could be compared with *TPM1* cohorts from other sequencing centers to definitive conclusions, since our enrichment analysis could be biased by the origin of our own population. For example, the fact of the high prevalence of the p.Arg21Leu and p.Met281Val hypertrophic cardiomyopathy-variants in the same region (head-tail overlapping junction between two subsequent tropomyosin variants) could be an specific finding in our population.

5.5: *TPM1* truncating and splicing zone variants

We have identified 24 *TPM1* variants that could produce a truncated tropomyosin protein: 19 intronic variants located within the consensus splicing zone (flanking positions -/+ 10) and five frameshift or nonsense-type in *TPM1*. Only variants previously identified in our center or those published in the literature, with an allelic frequency in the gnomAD database <0.1%, have been considered.

Data obtained in our study do not allow establishing familial cosegregation of any of these variants with the disease. Familial cosegregation of these variants was studied in only three families, and the data are inconclusive because there was another pathogenic/likely pathogenic sarcomeric variant in the carriers.

None of these genetic variants appear to be overrepresented in any phenotype, considering their prevalence in carriers versus other cardiovascular phenotypes identified among the 21,670 probands in whom this gene has been sequenced by next generation sequencing in our center.

Among the 24 variants, fifteen were identified in a single carrier. Most of these carriers had hypertrophic, dilated, or non-compaction cardiomyopathy – except three carriers with unrelated *TPM1* phenotypes, respectively, Brugada syndrome and arrhythmogenic cardiomyopathy (2 cases). Three *TPM1* variants had 2 carriers, but the carrier's phenotype was known in only one (hypertrophic cardiomyopathy). Other three *TPM1* variants had 3 carriers (all with hypertrophic cardiomyopathy) and one variant had 4 carriers (hypertrophic cardiomyopathy, dilated cardiomyopathy, another

carrier with unknown phenotype, and the last carrier with a phenotype not related to this gene - long QT syndrome). Only one *TPM1* variant had five carriers listed (all with hypertrophic cardiomyopathy) and, finally, there was a variant with nine carriers (2 hypertrophic cardiomyopathy, one dilated cardiomyopathy, 4 with unknown phenotype an two with phenotypes not related to this gene).

Among these carriers, 40% had an additional pathogenic/likely pathogenic variant in priority genes associated with the respective phenotypes. We could consider that the 60% carriers without an additional genetic variant would be a similar percentage of negative genetic studies in cardiomyopathies, considering the general yield of the genetic study in cardiomyopathies.

It is worth noting that the gnomAD database suggests tolerated haploinsufficiency in this gene (possibly benign). The probability of intolerance to loss of function in *TPM1* is equal to zero (pLI=0), according to this database.

Taking into account the variants registered in our database and in ClinVar, these variants could be classified as possibly benign or as having uncertain clinical significance. As exceptions, we have two variants in the literature; first, ClinVar cites an intronic variant described by England J et al (2020) identified in a patient with tetralogy of Fallot, but no cosegregation analysis was performed, this finding was not consistently reproduced by other groups to date. We consider that this information is insufficient to confirm the pathogenicity of this variant. Further, another variant (c.375-3C>T) was described in the literature in a patient with hypertrophic cardiomyopathy in a Spanish cohort with 104 unrelated cases of non-familial

hypertrophic cardiomyopathy (Núñez L et al, 2013). No specific clinical information on this carrier was published, including any cosegregation data.

In summary, we have considered that intronic variants located in the flanking regions (positions -/+ 10) could be affecting the splicing process; however, our data cannot establish a pathological role. We have identified only five frameshift or nonsense type variants in *TPM1* among more than 21,000 inherited heart diseases patients sequenced in our center. No familial cosegregation study or overrepresentation in affected vs control individuals were observed. Moreover, only two truncating variants were published in the literature; both with inconsistent information to really consider it as disease causing. Public databases with genetic information from the general population suggest that haploinsufficiency would be tolerated in *TPM1.* Therefore, the role of truncating variants in *TPM1* is still unknown. Our data suggest that these variants would be likely bening; however, additional clinical and functional investigations could be necessary to definitely determine their relation with the disease development. All *TPM1* truncating variants identified in this study are listed in table 5.6.

Table 5.6: TPM1 truncating and splicing-zone variants

Protein level	cDNA	Carriers (*n*, families, phenotype)	ClinVar database
p.Glu33Glyfs*10	c.97_98insG	Total carriers: 1 carrier (1 family). Affected or possibly affected: 1 carrier. Brugada Syndrome: 1 carrier.	
p.Glu63*	c.187G>T	Total carriers: 2 carriers (1 family). Affected or possibly affected: 1 carrier. Unknown: 1 carrier. Cardiomyopathy, Hypertrophic + Isolated Noncompaction of the Ventricular Myocardium: 1 carrier.	
p.His65Glnfs*21	c.195delC	Total carriers: 1 carrier (1 family). Affected or possibly affected: 1 carrier. Cardiomyopathy, Hypertrophic + Cardiac Conduction System Disease: 1 carrier.	
p.Glu40*	c.118G>T	Total carriers: 1 carrier (1 family). Affected or possibly affected: 1 carrier. Cardiomyopathy, Dilated: 1 carrier.	Uncertain significance, Conditions: Cardiomyopathy Hypertrophic cardiomyopathy not specified Cardiovascular

			phenotype not provided
p.Ile143Phefs*6	c.427delA	Total carriers: 1 carrier (1 family). Affected or possibly affected: 1 carrier. Cardiomyopathy, Hypertrophic: 1 carrier.	
	c.115-7C>A	Total carriers: 1 carrier (1 family). Affected or possibly affected: 1 carrier. Cardiomyopathy, Hypertrophic: 1 carrier.	
	c.241-6A>G	Total carriers: 5 carriers (5 distinct families). Affected or possibly affected: 5 carriers . Cardiomyopathy, Hypertrophic: 5 carriers.	
	c.375-3C>T	Total carriers: 1 carrier (1 family). Affected or possibly affected: 1 carrier. Cardiomyopathy, Hypertrophic: 1 carrier.	
	c.375-5T>C	Total carriers: 9 carriers (5 distinct families). Affected or possibly affected: 5 carriers . Unknown: 4 carriers . Cardiomyopathy, Hypertrophic: 2 carriers . Cardiomyopathy, Dilated: 1 carrier. Arrhythmogenic Right Ventricular Dysplasia: 1 carrier. Sudden Infant Death: 1 carrier.	Likely benign, Conditions: Cardiomyopathy Hypertrophic cardiomyopathy not specified not provided
	c.493-7G>A	Total carriers: 3 carriers (3 distinct families). Affected or possibly affected: 3 carriers . Cardiomyopathy, Hypertrophic: 3 carriers .	Uncertain significance, Conditions: Cardiomyopathy
	c.493-6C>T	Total carriers: 1 carrier (1 family). Affected or possibly affected: 1 carrier. Arrhythmogenic Right Ventricular Dysplasia: 1 carrier.	Uncertain significance, Conditions: Dilated cardiomyopathy 1Y Familial hypertrophic cardiomyopathy 3 not specified
	c.640-10C>G	Total carriers: 1 carrier (1 family). Affected or possibly affected: 1 carrier. Sudden death: 1 carrier.	
	c.773-6T>A	Total carriers: 1 carrier (1 family). Affected or possibly affected: 1 carrier. Isolated Noncompaction of the Ventricular Myocardium: 1 carrier.	
	c.773-3T>C	Total carriers: 3 carriers (2 distinct families). Affected or possibly affected: 3 carriers . Cardiomyopathy, Hypertrophic: 3 carriers .	Conflicting interpretations of pathogenicity, Conditions: Cardiomyopathy Hypertrophic cardiomyopathy
	c.852-7C>G	Total carriers: 2 carriers (2 distinct families). Affected or possibly affected: 2 carriers . Cardiomyopathy, Hypertrophic: 1 carrier. Cardiomyopathy, Dilated: 1 carrier.	
	c.852-5C>T	Total carriers: 1 carrier (1 family). Affected or possibly affected: 1 carrier. Cardiomyopathy, Dilated: 1 carrier.	
	c.665-4A>G	Total carriers: 1 carrier (1 family). Affected or possibly affected: 1 carrier. Cardiomyopathy, Hypertrophic + Bicuspid Aortic Valve: 1 carrier.	
	c.114+2T>C	Total carriers: 1 carrier (1 family). Affected or possibly affected: 1 carrier. Tetralogy of Fallot: 1 carrier.	Likely pathogenic, Conditions: Congenital heart disease
	c.240+3A>G	Total carriers: 1 carrier (1 family). Affected or possibly affected: 1 carrier. Cardiomyopathy, Hypertrophic: 1 carrier.	
	c.240+6T>C	Total carriers: 2 carriers (1 family). Affected or possibly affected: 1 carrier. Unknown: 1 carrier. Cardiomyopathy, Dilated: 1 carrier.	
	c.240+8C>T	Total carriers: 1 carrier (1 family). Affected or possibly affected: 1 carrier. Cardiomyopathy, Dilated: 1 carrier.	
	c.240+9A>G	Total carriers: 4 carriers (2 distinct families). Affected or possibly affected: 2 carriers . Unknown: 1 carrier. Unaffected or healthy: 1 carrier. Cardiomyopathy, Hypertrophic: 1 carrier. Long QT Syndrome: 1 carrier.	
	c.132+8A>T	Total carriers: 1 carrier (1 family). Affected or possibly affected: 1 carrier. Atrial Fibrillation: 1 carrier.	

	c.493-7G>A	Total carriers: 3 carriers (3 distinct families). Affected or possibly affected: 3 carriers . Cardiomyopathy, Hypertrophic: 3 carriers .	Uncertain significance, Conditions: Cardiomyopathy
	c.493-6C>T	Total carriers: 1 carrier (1 family). Affected or possibly affected: 1 carrier. Arrhythmogenic Right Ventricular Dysplasia: 1 carrier.	Uncertain significance, Conditions: Dilated cardiomyopathy 1Y Familial hypertrophic cardiomyopathy 3 not specified

5.6: *TPM1* variants prevalence by phenotype

We have identified 184 *TPM1* index cases among 21,670 probands with inherited heart disease sequenced in our center. It corresponds to less than 1% (0.85%) of the total probands. Index cases with hypertrophic cardiomyopathy were 143 among 9,113 probands in the cohort with this phenotype (1.56%). *TPM1* variants prevalence in our cohort was near to those reported for this phenotype in the literature. European guidelines on hypertrophic cardiomyopathy (Elliott PM et al 2014) have reported a prevalence of 1-5%. Higher prevalence (5%) was based on small cohorts of hypertrophic cardiomyopathy, in which the authors had suggested that it is likely related with some possible founder variants in specific populations (Yamauchi-Takihar et al, 1996).

In our dilated cardiomyopathy group, we have identified a prevalence of 0.6% (29/4,794 probands) that is lower to those reported by Lakdawala et al (2012) who studied a small cohort (1-2% in a small cohort with 264 patients). Taking into account only the left ventricle non-compaction cohort, we have identified 3/953 probands with this phenotype (0,31%).

Finally, we have identified a prevalence 0.31% (3/953 probands) in left ventricle non compaction cardiomyopathy, which is smaller than the prevalence reported by Kayvanpour E et al (2019). In this last publication, the authors have investigated a selected cohort of non-compaction cardiomyopathy associated with congenital heart defects with predominantly pediatric patients, so a higher *TPM1* variants prevalence (2%) observed by this group could be related with a selection bias. We have selected our *TPM1* carriers from a consecutive cohort with more than 21,000 sequenced probands with inherited cardiomyopathies composed of pediatric and adult patients, therefore, we have obtained a general prevalence differently from that in specific age groups, such as pediatric cohorts.

6. *TPM1* General clinical data

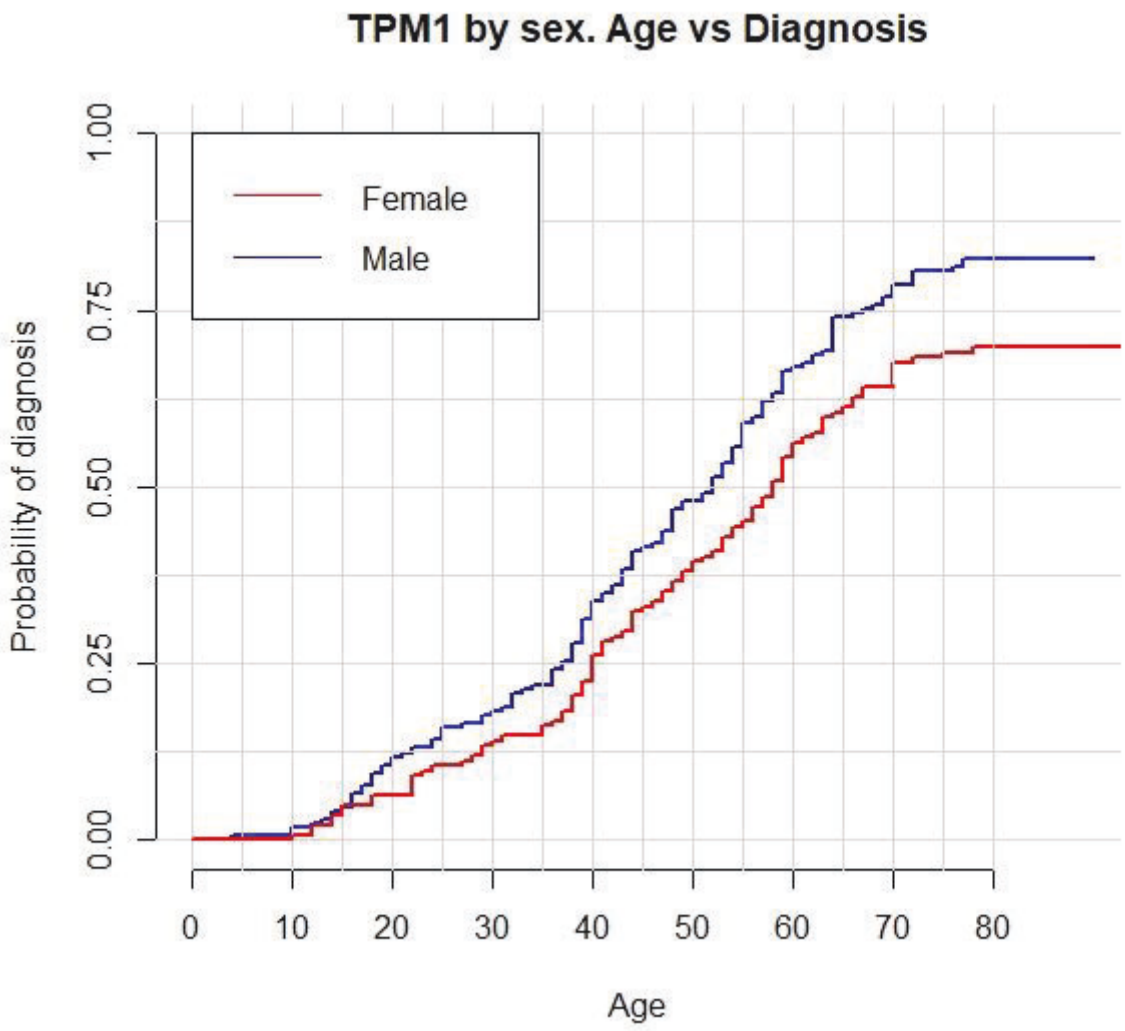
Clinical data on *TPM1* carriers are presented here exclusively based in the Supplementary Material - Excel table chapter 6. Detailed clinical features can be found in this appendix, except for the p.Arg21Leu variant carriers in which the genotype-phenotype analysis was described in the chapter 4 of this doctoral thesis.

Fifty-one centers from all Europe (mainly Spain) have participated in this project, sending for us essential genetic and clinical data to reach the main objective of our investigation. In total, we have identified 380 individuals carrying *TPM1* variants, including the p.Arg21Leu variant cohort.

We would like to start our clinical data analysis on *TPM1* carriers carrying out a general analysis of age of diagnosis and survival for all carriers, independently of the groups by phenotype (hypertrophic and dilated/left ventricle non compaction cardiomyopathy). Age of diagnosis graph presented in the next page (Fig. 6.1) shows probability of diagnosis for *TPM1* carriers by age. Very few carriers were diagnosed under the age of 10 years old, and in both groups we can observe an increase of the diagnosis starting at the second decade of life. From the age of 15 years, there is a higher probability of diagnosis in males than female carriers. This tendency of a higher percentage of diagnosis in men apparently continues until advanced ages (p value < 0.05). At the age of 20 years old, 12.5% of the male carriers had been diagnosed, which was around 5% more than in females. This difference keeps similar until the age of 50 years old, when the percentage of difference rose 10%. At older ages,

approximately 14% of the male carriers and 27.5% of females had no diagnosis, suggesting incomplete penetrance.

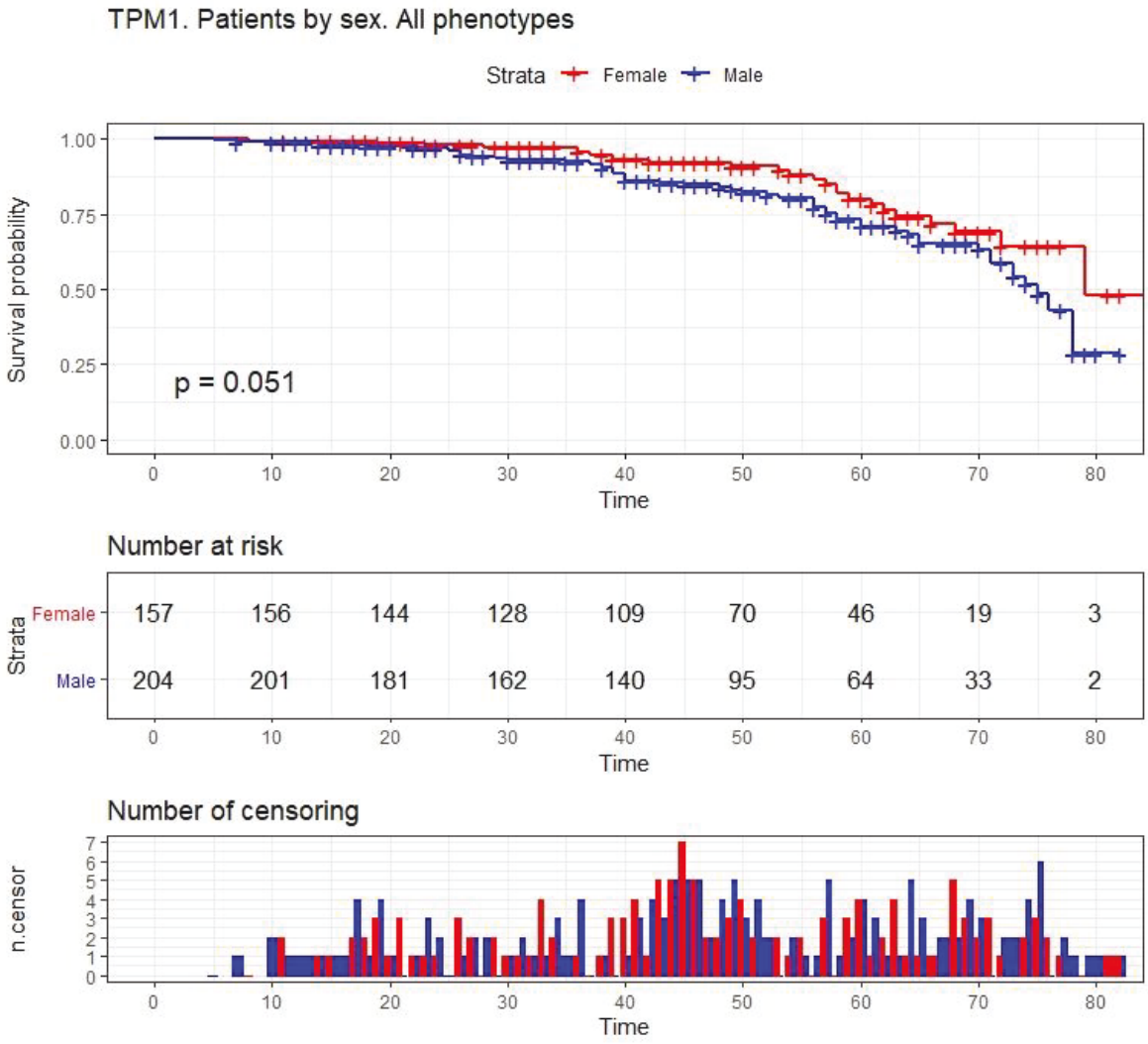
Figure 6.1: TPM1 General age of diagnosis



Survival analysis by Kaplan-Meyer method is presented in the figure 6.2 (next page). No statistically significant difference was observed between male and female carriers (p value = 0.051). Mortality at the age of 40 years is 12.5%, and this rate is lower than 25% at the age of 50 years. Curve inclination in the graph after the 55 years old could suggest a

higher incidence of cardiovascular events between 55-65 years old approximately. Mortality at the age of 70 years is close to 37.5%.

Fig. 6.2: TPM1 General survival analysis



6.1: Clinical data analysis by phenotype

Can we say that there is a specific cardiomyopathy-type for each *TPM1* variant? Several sarcomeric genetic variants have been associated with the development of inherited cardiomyopathies. Our experience has demonstrated that most of the variants (not all) in these genes have been identified in patients with a specific cardiomyopathy presentation,

for example hypertrophic or dilated cardiomyopathy; nonetheless, several of these genetic variants have been reported in association with both cardiac phenotypes. Therefore, how would be the behavior in *TPM1*? We have divided our *TPM1* clinical analysis in two different phenotype groups, hypertrophic cardiomyopathy and dilated/left ventricle non-compaction cardiomyopathy. It could be considered initially a mistake, but we would like to do some particular considerations in order to embassy this division.

First, we need to consider that the risk stratification and familial screening parameters on hypertrophic cardiomyopathy or dilated cardiomyopathy have been performed taking into account different clinical markers, since these are distinct pathologies with different clinical courses. So, clinical data analysis should be carried out with particular clinical criteria by phenotype, and two different subgroups of patients can offer a better clinical characterization. Further, evidence presented in the literature from clinical and functional studies (such as we have shown in the first chapter) have suggested the existence of two phenotype groups associated with *TPM1* variants. Several functional studies have suggested that *TPM1* variants previously associated with hypertrophic cardiomyopathy have opposite effects on cardiac contractility when compared with variants identified in dilated cardiomyopathy patients (differences explained through calcium-sensitivity mechanism). Additionally, we also have observed in the chapter 1 of this Phd thesis that no clinical report with the same *TPM1* variant was described to date with a pedigree presenting different forms of cardiomyopathy. Rare *TPM1* hypertrophic cardiomyopathy pedigrees were reported with individuals with left ventricle dilatation; however, they were clearly reported as end-stage (burn-out) hypertrophic cardiomyopathy. Similar profile was observed among the

carriers from our cohort. No patient was reported as dilated cardiomyopathy in families having hypertrophic cardiomyopathy as main phenotype, and vice versa.

Moreover, we have documented ventricular hypertrabeculation in some patients with hypertrophic cardiomyopathy, but they did not fulfill left ventricle non-compaction cardiomyopathy diagnosis criteria. We have described just a few carriers with the diagnosis of non-compaction cardiomyopathy, and they had left ventricular dilatation/dysfunction or they were carrying genetic variants also identified in other families with dilated cardiomyopathy. So, we have added these patients into the dilated cardiomyopathy cohort, constituting the dilated/left ventricle non compaction cardiomyopathy group.

Finally, we can observe in our list containing all *TPM1* variants (Supplementary Material - Excel table chapter 5) that some of these genetic variants are described in ClinVar database with different forms of cardiomyopathy. It could be an argument against our division by groups; nonetheless, we need to consider that this information has some uncertainties. ClinVar does not report clinical details from each subscriptor, sometimes reflecting in their clinical phenotype section the information regarding the NGS panel applied in each case by the laboratory center. These contradictions were not found in our cohort, or in the literature.

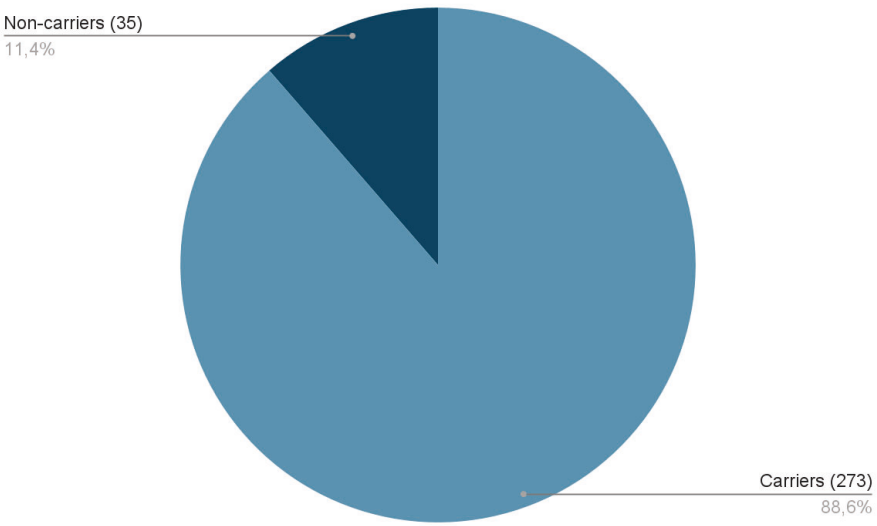
Following the tendency of the previous publications about the *TPM1* gene, we have divided our general cohort into two different groups: (a.) hypertrophic cardiomyopathy, and (b.) dilated/left ventricle noncompaction cardiomyopathy. In the hypertrophic cardiomyopathy group, 308 individuals were listed. Other 72 individuals were added to the dilated/left ventricle noncompaction cardiomyopathy group. No patient was reported with

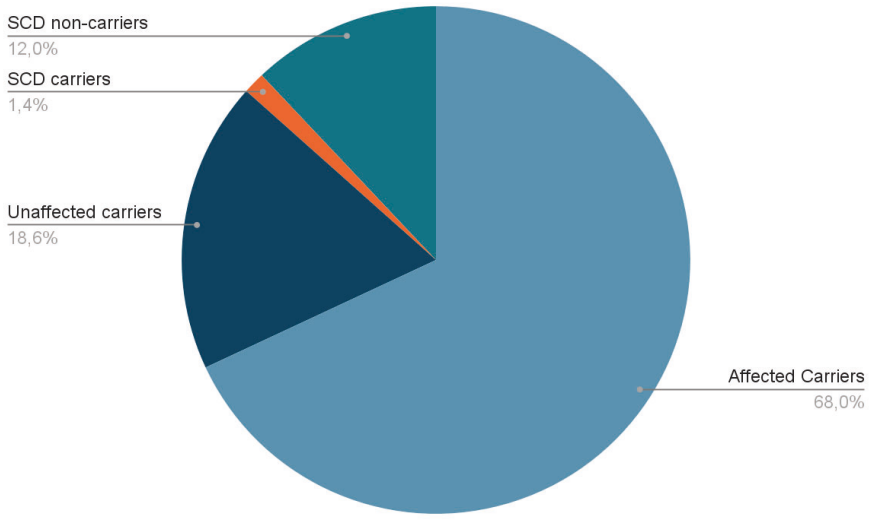
arrhythmogenic cardiomyopathy or isolated congenital heart defect, although this last phenotype had been described in association with a cardiomyopathy phenotype in both groups.

6.2: Hypertrophic Cardiomyopathy patients

We have identified 308 individuals from different 115 families with hypertrophic cardiomyopathy, totalizing 273 carriers (256 with available clinical data). Among those reported with hypertrophic cardiomyopathy diagnosis, 198 (77%) were affected carriers. Only a single carrier (0,45%) was reported as restrictive cardiomyopathy due an important diastolic dysfunction; however, he had hypertrophy of specific myocardial segments. Fifty-four (21%) individuals (family members) were unaffected carriers. Four carriers (1,55%) were reported with unexplained sudden cardiac death phenotype. Additionally, 35 first- and second degree relatives with unknown genotypes were also reported as unexplained sudden cardiac deaths.

Fig. 6.3: TPM1 Hypertrophic cardiomyopathy patients overview.





Congenital heart defects in hypertrophic cardiomyopathy phenotype. Positive family history of heart defects was reported in 9.5% of the families (11/115). Five carriers (1,9%; 5/257) were identified with a heart defect associated with the main phenotype. One patient diagnosed with hypertrophic cardiomyopathy at the age of 17 years carrying the p.Gly126Val variant, and an asymptomatic 40 years old hypertrophic cardiomyopathy patient with the p.Asp175Asn variant were described with a bicuspid aortic valve. Another carrier of the p.Asp175Asn variant was diagnosed with hypertrophic cardiomyopathy at the age 47 years old, and he had a congenital ventricular septal defect. Two atrial malformations were also reported among the patients from our hypertrophic cardiomyopathy cohort; an interatrial aneurysm (p.Gln210Arg), and an atrial septal defect (p.Tyr221Cys) in hypertrophic cardiomyopathy patients diagnosed with 68 and 32 years old, respectively.

Hypertrophic cardiomyopathy variants. Among these 115 hypertrophic cardiomyopathy families, thirty-one *TPM1* variants were identified, all missense-type variants. Three variants were particularly more prevalent; p.Arg21Leu (67 carriers), p.Met281Val (53 carriers), and p.Asp175Asn (36 carriers). Clinical data about the carriers

with these three variants versus all *TPM1* carriers can be compared in Table 6.1. No significant statistical difference could be identified among these different specific variants, and in the group composed by all TPM1 variants.

Table 6.1: Clinical features of TPM1 carriers by variant in hypertrophic cardiomyopathy.

	n=67		*n*=53		*n*=36		*n*=257	
	p.Arg21Leu	**%**	**p.Met281Val**	**%**	**p.Asp175Asn**	**%**	**All variants**	**%**
Affected carriers	53/67	79.1	35/53	66	17/36	47.2	186/257	72.4
Probands	31/67	46.3	27/53	50.9	8/36	22.2	115/257	44.7
Female sex	33/67	49.4	23/53	43.4	14/36	38.8	127/257	49.4
Age (years)								
At diagnosis	46.4 (±19.2)	range 11-73	51.4 (±13.8)	range 13-78	44 (±16.9)	range 16-69	45.1 (±17.1)	range 11-78
Symptons								
NYHA II	9/53	17	8/35	22.8	3/14	21,4	39/186	20.9
NYHA III/IV	5/53	9.4	2/35	5.7	0		16/186	8,6
Syncope	5/53	9.4	0		2/14	14.2	15/186	8.1
ECG								
Atrial fibrillation	6/53	11.3	6/35	17	3/14	21.4	35/186	18.8
AVB	6/53	11.3	2/35	5.7	2/14	14.2	11/186	5.9
NSVT	8/53	15.1	3/35	8.5	5/14	35.7	40/186	21.5
IMAGING								
LVH (mm)	44/53 21.4 (±7.65)	83	31/35 17 (±6.5)	88.6	12/14 15.7 (±4.1)	85.7	144/186 18 (±4.3)	77.4
Left atrial dilatation (mm)	26/44 40.38 (±6.7)	59.1	16/31 45 (±10.5)	51.6	6/12 36 (±7.5)	50	88/144 45.7 (±6.5)	61.1
LVOTO (mmHg)	15/44 67.4 (±33.8)	34.1	7/31 46 (±13.7)	22.5	1/12 75	7.1	36/144 55.9 (±21.2)	25
LGE on MRI	18/44	38.9	7/31	22.5	1/12	8.3	62/144	43
Diastolic dysfunction								
Grade I	14/44	31.8	9/31	29	5/12	41.6	29/144	20.1
Grade II/III	8/44	18.2	5/31	16.1	1/12	8.3	37/144	25.7
Abnormal exercise testing	5/44	11.4	1/16	6.2	0/12		9/144	6.25

TREATMENT								
Pacemaker	1/53	1.9	1/35	2.85	4/14 *	28.5	16/186	11.1
ICD	4/53	7.5	3/35	8.5	2/14	14.2	34/186	18.2
Primary prevention	4/4	100	3/3	100	2/2	100	26/34	76.5
Appropriate discharge	0		0		0		5/34	14.7
Cardiac surgery	4/53	7.5	2/35	5.7	2/14	14.2	13/186	
Heart transplant	1/53	1.9	0		0		2/186	

AVB = atrioventricular block. ECG = electrocardiogram. ICD = implanted cardio defibrillator. LGE = Late gadolinium enhancement. LVH = left ventricle hypertrophy. LVOTO = Left ventricle outflow tract obstruction. MRI = Cardiac magnetic resonance. NSVT = nonsustained ventricular tachycardia. NYHA = New York Heart Association. Cardiac surgery: Heart transplant or septal reduction therapy. (*) Two pacemaker implants were reported as “old HCM treatment” without cardiac conduction disease.

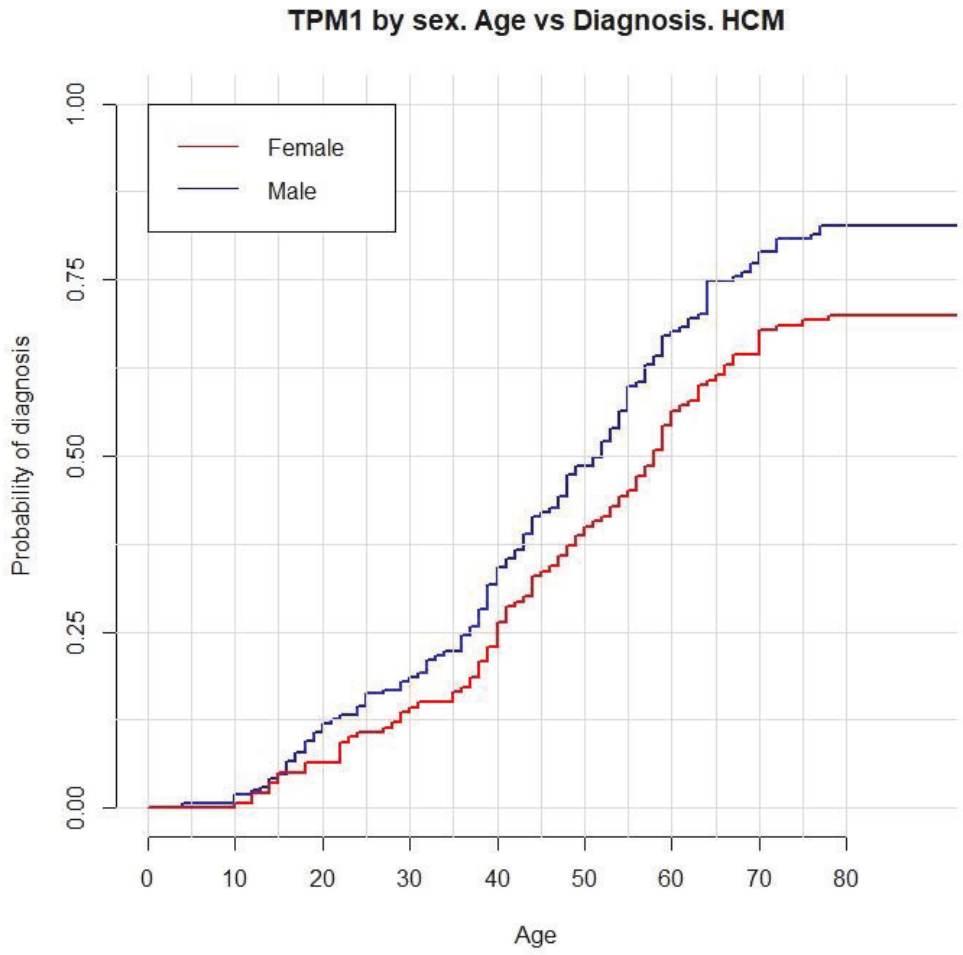
Additional genetic variants in the hypertrophic cardiomyopathy group. Thirty nine carriers were diagnosed with an additional genetic variant. Only two [5,1%] of them had 2 or more genetic variants) in other relevant cardiomyopathy and sudden cardiac death genes. No homozygous carrier was identified, except the four previously described with the *TPM1* p.Arg21Leu variant. Among them, five (12.8%) were diagnosed under the age of 35 years. Left ventricle hypertrophy greater than 25 mm was observed in four (10.2%) carriers with an additional genetic variant. Major cardiovascular events were reported in only two (5%) carriers with an additional genetic variant. Both had multiple appropriated cardiac defibrillator discharges. Minor cardiovascular events were reported in three (7.6%) other carriers from this group: two non-fatal strokes, and one septal reduction therapy.

6.2.1: Hypertrophic cardiomyopathy age of diagnosis

Age of diagnosis in hypertrophic cardiomyopathy carriers by age is shown in Figure 6.4. This curve is very similar with the age of diagnosis curve above presented for all carriers, because most of the carriers in our cohort had hypertrophy ventricular. Probability of diagnosis was 12.5% in men at the age of 20 years. Only one individual (a non-genotyped

relative) suffered a sudden cardiac death at an age lower than 10 years. At the age of 40 years old, 30% of the male carriers had been diagnosed with hypertrophic cardiomyopathy versus 26% in female carriers. Mean age at diagnosis was 45.±17 years (range 11-78) taking into consideration both genders together.

Figure 6.4: HCM Age of diagnosis

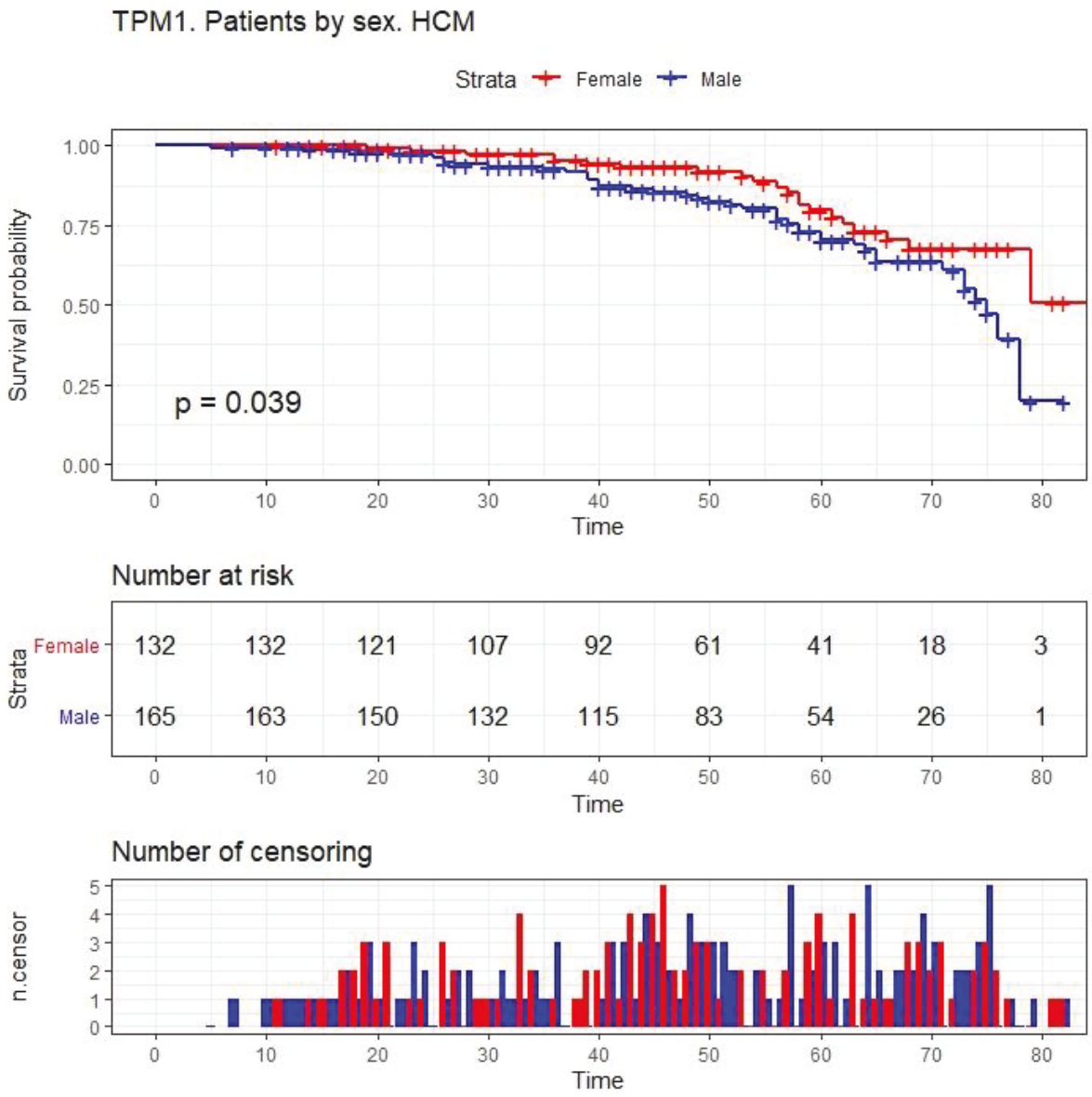


6.2.2: Hypertrophic cardiomyopathy survival analysis

Figure 6.5 shows survival analysis by Kaplan-Meier method. We can observe less than 5% mortality at the age of 20 years old, and this percentage rises 12.5% in males at the age

of 40 years old, and 6% in female carriers. At 60 years old, the mortality rate is at 30% for male carriers, and 23% for female carriers. Most of the cardiovascular events were reported between the ages 50 and 60 years in both sexes, with another significant increase in mortality after 70 years old in males. Significant statistical difference was observed between male and female carriers (p value = 0.039).

Figure 6.5: HCM Survival analysis



Eighteen cardiovascular events were reported among *TPM1* carriers (see table 6.2) from our hypertrophic cardiomyopathy cohort. Three heart failure-related events and twelve arrhythmic-related events were reported in carriers. Other two unspecified cardiac deaths and one stroke-related death were also reported in this group. Fifteen minor cardiovascular events in carriers are listed in the same table too. Cardiovascular events in *TPM1* p.Arg21Leu carriers previously presented in chapter 4 were also included in the table below.

Table 6.2: Major and minor adverse cardiovascular events reported in the TPM1 HCM-carriers.

Sex	Variant	Major CV event (age)	Observations
CARRIERS			
1. Male	p.Gln9Lys	ICD appropriated discharge (41)	Echo 14 mm *FHOD3* p.Arg637Gln, *DES* p.Ser72Arg (Homoz)
2. Male	p.Arg21Leu	Sudden death (55)	No left ventricle hypertrophy.
3. Male	p.Arg21Leu	Heart transplant (48)	
4. Female	p.Arg21Leu	Heart failure death (68)	Systemic sclerosis (Pulmonary fibrosis).
5. Obligated carrier, female	p.Arg21Leu	Unspecified cardiac death (79)	Unavailable clinical data.
6. Male	p.Glu25Asp	Sudden death (26)	Sudden death, first cardiac manifestation.
7. Male	p.Ala63Val	Sudden death (28)	
8. Male	p.Leu71Val	Sudden death (77)	Restrictive left ventricular filling pattern.
9. Male	p.Arg91Cys	Sudden death (26)	Sudden death, first cardiac manifestation.
10. Male	p.Ile172Thr	Sudden death (72)	Restrictive left ventricular filling pattern. Previous ICD appropriate discharge.
11. Female	p.Asp175Asn	Sudden death (64)	
12. Male	p.Asp175Asn	Sudden death (25)	Autopsy suggested HCM.
13. Female	p.Glu192Lys	Heart failure death (51)	Previous heart transplant. Previous non-fatal stroke.
14. Female	p.Glu192Lys	Sudden death (63)	
15. Male	p.Glu192Lys	ICD appropriated discharge (<64)	
16. Male	p.Tyr221Cys	Unspecified death cause (33)	Died in sleep. ICD check no arrhythmias.
17. Female	p.Asn267Ser	ICD appropriated discharge (46)	Truncating MYBPC3 variant

18.	Male	p.Met281Val	Stroke-related death (75)	Extensive ischemic stroke. *PKP2* p.Val590Ile without arrhythmogenic phenotype.
Minor adverse CV events (age) **				
CARRIERS				
1.	Female	p.Arg21Leu	Septal myectomy (23)	Echo 33,5 mm
2.	Male	p.Arg21Leu	Septal myectomy (32)	Echo 51 mm
3.	Female	p.Arg21Leu	Mitral v. replacement (41)	Systolic anterior motion of mitral valve.
4.	Male	p.Arg21Leu	Non-fatal stroke (65)	
5.	Female	p.Arg21Leu	Non-fatal stroke (73)	
6.	Male	p.Ile172Thr	Non-fatal stroke (50)	
7.	Male	p.Asp175Asn	Septal myectomy (46)	Echo 24 mm, basal gradient 75 mmHg. Pacemaker.
8.	Male	p.Asp175Asn	Septal myectomy (65)	Basal gradient 70 mmHg.
9.	Female	p.Glu192Lys	Non-fatal stroke (75)	End-stage HCM. TNNT2 p.Arg286His.
10.	Female	p.Ser215Leu	Septal myectomy (40)	
11.	Male	p.Ala262Thr	Septal myectomy (45)	Echo 22 mm, basal gradient 69 mmHg.
12.	Male	p.Met281Val	Non-fatal stroke (60)	Restrictive cardiomyopathy without hypertrophy.
13.	Male	p.Met281Val	Septal myectomy (58)	Echo 21 mm
14.	Male	p.Met281Val	Septal myectomy (<70)	Echo 31 mm
15.	Male	p. Ile284Val	Septal myectomy (57)	Echo 22 mm, basal gradient 74 mmHg.

** Minor adverse cardiovascular events were not included in the survival curves.

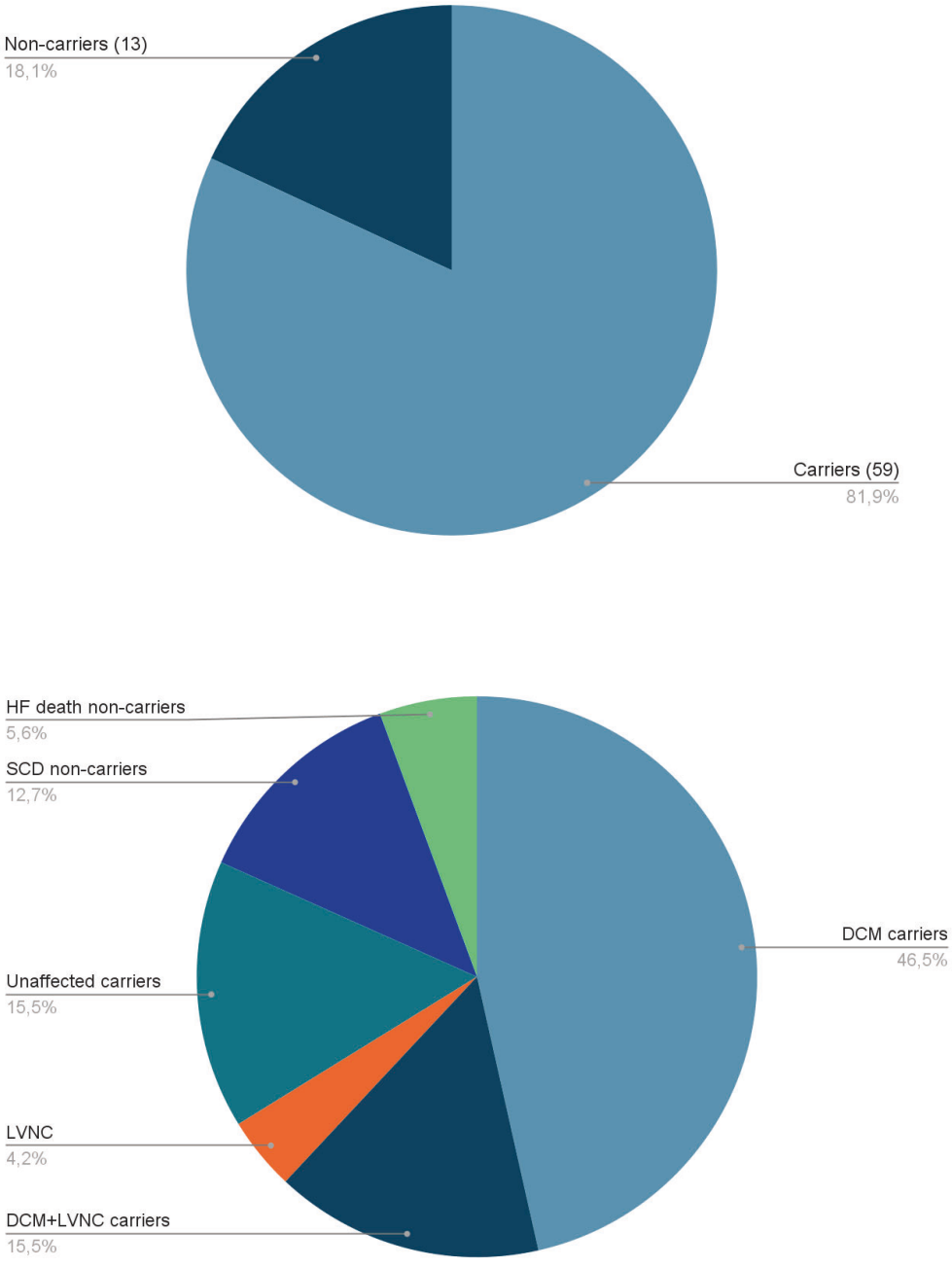
Other 35 sudden cardiac deaths were reported in first- and second-degree relatives. Nonetheless, clinical data on these patients is very limited for a detailed analysis. Five of them were diagnosed in early ages (under 35 years old) with 5, 14, 18, and 35 years in male relatives, and 23 years old in a female relative, respectively.

6.3: Dilated/Left ventricle non-compaction cardiomyopathy

We have identified 72 individuals from different with dilated or left ventricle non-compaction cardiomyopathy phenotype, totalizing 59 carriers from 29 families (twenty-four probands with dilated cardiomyopathy, four with dilated/left-ventricle non-compaction overlapping phenotype, and a single proband with left ventricle non-compaction cardiomyopathy without ventricular dilatation). Thirty relatives were

carriers, 19 affected and 11 unaffected relatives. Further, four individuals were reported with heart failure death, and nine were reported with cardiac sudden/arrhythmic death without genetic testing, in total 13 non-carriers relatives. Overview on dilated/left ventricle non-compaction cardiomyopathy group is represented in the figure 6.6 (next page).

Fig. 6.6: TPM1 Dilated/left ventricle non-compaction cardiomyopathy patients overview.



Congenital heart defects in dilated/left ventricle non-compaction cardiomyopathy phenotype. Positive family history of heart defects was reported in nine probands (15,2%). Four carriers (6,8%) were identified with a heart defect associated with the main phenotype. One proband diagnosed with dilated cardiomyopathy at the age of 25 years carrying the p.Asp84Asn was described with bicuspid aortic valve, and anomalous coronary arteries. Two dilated cardiomyopathy probands were diagnosed with atrial septal defect (p.Ile130Thr and p.Glu180Gly), one of them diagnosed at the first year of life with pulmonary valve stenosis in association. Ebstein's anomaly was observed in a single left ventricle non-compaction cardiomyopathy without ventricular dilatation proband (p.Lys152Glu) diagnosed at adult age.

Clinical Features of carriers with dilated or left ventricle noncompaction cardiomyopathy is summarized in Table 6.3.

Table 6.3: DCM/LVNC Clinical Features (*n*=59)

	All carriers *n*=59			Index cases *n*=29		
		%	Standard desviation		%	Standard desviation
Female sex	24/59	40.6		9/29	31	
Age (years)						
At diagnosis	42	range 1-77	±20	39	range 1-77	±21
Last evaluation	45,7	range 7-80	±19.1	50	range 11-80	±19
NYHA II						
First evaluation	14/59	23.7		10/29	34.5	
Last evaluation	16/53	30.1		12/28	42.8	
NYHA III/IV						
First evaluation	9/59	15.2		5/29	17.2	
Last evaluation	4/53	7.5		3/28	10.7	
Syncope						

First evaluation	1/58	1.7		1/28	3.5	
Last evaluation	1/52	1,92		0/28		
ECG						
Atrial fibrillation						
First evaluation	8/59	13.55		4/29	13.8	
Last evaluation	9/53	17		4/28	14.2	
AVB 2º-3º	0/29			0/29		
NSVT						
First evaluation	9/43	20.9		5/24	20.8	
Last evaluation	12/53	22.6		8/27	29.6	
IMAGING						
LVEDD (mm)						
First evaluation		55.3	±12.1		59	±10.3
Last evaluation		56.4	±7.9		57,85	±8,9
LVEF (%)						
First evaluation		44	±17		35	±14
Last evaluation		45	±11		42	±11
LGE on MRI *	8/15	53.3		5/12	41.6	
Hypertrabeculation **	8/52	15.4		5/29	17.2	
TREATMENT						
Pacemaker	2/57	3.5		1/28	3.5	
ICD	14/57	24.5		11/28	39.2	
Primary prevention	14/57	24.5		11/28	39.2	
Appropriate discharge	1/49	2		1/28	3.5	
CRT	2/57	3.5		1/28	3.5	
HF admission	9/57	15.7		7/28	25	
LVAD	0/57			0/28		
Cardiac transplant	1/57	1.7		1/28	3.5	
FAMILY HISTORY						
Family History of CMP				8/29	27.5	
Family History of CHD				9/29	31	
Family History of SCD						
1º degree				9/29	31	
1º degree >35 yo				6/29	20.6	
Other				6/29	20.5	

[*] At the moment of diagnosis. [**] Hypertrabeculation without left ventricle noncompaction cardiomyopathy diagnosis criteria.

AVB = Atrioventricular block. CHD = Congenital Heart defect. CMP = Cardiomyopathy. CRT = Cardiac rechronization therapy. ECG = electrocardiogram. ICD = implanted cardio defibrillator. HF = Heart Failure. LGE = Late gadolinium enhancement. LVEDD = Left ventricle diastolic diameter. LVEF = left ventricle ejection fraction. LVAD = Left ventricle assistance device.. LVOTO = Left ventricle outflow tract obstruction. MRI = Cardiac magnetic resonance. NSVT = nonsustained ventricular tachycardia. NYHA = New York Heart Association. SCD = Sudden cardiac death.

Additional genetic variants in the dilated/left ventricle non-compaction cardiomyopathy group. Among these 29 families, twenty-three *TPM1* variants were identified, all missense-type variants (see table 6.4). Five variants were recurrents in different families; p.Asp84Asn, p.Ala109Val, p.Leu113Val, p.Ala120Val, and p.Lys128Gln. Seven families were reported with an additional genetic variant in relevant cardiomyopathy genes. Some particularities on genotype-phenotype correlation must be highlighted among these cases with a second genetic variant:

1) *TPM1* p.Gln47Lys was identified in a 7-years-old proband with dilated cardiomyopathy/left ventricle non-compaction overlapping. He was a homozygous carrier of the variant *SCN5A* p.Ser1103Tyr, of unknown clinical significance. No cardiac conduction disease or sudden cardiac death was documented in the family. His 37-years-old mother had left ventricle non-compaction without ventricular dilatation. She was a heterozygous carrier of both variants.

2) Three dilated cardiomyopathy probands were identified carrying the *TPM1* p.Asp84Asn variant, and no cosegregation analysis was possible to be performed. One proband was reported with a relative that suffered a sudden cardiac death at the age of 38 years; however, he also was a carrier of the *DSP* p.Arg2123Ser, a unknown significance desmosomal variant that could be increasing the arrhythmogenic potential in this family.

3) *TPM1* p.Ala109Val variant was identified in two unrelated families, both probands presenting with dilated cardiomyopathy. In one of these families, the index case was a carrier only of this *TPM1* variant, nonetheless, four relatives were reported with an additional genetic variant, the pathogenic *LMNA* p.Arg298Cys. Two of them were unaffected at the ages 34 and 41 years, respectively. Furthermore, two sudden cardiac deaths were reported in relatives without genetic testing.

4) Left ventricle non compaction associated with dilated cardiomyopathy was observed in eight individuals from two unrelated families, carrying the *TPM1* p.Leu113Val variant. One proband was diagnosed at the age 12 years old, and he had two affected relatives at pediatric ages and other two affected relatives older than 30 years old. Some of these individuals were carriers of the *DSP* p.Arg1458Gly variant, and some of them only carriers of the *FHOD3* p.Ser1337Leu variant, both of uncertain significance. The association of a second variant seems not to be related with earlier onset, malignant arrhythmias, right ventricle systolic dysfunction or a greater myocardial hypertrophy as well as no cardiovascular was reported.

5) The third *DSP* variant identified in our dilated cardiomyopathy cohort was the p.Asp1732Asn variant of uncertain diagnosis. The index case was a female carrier of the *TPM1* p.Lys128Gln variant, and she was 68 years old at her last evaluation. Both familial variants were also identified in an unaffected carrier with 39-years-old. A heart failure death was reported in a 29-years-old male relative (unknown genotype). No sign of arrhythmogenic cardiomyopathy was identified among these patients.

Table 6.4: Number of DCM/LVNC affected and unaffected carriers by variant and phenotype.

TPM1 Variant	Affected / Unaffected carrier (Families)	Phenotype
p.Glu23Gln	1 (1)	DCM
p.Ser36Asn	2 / 1 (1)	DCM
p.Ser36Gly	1 (1)	DCM
p.Leu43Val	1 (1)	DCM
p.Gln47Lys	2 (1)	LVNC/DCM
p.Ala63Pro	3 (1)	LVNC/DCM
p.Glu75Gln	1 (1)	DCM
p.Asp84Asn	3 (3)	DCM
p.Val85Leu	1 (1)	DCM
p.Ala109Val	4 / 3 (2)	DCM
p.Leu113Val	8 (2)	LVNC/DCM
p.Ala120Val	3 (2)	DCM
p.Ser123Thr	2 (1)	DCM
p.Met127Thr	1 (1)	DCM
p.Lys128Gln	2 / 3 (2)	DCM
p.Ile130Thr	1 (1)	DCM
p.Lys152Glu	2 (1)	LVNC/no DCM
p.Ile171Val	1 (1)	DCM
p.Glu180Gly	1 (1)	DCM
p.Thr201Met	2 / 4 (1)	DCM
p.Asp230Asn	3 (1)	DCM
p.Thr237Ser	2 (1)	DCM
p.Asp275Tyr	1 (1)	DCM

DCM = Dilated cardiomyopathy. LVNC = Left ventricle noncompaction cardiomyopathy.

6) Two family members affected with dilated cardiomyopathy were diagnosed at the ages 32 and 45 years, respectively. They are carriers of the *TPM1* p.Thr237Ser variant, and also of the likely pathogenic variant in *TTN* p.Arg19338*. No cardiovascular event was reported in this pedigree.

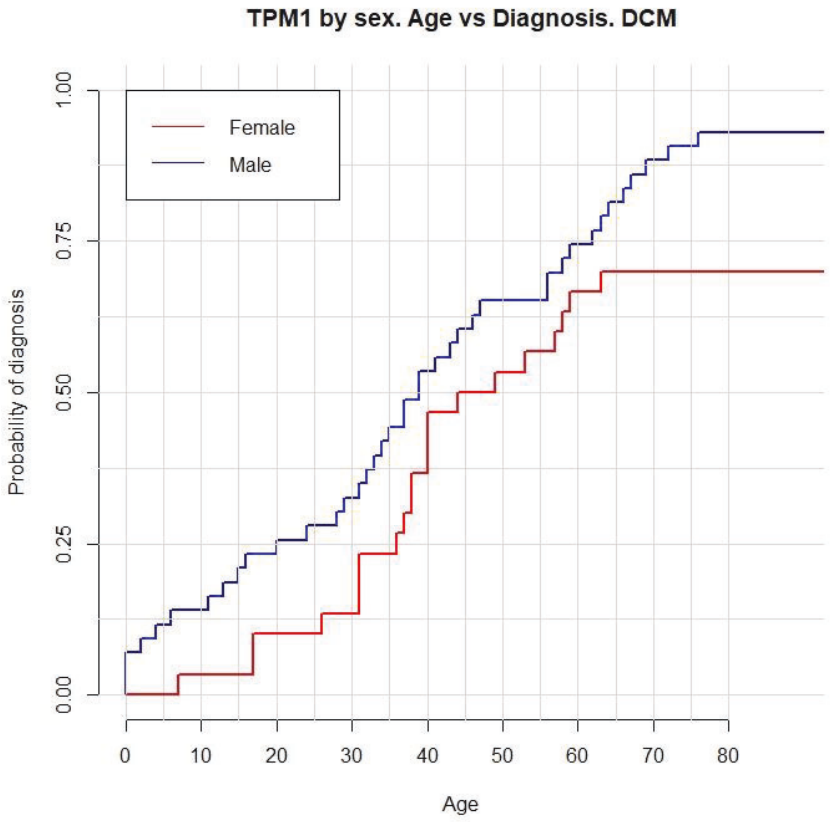
7) Finally, we have identified a female proband carrying *TPM1* p.Asp275Tyr and *MYH7* p.Gly1154Ser, likely pathogenic. She was diagnosed with dilated

cardiomyopathy and severe systolic dysfunction at the age of 18 years. Her left ventricle had hypertrabeculations without non-compaction cardiomyopathy.

6.3.1: DCM/LVNC Age of diagnosis

We have analyzed the age of diagnosis through cumulative probability of diagnosis (see Figure 6.5) in the group of dilated/left ventricle cardiomyopathy patients. Several papers in the literature have reported *TPM1* carriers affected with dilated or non-compaction cardiomyopathy in patients with pediatric ages, especially female carriers. In fact, we can observe a significant part of our cohort have been diagnosed at younger ages, although male carriers mainly.

Figure 6.7: DCM/LVNC Age of diagnosis



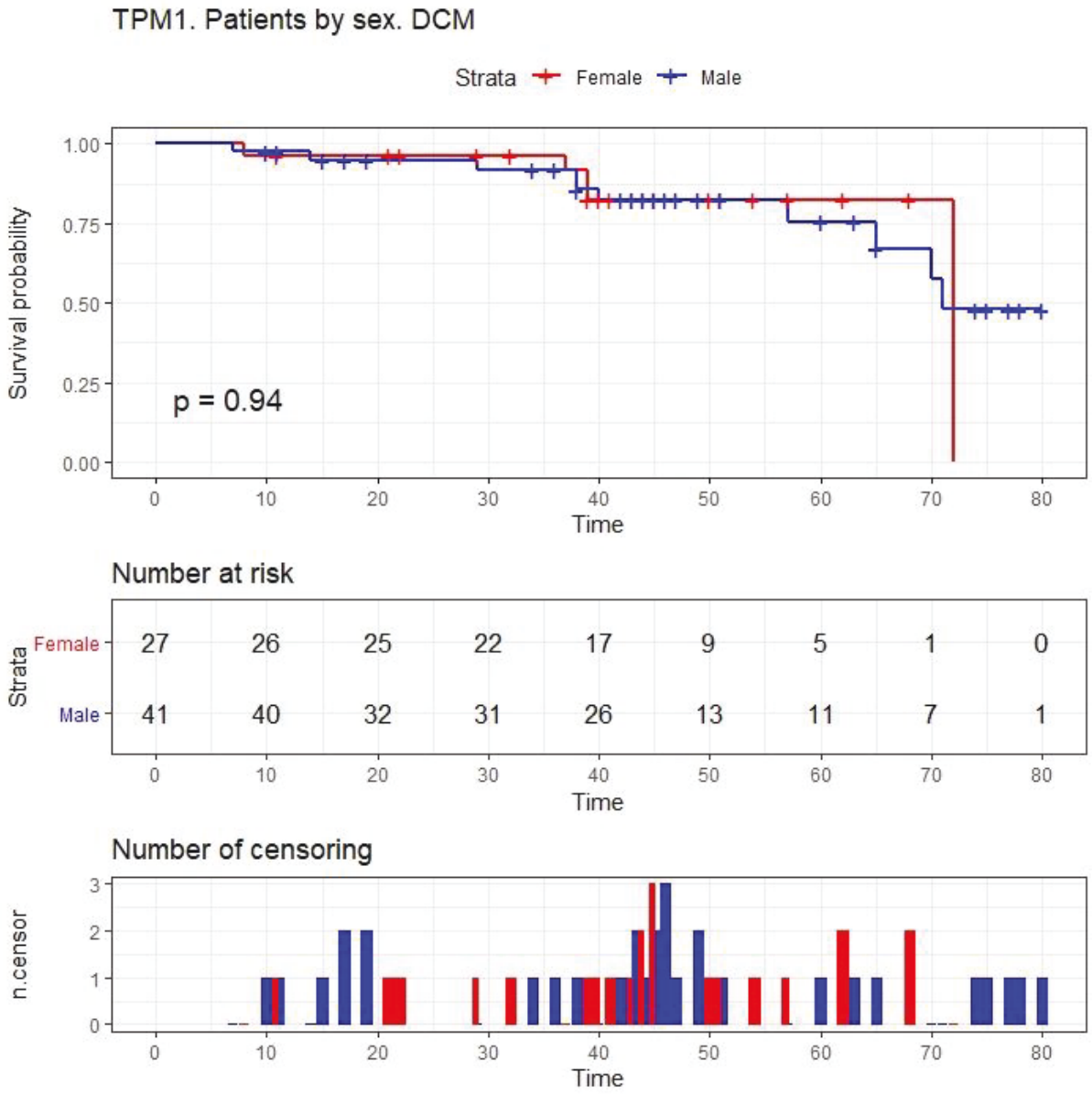
Age of diagnosis graph in the dilated/non compaction cardiomyopathy group shows a different pattern than in the hypertrophic cardiomyopathy group. Diagnosis here is also more common in males than females, but we see diagnosed patients at the first decade of life. Thirteen percent of the male carriers were diagnosed under the age of 10 years, and 25% of them at the age of 20 years versus 4% and 10% in females, respectively. Curves rises approximately 50% at the age of 40 years old in both groups. Mean age at diagnosis was 42±20 years (range 11-77) taking into consideration both groups together.

6.3.2: DCM/LVNC Survival analysis

Figure 6.6 shows survival analysis by Kaplan-Meier method. No significant statistical difference was found between male and female groups ($p = 0.94$). As in the analysis of age at diagnosis, our data contrast with that in the literature, where most of the cardiovascular events have been documented in pediatric ages, while adult patients have a favorable prognosis.

We can observe 5% mortality at the age of 15 years old, with few cardiovascular events registered at the early childhood stage. This proportion remains practically the same until the age of 35 years. At this point, mortality observed rose 20%, and this value keeps the same until the sixth-seventh decade of life.

Figure 6.8: DCM/LVNC Survival analysis



Among the 59 carriers, we observed two heart failure deaths at the ages of 7 and 72 years, respectively, and a single heart transplant. First patient, carrier of the variant p.Leu63Pro, was diagnosed with a severe overlapping phenotype with left ventricle non-compaction and dilated cardiomyopathy at the age of 5 years. He had at the moment of diagnosis a left ventricular ejection fraction of 38% with restrictive pattern, moderate pulmonary hypertension, and a left ventricle apical aneurysm. He partially responded to drug therapy, with functional class improvement (NYHA II) and ejection fraction 46%;

however, its ventricular end-diastolic diameter increased. His death was registered at the age of 7 years. Second patient with a heart failure death was a male dilated cardiomyopathy patient, carrying the variant p.Asp230Asn. He was diagnosed at 60 years old with severe left ventricular systolic dysfunction. He received a cardiac defibrillator implant due a non-sustained ventricular tachycardia at the first evaluation. Medical therapy was not effective in the echo parameters, and heart failure death was reported at 72 years old.

Heart transplantation was reported in only one carrier with dilated cardiomyopathy phenotype, a 46 years old patient carrying the p.Leu113Val variant. He had an overlapped phenotype with left-ventricle non compaction, and he was diagnosed with severe systolic dysfunction at 21 years old.

7. Final considerations

Here in this PhD thesis, it was the first time that a research group has established a large study population with patients carrying *TPM1* variants. It represents a paradigm break in the respective literature because available clinical information on this gene to date was limited to publications describing a single pedigree or a small number of index cases for each variant (many times without individual clinical features). In total, 380 new individuals from *TPM1* families were described in this study, constituting the widest cohort even published about this gene. Further, we also have analyzed in detail the genotype-phenotype correlations of the largest population in the literature carrying the single TPM1 variant, p.Arg21Leu. *TPM1* variants prevalence in different cardiomyopathy-types was similar with those reported in the literature, and seems to be lower in left ventricle non compaction cardiomyopathy phenotype, since we used an unselected population with inherited heart conditions sequenced in our center.

In the current days, next generation sequencing for patients with inherited heart diseases have been performed by several laboratorial centers, most of them, reporting genetic variants in an automatic process which results in simple communications of the genetic findings and related-pathogenicity. What we could suppose to be simple ends up becoming confusing for clinical practice many times, because physicians and patients need a genetic interpretation that enhances their clinical decision making process and genetic counseling. *TPM1* p.Arg21Leu analysis was carried out based on the reporting model developed by the Health in Code team, which was born with a deep scientific bias from the Universidade da Coruña. This analysis shows how genetic variants on cardiomyopathies can

be explored, bringing several insights into clinical practice and genetic results interpretation.

The availability of a large number of carriers with this same *TPM1* variant has confirmed that this genotype is a founder effect in a defined area composed by Galicia/Extremadura and Northern Portugal. Detailed genotype-phenotype correlation on this variant revealed that it is a pathogenetic genotype associated with late onset hypertrophic cardiomyopathy, incomplete penetrance, and generally a favorable clinical course. In the chapter 4 of this thesis we have made several conclusions on *TPM1* p.Arg21Leu which we will not repeat here. Nonetheless, some of these more refined conclusions can be considered not only for this specific variant, but also for general interpretation of genetic findings on cardiomyopathies. One of these specific topics is that our data suggests that the interpretation of an additional genetic variant in cardiomyopathies must be done considering the clinical features and penetrance associated specifically to each one of the identified variants. There is a consolidated genomic principle that the burden of additional genetic variants would represent a more severe phenotype ("dosage-dependent effect"); however, we did not identify this behavior in our cohort. So, this observation can be likely applied to several genes related with cardiomyopathies since these disorders are associated with variants of late onset and incomplete penetrance, reinforcing the need to know the age of diagnosis and the clinical features referring to each genetic variant.

This clinical profile can probably be applied for all hypertrophic cardiomyopathy variants identified in this gene. Comparing this variant with other prevalent variants in this gene (p.Asp175Asn and p.Met281Leu), as well as for all variants related to hypertrophic

cardiomyopathy, we do not identify significant statistical differences in the clinical features and cardiovascular events. Age of diagnosis and prognosis were similar with those descriptions of unselected cohorts with this disorder. Hypertrophic cardiomyopathy diagnosis started mainly at the second decade of life, being more frequent in male carriers. A higher incidence of diagnosis by year was observed between the ages 35-60 years, suggesting late onset and incomplete penetrance at older ages for *TPM1* carriers. Further, survival function for all hypertrophic cardiomyopathy variants presented also a favorable prognosis in general, similarly with hypertrophic cardiomyopathy by itself (less than 1% mortality by year in the current days), but here no difference was observed between genders.

Nevertheless, some *TPM1* carriers were described with severe clinical presentation, which could be considered a challenge in risk stratification of these carriers. Inter- and intra-familial clinical heterogeneity has been observed in pedigrees carrying the same genetic variant, as well as variants in the same gene, and it is a behavior found in hypertrophic cardiomyopathy by itself. In *TPM1*, we consider that clinicians should have in mind that these are single cases occurring in a few families, and if a sudden cardiac death or severe phenotype is identified in a pedigree, it is probably an isolated fact - that definitely does not happen in the most of the families.

On the other hand, our dilated/left ventricle non compaction cardiomyopathy patients group have shown some disparities in relation to the hypertrophic cardiomyopathy group like a lower age of diagnosis, with several cases being diagnosed at the first decade of life. In the dilated cardiomyopathy variants, we observed the presence of patients fulfilling

left ventricle non compaction cardiomyopathy diagnosis criteria (not only hypertrabeculations, such as in the hypertrophic cardiomyopathy group). Furthermore, a higher prevalence of congenital heart defects was observed in the dilated cardiomyopathy group. Survival function in this group does not also show differences between genders; however, most of the cardiovascular events in our cohort were reported in adult ages, a fact contrasting with the literature, where the events were reported predominantly in the childhood.

Our thesis has systematized all *TPM1* variants described in the literature, public genomic databases, and those identified in our center. This work allows changes in the pathogenicity of the variants, and identified enrichment functional domains by phenotype. No heptad position-phenotype correlation, and the role of truncating and splicing-zone variants was defined in our study. We believe that our analysis on *TPM1* variants will enable a more precise classification of the genetic findings, making the difference for their medical management and familial screening.

8. *TPM1* Conclusions

1. Genotype-phenotype correlations studies in collaboration with several medical centers is a research model fundamental to approach rare etiologies in inherited heart diseases, in which it has provided the widest cohort ever published about the *TPM1* gene, and the largest population carrying the same variant in this gene, p.Arg21Leu.

2. *TPM1* variants prevalence in different cardiomyopathy-types was similar with those reported in the literature, and seems to be lower in left ventricle non compaction cardiomyopathy phenotype in our study, since we used an unselected population with inherited heart conditions.

3. The availability of a large number of *TPM1* p.Arg21Leu carriers revealed that it is a pathogenetic variant associated with late onset hypertrophic cardiomyopathy, incomplete penetrance, and generally a favorable clinical course is crucial, in addition to confirming this genotype is a founder effect in a defined area composed by Galicia/Extremadura and Northern Portugal.

4. Clinical profile observed in this founder variant can probably be expanded to all hypertrophic cardiomyopathy variants identified in this gene, constituting the associated-phenotype observed in *TPM1* for this disorder.

5. Severe disease expression in TPM1 carriers even in the absence of identifiable risk factors (inter- and intra-familial clinical heterogeneity) is probably the major

challenge in risk stratification of these carriers. However, in *TPM1* we consider that the clinicians should have in mind that these are sporadic cases occurring in only few families, and if a sudden cardiac death or severe phenotype is reported in a pedigree, it is probably an isolated fact - that definitely does not happen in the most of the families.

6. We have not observed the consolidated genomic principle that the burden of additional genetic variants would represent a more severe phenotype ("dosage-dependent effect"); our work suggests that the interpretation of an additional genetic variant in cardiomyopathies must be done considering the clinical features and penetrance associated specifically to each one of the identified variants, reinforcing the need to know the age of diagnosis and the clinical features associated to each specific genetic variant.

7. Dilated/left ventricle non compaction cardiomyopathy patients group have shown some disparities in relation to the hypertrophic cardiomyopathy group like a lower age of diagnosis, with several cases being diagnosed at the first decade of life, a higher prevalence of congenital heart defects, and the presence of patients fulfilling left ventricle non compaction cardiomyopathy diagnosis criteria.

8. Systematized analysis on all *TPM1* variants described in the literature, public genomic databases, and those identified in our center allowed changes in the pathogenicity of several variants, and the identification of enrichment functional domains by phenotype. No heptad position-phenotype correlation, and no pathogenic role for truncating and splicing-zone variants was established in our study.

9. We believe that our analysis on *TPM1* variants will enable a more precise classification of the genetic findings, beyond providing useful clinical information for the medical management and familial screening of these carriers.

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Supplementary Material

Appendix A

Publications derived from this thesis.

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Original article

Genotype-phenotype correlations in hypertrophic cardiomyopathy: a multicenter study in Portugal and Spain of the *TPM1* p.Arg21Leu variant

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ABSTRACT

Introduction and objectives: *TPM1* is one of the main hypertrophic cardiomyopathy (HCM) genes. Clinical information on carriers is relatively scarce, limiting the interpretation of genetic findings in individual patients. Our aim was to establish genotype-phenotype correlations of the *TPM1* p.Arg21Leu variant in a serie of pedigrees.

Methods: *TPM1* was evaluated by next-generation sequencing in 10 561 unrelated probands with inherited heart diseases. Familial genetic screening was performed by the Sanger method. We analyzed *TPM1* p.Arg21Leu pedigrees for cosegregation, clinical characteristics, and outcomes. We also estimated the geographical distribution of the carrier families in Portugal and Spain.

Results: The *TPM1* p.Arg21Leu variant was identified in 25/4099 (0.61%) HCM-cases, and was absent in 6462 control individuals with other inherited cardiac phenotypes ($P < .0001$). In total, 83 carriers (31 probands) were identified. The combined LOD score for familial cosegregation was 3.95. The cumulative probability of diagnosis in carriers was 50% at the age of 50 years for males, and was 25% in female carriers. At the age of 70 years, 17% of males and 46% of female carriers were unaffected. Mean maximal left ventricular wall thickness was 21.4 ± 7.65 mm. Calculated HCM sudden death risk was low in

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34 carriers (77.5%), intermediated in 8 (18%), and high in only 2 (4.5%). Survival free of cardiovascular death or heart transplant was 87.5% at 50 years. Six percent of carriers were homozygous and 18% had an additional variant. Family origin was concentrated in Galicia, Extremadura, and northern Portugal, suggesting a founder effect.

Conclusions: *TPM1* p.Arg21Leu is a pathogenic HCM variant associated with late-onset/incomplete penetrance and a generally favorable prognosis.

Correlación genotipo-fenotipo en miocardiopatía hipertrófica: un estudio multicéntrico en Portugal y España sobre la variante p.Arg21Leu de *TPM1*

Palabras clave:
Miocardiopatía hipertrófica
Secuenciación de nueva generación
Tropomiosina
TPM1
Efecto fundador
Correlación genotipo-fenotipo

RESUMEN

Introducción y objetivos: *TPM1* es uno de los principales genes en la miocardiopatía hipertrófica (MCH). La información clínica sobre portadores es relativamente escasa, lo cual limita la interpretación de los estudios genéticos. Nuestro objetivo es establecer la correlación genotipo-fenotipo de la variante p.Arg21Leu de *TPM1* en una serie de familias.

Métodos: Se evaluó el *TPM1* mediante secuenciación de nueva generación en 10.561 probandos con cardiopatías hereditarias. Se genotipificó a los familiares mediante Sanger. Se analizaron la cosegregación, las características clínicas y los eventos cardiovasculares. Se estimó la distribuición geográfica de las familias en Portugal y España.

Resultados: Se identificó la variente p.Arg21Leu de *TPM1* en 25/4.099 (0,61%) casos con MCH y estaba ausente en 6.462 controles con otras cardiopatías familiares ($p < 0{,}0001$). Se identificó a 83 portadores (31 probandos). La LOD *score* combinada para cosegregación fue 3,95. La probabilidad acumulada de diagnóstico en portadores a los 50 años fue del 50% para los varones y el 25% para las mujeres. El 17 de los varones y el 46% de las mujeres no estaban afectadas a los 70 años. El grosor medio del ventrículo izquierdo fue 21,4 ± 7,65 mm. El riesgo de muerte súbita-MCH fue bajo en 34 (77,5%), intermedio en 8 (18%) y alto en 2 (4,5%) de los portadores. La supervivencia libre de eventos cardiovasculares fue del 87,5% a los 50 años. El 6% de los portadores eran homocigotos y el 18% tenían una variante adicional. El origen de las familias se concentró en Galicia, Extremadura y norte de Portugal, lo que indica un efecto fundador.

Conclusiones: P.Arg21Leu es una variante patogénica de *TPM1* asociada con MCH de penetrancia tardía/incompleta y pronóstico generalmente favorable

Abbreviations

ECG: electrocardiogram
HCM: hypertrophic cardiomyopathy
LVH: left ventricular hypertrophy
SCD: sudden cardiac death

INTRODUCTION

Hypertrophic cardiomyopathy (HCM) is a common genetic disorder (prevalence > 1/500), with wide phenotypic and *locus* heterogeneity.[1–3] The *TPM1* gene (encoding α-tropomyosin) is considered one of the main causative HCM genes; nevertheless, it is a relatively rare etiology, accounting for 1% to 5% of the cases with this phenotype.[3]

Available clinical information on most of the patients carrying *TPM1* variants is restricted to a single pedigree or a few index cases for each variant,[4–13] which limits assertive clinical interpretation of the genetic findings. The exception to this rule is the *TPM1* p.Asp175Asn variant identified in several Finnish HCM patients because it is the single founder effect described in this gene to date.[14,15] Genotype-phenotype correlation studies of founder variants in HCM populations have contributed to a better understanding of the clinical course and prognosis associated with this variant.[16–22]

At our center, we identified the variant *TPM1* p.Arg21Leu by next-generation sequencing in several individuals with HCM, including homozygous carriers. Although we undertook genetic studies from several countries around the world, the p.Arg21Leu variant cases predominantly came from hospitals in northwest Spain and Portugal, raising the hypothesis that it could be a founder effect. To date, this variant has been classified in public databases as of uncertain clinical significance (5 independent submitters) and likely pathogenic (only 1 submitter),[23] while the associated-phenotype is still unknown. Our main objective in this study was to describe the phenotypic features and associated prognosis of the *TPM1* p.Arg21Leu variant in a series of pedigrees.

METHODS

This is a descriptive study of a series of families carrying the variant *TPM1* NP_001018005.1: p.Arg21Leu (c.62G > T). This study was performed in accordance with the principles of the Helsinki Declaration, and is part of the research line registered with the number 2012/139 in the Research Ethics Committee of Galicia, Spain. Informed consent was obtained for all participants.

Study population and bioinformatic analysis

From March 2008 to September 2018, the *TPM1* gene was sequenced in 10 561 consecutive index cases with different

inherited heart diseases from distinct hospitals around the world referred to our center for molecular diagnosis.

All exons and intronic boundary regions of 213 genes (table 1 of the supplementary data) related to cardiomyopathies and sudden cardiac death (SCD) were studied by next-generation sequencing in the index cases. The minimum read-depth obtained was > 30 (average from 250 x to 400 x) in deoxyribonuclei acid (DNA). Fragments that did not fulfill these criteria were sequenced by Sanger. Familial cascade genetic screening in relatives was performed using Sanger sequencing. A multidisciplinary team performed bioinformatics analysis and clinical interpretation. Information regarding allelic frequency in the general population (controls) was considered based on Genome Aggregation Database (gnomAD) and TOPMed Program populations. The pathogenicity classification of the variants was in accordance with current recommendations, which consider the prevalence of the variant in affected vs control individuals, familial cosegregation data, functional studies, and in silico predictors, among other criteria.[24] Pathogenic/likely pathogenic variants in any of the sequenced genes were described, as well as those of unknown clinical significance in 18 priority HCM genes (table 1 of the supplementary data).

Additionally, contact was made during the first Iberian meeting of cardiomyopathies (Óbidos, Portugal, March, 2017) to collect information from *TPM1* p.Arg21Leu carriers identified at other Portuguese and Spanish centers.

Phenotype characterization

Clinical data from index cases and family members were reviewed, and their pedigrees were constructed. All carrier families were included, regardless of the length of follow-up or the number of family members evaluated. We analyzed the factors related to penetrance and disease expression in HCM: age, sex, genotype, clinical features, and SCD risk factors. Early onset was defined as diagnosis in persons younger than 35 years.[25] The diagnostic criteria and SCD risk stratification model for HCM followed the recommendations of the European Society of Cardiology (ESC).[3] All probands fulfilled conventional diagnostic criteria for HCM with ≥ 1.5 cm wall thickness on echocardiography in at least 1 myocardial segment. Carrier relatives with left ventricular wall thickness ≥ 1.3 cm were considered to be diagnosed with HCM. Relatives with only minor electrocardiogram (ECG) changes or normal ECG and normal echocardiography, regardless of their carrier status, were considered clinically unaffected. Relatives who had not undergone diagnostic evaluation were classified as nonclinically evaluated. SCD risk score (ESC calculator) was obtained for carriers with an HCM diagnosis and available clinical data. Other SCD risks were also considered[2,26]: left ventricular apical aneurysm, end-stage HCM, and extensive late gadolinium enhancement (LGE) (defined here as ≥ 3 affected cardiac segments) on magnetic resonance imaging.

The cumulative probability of occurrence of cardiovascular death or equivalent (SCD, appropriate defibrillator shock, heart failure death, stroke-related death, and unspecified cardiac death) and heart transplant was estimated with the Kaplan-Meier method, and factors were compared using the log-rank (Mantel-Cox) method. Survival was calculated from birth. A 2-sided P value < .05 was considered to indicate statistical significance. *TPM1* p.Arg21Leu carriers (index cases and relatives) and all clinically affected first- and second-degree relatives without genetic testing were included for this evaluation. The cumulative probability of an HCM diagnosis in carriers by age was estimated also by the Kaplan-Meier method. A 2-point logarithm of the odds (LOD scores) was calculated in all families using the PARAMLINK package for R software. The model was set with θ = 0, phenocopy rate = 0.005 and 2 different penetrance values: 0.80 and 0.95. An indeterminate status was assigned to family members who were reported only with diagnostic suspicion, as well as to males younger than the age of 50 years and females younger than age 55 years who did not fulfill the clinical criteria for HCM and could subsequently develop the disease.

Information about the region of origin of each index case was also collected to estimate the number of carrier families in different regions and the geographic distribution of the variant in Portugal and Spain.

Statistical analyses were performed with the software R version 3.4.3 (R Foundation for Statistical Computing, Vienna, Austria) and Health in Code (HiC) mutations version 7.7.6 (Health in Code S.A., A Coruña, Spain).

RESULTS

TPM1 p.Arg21Leu was identified in 25/10 561 (0.23%) consecutive index cases with different inherited heart diseases sequenced in our center. The variant was present in 25/4099 (0.61%) HCM-probands, and absent in 6462 index cases with other cardiac phenotypes (0/3830 dilated, left ventricular noncompaction, and arrhythmogenic cardiomyopathies; 0/1590 channelopathies, and 0/1042 other inherited heart diseases) ($P < .0001$). This variant appeared in simple heterozygosis in 10/62 784 (0.016%) individuals from the TOPMed program population, and in 5/120 158 (0.004%) individuals (age range 55-65 years) from the gnomAD population (non-TOPMed samples).

Further, we included 6 additional index cases identified in other collaborating hospitals. In total, 83 carriers (50.6% male) were identified—31 index cases. Detailed clinical data were described for 67 carriers; 44 (65.7%) were clinically affected (table 2 of the supplementary data and figure 1 of the supplementary data).

Twenty-eight pedigrees were constructed (familial data were not reported in 3 probands), and familial cosegregation of the variant was documented with an autosomal dominant inheritance pattern (combined LOD score = 3.95). All pedigrees, study population, specific LOD scores, individual clinical data, and SCD risk scores are available in the supplementary data.

Index cases were evaluated from 13 hospitals (4 Portuguese and 9 Spanish). The reported origin of the families was concentrated in the western part of the Iberian Peninsula (the region of Braga in northern Portugal, as well as the Spanish regions of Galicia and Extremadura) (figure 1). We did not identify the *TPM1* p.Arg21Leu variant in samples referred from hospitals of other parts of the world.

Age at diagnosis

The cumulative percentage of diagnosis in carriers by age and sex is shown in figure 2. At the age of 30 years, 25% of male and 6% of female individuals had been clinically diagnosed with HCM. The percentage rose to 50% at the age of 50 years for male vs 25% for female carriers. At the age of 70 years, approximately 17% of males and 46% of females were still unaffected. The mean age at diagnosis was 47.3 ± 18.8 [range 11-73] years. Individuals diagnosed under the age of 35 years were 18% (12/67) of the carriers with detailed data.

Phenotypic features

Clinical data obtained from 67 carriers (31 index cases and 36 relatives) are summarized in table 1. Forty-four carriers (44/67, 66%; 31 index cases and 13 relatives) met the diagnostic criteria for

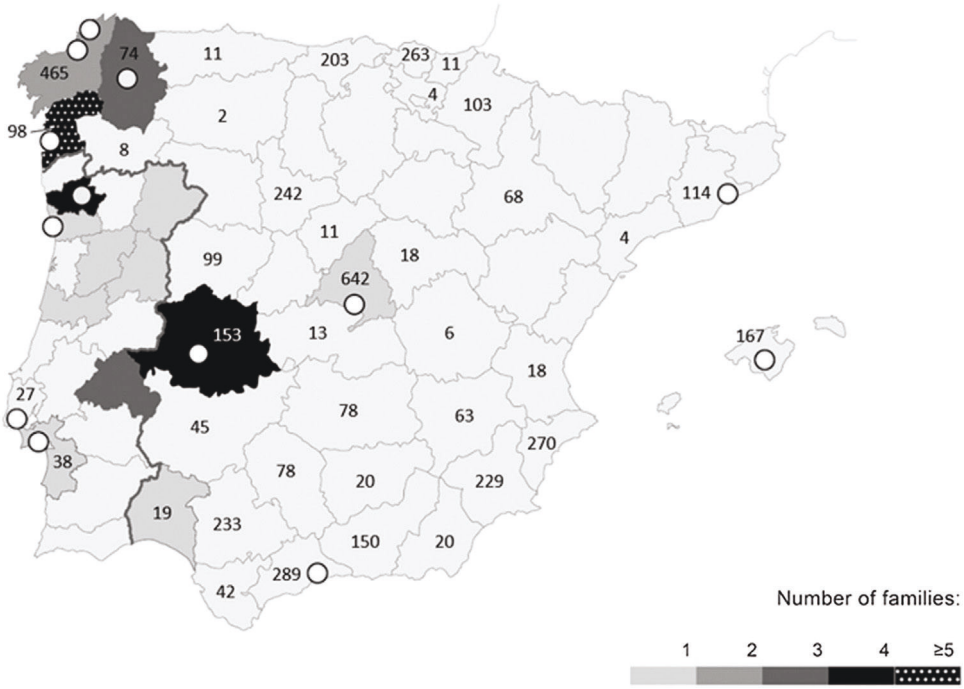

Figure 1. Geographic distribution (by origin) of the families carrying the *TPM1* p.Arg21Leu variant. Portugal and Spain maps divided by region. Concentration of families (origin) is represented in gray scale. White circles indicate the reference hospitals where the index cases were identified. Numbers (n) represent the hypertrophic cardiomyopathy studies requested by each region.

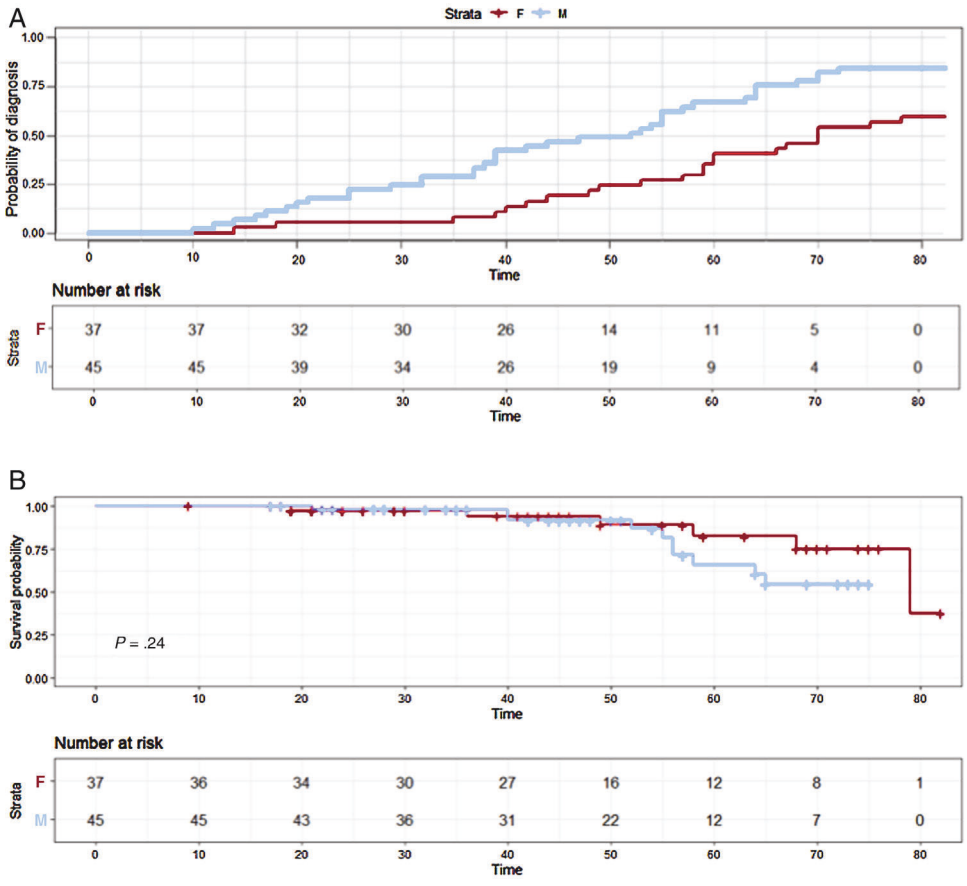

Figure 2. Cumulative probability of hypertrophic cardiomyopathy diagnosis in *TPM1* p.Arg21Leu carriers (at the top) and survival function (below), both by age and sex. Male individuals are in blue and female in red. F, female; M, male.

Table 1
TPM1 p.Arg21Leu clinical features (n = 67)

Carriers with data	Total *n* = 67	%	Mean ± SD	Index cases *n* = 31	%	Mean ± SD	Relatives *n* = 36	%	Mean ± SD
Unaffected	23/67	34.3		0/31			23/36	63.8	
Affected	44/67	65.7		31/44	70.4		13/44	29.6	
Male:female	1.01			1.38			0.53		
Age at diagnosis, y			47.3 ± 18.8 [range 11-73]			46.6 ± 19.3			48 ± 16.5
Mean follow-up length, y	7.7 ± 7.1								
Symptoms									
Dyspnea	14/44	31.8		12/31	38.7		2/13	15.4	
NYHA II	9/44	20.45		7/31	22.6		2/13	15.4	
NYHA III - IV	5/44	11.4		5/31	16.1		0/13		
Syncope	5/44	11.4		5/31	16.1		0/13		
Chest pain	4/44	9.1		4/31	12.9		0/13		
Imaging									
LVH									
Apical	7/44	15.9		5/31	16.1		2/13	15.4	
Asymmetric septal	34/44	77.3		24/31	77.4		10/13	76.9	
Concentric	1/44	2.3		1/31	3.2		0/13		
Atypical	2/44	4.5		1/31	3.2		1/13	7.7	
Maximal LV wall thickness, mm			21.4 ± 7.65			22.2 ± 8.1			15.1 ± 6.6
LV mass, g	11/44	25	290.5 ± 168.3	5/31	16.1	338 ± 173.9	6/13	46.2	251 ± 168.3
LVOTO	15/44	34.1		12/31	38.7		3/13	23.1	
Mean peak value, mmHg			67.4 ± 33.8			65.1 ± 35.6			73.2 ± 33.1
Abnormal response to exercise testing	5/44	11.4		4/31	12.9		1/13	7.7	
Midventricular obstruction	0/44			0/31			0/13		
LV apical aneurism	0/44			0/31			0/13		
Ejection fraction, %			68.3 ± 8.6			68.2 ± 9.2			68.5 ± 7.4
Systolic dysfunction	0/44			0/31			0/13		
LV diastolic dysfunction	22/44	50		16/31	51.6		6/13	46.2	
Grade I	14/44	31.8		10/31	32.2		4/13	30.8	
Grade II	7/44	15.9		5/31	16.1		2/13	15.4	
Restrictive pattern	1/44	2.3		1/31	3.2		0/13		
Left atrial dilatation, mm[a]	26/44	59.1	40.38 ± 6.7	21/31	67.7	41.64 ± 6.5	5/13	38.5	37.86 ± 6.62
MRI	18/44	40.9		15/31	48.4		3/13	23.1	
No LGE	5/18	27.8		3/15	20		2/3	66.7	
LGE 1-2 segments	9/18	50		9/15	60		0/3		
LGE ≥ 3 segments	4/18	22.2		3/15	20		1/3	33.3	
ECG									
Minor ECG changes	40/44	90.9		27/31	87.1		13/13	100	
High voltages **	23/41	56.1		21/27	77.8		2/13	15.4	
T-wave inversion	14/41	34.1		10/27	37		4/13	30.7	
Pathologic Q waves	14/41	34.1		10/27	37		4/13	30.7	
Nonspecific repolarization changes	13/41	31.7		8/27	29.6		5/13	38.4	
Atrial fibrillation	6/44	13.6		5/31	16.1		1/13	7.7	
Conduction disease	5/44	11.3		5/31	16.1		0/13		
Ventricular arrhythmias	8/44	18.2							
NSVT on 24-Holter	5/8	62.5		5/31	16.1		0/13		
Premature ventricular beats	4/8	50		2/31	6.5		2/13	15.4	
Exercise-induced arrhythmias	0/44			0/31			0/13		
Treatment									
Surgery[b]	4/44	9.1		3/31	9.7		1/13	4.5	
Pacemaker	1/44	2.3		0/31			1/13	4.5	
ICD[c]	4/44	9.1		4/31	11.7		0/13		
Appropriate shocks	0		Mean follow-up, 3.8 y						
LVH predisposing factors									
Competitive sports	6/44	13.6		3/31	9.7		3/13	23.1	

Table 1 *(Continued)*
TPM1 p.Arg21Leu clinical features (n = 67)

Carriers with data	Total *n* = 67	%	Mean ± SD	Index cases *n* = 31	%	Mean ± SD	Relatives *n* = 36	%	Mean ± SD
Hypertension	7/44	15.9		6/31	19.4		1/13	7.7	
Moderate-severe	2/7	28.6		2/31	6.5		0/22		

ECG, electrocardiogram; ICD, implanted cardiodefibrillator; LGE, late gadolinium enhancement; LV, left ventricle; LVH, left ventricular hypertrophy; LVOTO, left ventricular outflow tract obstruction; MRI, cardiac magnetic resonance; NSVT, nonsustained ventricular tachycardia; NYHA, New York Heart Association; SD, standard deviation.
P value Fisher exact test for the subpopulations index cases and relatives, only shown [**] when *P* value < .05 comparing index cases and relatives.
[a] Statistical difference in left atrial dilatation is restricted for ratios (%) (dichotomous variable), not for average (SD) values (contiguous variable).
[b] Two myectomy, 1 valve mitral prosthesis surgery + myectomy, and 1 heart transplant.
[c] All cases for primary prevention.

HCM by left ventricular hypertrophy (LVH) criteria, and 23 carriers (23/67; 34%) were unaffected.

The predominant left ventricular morphological pattern was asymmetric septal (34/44; 77%) and 16% (7/44) had apical hypertrophy. Figure 2 of the supplementary data shows the distribution of maximum left ventricular wall thickness (in mm) by sex, genotype, and age at the last follow-up. Carriers were concentrated between 15 to 25 mm of LVH and at advanced ages. Mean maximum left ventricular wall thickness was 21.4 ± 7.65 mm. Among the carriers diagnosed at < 35 years, mean maximum left ventricular wall thickness was 27.04 ± 11.2 mm. Left ventricular outflow tract obstruction was reported in 34% of cases (15/44) and abnormal blood pressure response to exercise testing in 11% (5/44). Sixteen percent (16%; 7/44) had a pseudonormalization pattern of the mitral valve inflow, and only 1 case had a restrictive pattern. No left ventricular midventricular obstruction was observed.

ECG abnormalities were reported in 91% of the affected carriers; 13.6% (6/44) of them had atrial fibrillation, and 5 carriers (11.3%; 5/44) were reported to have conduction system disorders, 5 cases of first-degree atrioventricular block. One carrier with complete atrioventricular block after mitral valve surgery was not included in this subgroup because his atrioventricular block was considered secondary to surgery. He underwent pacemaker implant. Syncope was reported in 11.5% (5/44) of the affected carriers. Eight (8/44; 18%) of the affected carriers had ventricular arrhythmias; 5 of them with nonsustained ventricular tachycardia recorded on 24-hour Holter monitoring. There were no arrhythmias induced by the exercise stress test. There were no reports of appropriate shocks in a mean follow-up of 3.8 years among the 4 male patients who had implanted cardioverter-defibrillators for primary prevention; 3 of them had been diagnosed in adolescence and received the devices approximately 10 years after diagnosis. Two carriers with a cardioverter-defibrillator had high SCD risk (9.94%, and 6.37% over 5 years) and the other 2 had intermediate risk scores (both with 4.59% over 5 years), without additional SCD risk markers. SCD risk scores were obtained at the time of clinical decision-making.

Four carriers with early onset HCM (33%; 4/12) were competitive athletes (all football players) vs 2/32 (6.2%) carriers with late onset (*P* = .074) (table 3 of the supplementary data, clinical features of the *TPM1* p.Arg21Leu carriers diagnosed under the age of 35 years).

Only 2 clinical features showed significant statistical differences between index cases and relatives (table 1). Index cases had higher ECG voltages and more atrial dilatation than relatives.

Risk stratification and cardiovascular events

Survival analysis (figure 2) showed that less than 5% of our population had a cardiovascular death or heart transplant at the age of 30 years, this figure being 10% at 50 years for both sexes. Twenty-five percent of female individuals and 44% of males had had a major cardiovascular event at 70 years. No statistical difference between sexes was observed (*P* = .24).

Three cardiovascular deaths (1 SCD, 1 heart failure death, and 1 unspecified cardiac death) and 1 heart transplant (in total, 4/83; 5%) were reported in *TPM1* p.Arg21Leu carriers. During a mean follow-up time of 7.7 ± 7.1 years, no SCD or heart transplant was additionally reported. Twelve other cardiovascular events were reported in first- or second-degree relatives without genetic testing; 5 SCD, 2 heart failure deaths, 2 stroke-related deaths, and 3 unspecified cardiac deaths. Five carriers were reported to have had nonmajor cardiovascular events that were not included in the survival curves; 2 myectomies, 1 mitral valve replacement for left ventricular outflow tract obstruction due to systolic anterior motion, and 2 nonlethal strokes. Each event, age at occurrence and patient sex are specified in table 4 of the supplementary data.

ESC SCD risk was calculated for all affected carriers (n = 44). Most of them (77.3%; 34/44) had a low risk score (< 4% risk over 5 years), 18.2% (8/44) had intermediate values (4-6% risk over 5 years), and only 4.5% (2/44) had high risk (> 6% risk over 5 years). Figure 3 of the supplementary data shows that carriers were concentrated at ranges with low SCD risk and advanced ages. Eighteen affected carriers (18/44; 41%) had a magnetic resonance imaging scan; 5/18 (27.8%) were reported without LGE, 9/18 (50%) had LGE in 1 or 2 cardiac segments, and 4/17 (22.2%) had LGE in ≥ 3 segments. No left ventricular apical aneurysm or end-stage HCM was observed.

Homozygous carriers and additional genetic variants

Fifteen individuals (15/67; 17.9%) from 10 pedigrees had complex genotypes. Four had *TPM1* p.Arg21Leu in homozygosity (4/67; 6%), and 12 had an additional genetic variant (12/67; 17.9%); 1 of the homozygous carriers also had the pathogenic variant *MYBPC3* p.Asp75Asn. Clinical features of the homozygous carriers are described in table 5 of the supplementary data.

Five genetic variants in the *MYH7* gene were identified (p.Gly741Arg and p.Thr1019Asn, respectively classified as pathogenic/likely pathogenic variants; p.Lys351Asn, p.Tyr582Cys and p.Leu1333Val as variants of unkown clinical significance). Other pathogenic sarcomeric variants were identified: *TPM1* p.Met281Thr, *TNNT2* p.Arg278Cys, and *MYL3* p.Met173Val. Each of these variants was identified in different pedigrees. All these variants have been reported with very low allelic frequency (< 0.01%) in the general population, except *MYH7* p.Lys351Asn. See complementary description of each variant in table 6 of the supplementary data, showing clinical features of carriers with an additional genetic variant.

No cardiovascular death or heart transplant was reported in confirmed carriers with an additional genetic variant. Two SCD in first-degree relatives without genetic testing were reported in

pedigrees with an additional pathogenic genetic variant (*MYH7* p.Gly741Arg, and *TPM1* p.Met281Val, respectively).

DISCUSSION

Here we present the largest HCM population carrying the same *TPM1* variant described in the literature to date. This study shows that the *TPM1* p.Arg21Leu variant was significantly overrepresented in our HCM cohort compared with control populations, and familial cosegregation of the variant with the disease was documented with a significant LOD score.[27] Taking into account these results, there are sufficient criteria to now classify p.Arg21Leu as clearly pathogenic (table 7 of the supplementary data).

TPM1 p.Arg21Leu was identified exclusively in patients with the HCM phenotype. Most of the individuals in our population were simple heterozygous carriers with late onset HCM, mild to moderate phenotype, asymptomatic clinical course, and a low number of cardiovascular deaths and heart transplants. The annual incidence of cardiovascular events was 0.25% in the survival analysis, which suggests a better prognosis for TPM1 p.Arg21Leu than expected for the overall HCM population (≅ 0.5%/y).[28,29] SCD risk scores were predominantly in the low risk range, followed by approximately 20% of the cases with intermediate scores. Only 2 carriers had high SCD risk.

In comparison, the Finnish *TPM1* founder variant, p.Asp175Asn, was described in the literature as being associated with mild-moderate HCM phenotype and favorable prognosis; nonetheless, a higher penetrance in adulthood (91%-95%) was reported, based on 2 unrelated smaller cohorts.[13,14] Other variants in the *MYBPC3* and *MYH7* genes previously reported as founder effects in HCM populations have shown a worse prognosis than *TPM1* p.Arg21Leu.[17–21] Mean age at diagnosis in individuals carrying some of these variants was also lower, approximately 1 decade less.

TPM1 p.Arg21Leu carriers with early onset HCM were predominantly male. No additional pathogenic genetic variant (except 1 who had a *MYH7* variant of unknown clinical significance) was reported in this group. Their SCD risk scores were higher than the overall average, and they had more prominent LVH (both myectomies described in our study were in individuals from this group), but no cardiovascular death or heart transplant was documented among the carriers diagnosed under the age of 35 years. Other groups have described similar marked intrafamilial clinical heterogeneity in other *TPM1* HCM-variants,[4–11] with SCD episodes in individuals with early onset HCM. In contrast, there were no major CV events among our carriers with early onset HCM; however, there was an SCD episode in a nongenotyped first-degree relative at age 19 years (note that an additional relevant pathogenic *MYH7* variant was identified in this pedigree #7), and another case of SCD was reported in a nongenotyped second-degree relative at the age of 21 years. No HCM was reported in either carrier.

In this study, we report a significant number of unaffected carriers at advanced ages in our pedigrees, and also in the general population, which suggests incomplete penetrance. This late onset of the disease and the incomplete penetrance of the variant could represent a challenge when attempting to demonstrate familial cosegregation of a rare variant, such as *TPM1* p.Arg21Leu, requiring the grouping of a larger number of families, as we have done here.

Homozygous carriers and additional genetic variants

Almost 18% of the carriers had a second genetic variant, a percentage higher than that described in the literature (5%) for HCM.[2] Previous studies have demonstrated that the number of genetic variants may be a determinant of disease severity.[30,31] However, our data demonstrate that the presence of a second variant is not necessarily associated with a more severe phenotype. The majority of carriers with an additional genetic variant had mild phenotypes or were unaffected, which could be explained by the presence of 2 variants with late or incomplete penetrance. The clinical interpretation of genetic findings requires consideration of the clinical features and penetrance described specifically for each of the identified variants.

Further, we could also hypothesize that the presence of an additional genetic variant would be necessary for the penetrance (disease manifestation) of a rare variant identified in the general population, but it was not possible to verify this association statistically in our population.

Several studies have associated sarcomere variants in homozygosis to more severe presentations in HCM than when these variants are identified in simple heterozygozity.[32,33] *TPM1* p.Arg21Leu homozygous carriers in our study showed in general a more pronounced phenotype than simple heterozygous carriers (especially considering the existence of moderate-severe diastolic dysfunction, and ventricular arrhythmias); however, these homozygous carriers showed late onset disease manifestations, supporting the view that this variant is probably not be associated with a life threatening phenotype.

TPM1 p.Arg21Leu as a founder variant

The geographical distribution of the carrier families showed a prevalent concentration in the western part of the Iberian Peninsula, especially in the Spanish regions of Galicia and Extremadura, and northern Portugal (figure 1). These territories share common historical and geopolitical factors dating back to the 11th century, or even earlier.[34] Four index cases were identified in collaborating hospitals located outside these zones, but the origin of the families was reported in the founder region. Furthermore, the identification of homozygous carriers with nonconsanguineous progenitors would also reflect the greater prevalence of the variant in specific regions.

A recent study has already described a founder variant for the *GLA* gene in the same northern Portuguese region, where we also identified a high number of families carrying the *TPM1* p.Arg21Leu variant.[35] The authors demonstrated that it was related to cultural and socioeconomic particularities (ie, a high level of endogamy due to marriages within the same social stratum) existing since the 17th until 19th century in this region. They considered that social factors and the late penetrance of the variant perpetuated disease transmission in that region until the contemporary era. These characteristics could be hypothesized to explain the distribution observed for the *TPM1* p.Arg21Leu variant.

The presence of our variant exclusively in Latin individuals from gnomAD and TOPMed populations could reflect a possible common ancestor from Spain and Portugal, given that these countries had a central role in the colonization of Latin America. In comparison, another *TPM1* variant, p.Asp175Asn, described as a founder effect in Finland, is reported to be found predominantly in Finnish European individuals in the gnomAD database.

Limitations

This genotype-phenotype correlation study has some potential limitations. First, no clinical or genetic evaluation was reported in a high number (n = 106) of first-degree relatives identified in the pedigrees. This may be related to the low clinical impact of the variant and the risk perception of carrier families and physicians. Some tests were done only in a limited number of cases, such as cardiac magnetic resonance imaging, limiting our ability to assess additional risk markers. Further, the Kaplan-Meier curve presented in this study showing age at diagnosis should not

be considered the real age at onset of disease expression, but only the moment when the diagnosis was made.

Although we describe a relatively large population carrying the same variant, the numbers are still too small to allow definitive conclusions. In this regard, a comparative study of the *TPM1* p.Arg21Leu clinical features vs others genetic variants in this same gene may provide additional evidence for more assertive risk stratification in HCM. Finally, there is a need for further studies to better characterize the founder effect suggested here, such as haplotype studies. However, we believe that the available epidemiological data are sufficient to support a founder effect.

CONCLUSIONS

The availability of a large number of carriers with the same *TPM1* variant from a defined geographic area in Galicia/Extremadura/Northern Portugal enabled genotype-phenotype evaluation, which revealed the p.Arg21Leu variant to be pathogenic and associated with late onset HCM, incomplete penetrance, and a generally favorable clinical course.

WHAT IS KNOWN ABOUT THE TOPIC?

- TPM1 is one of the main HCM genes, accounting for 1% to 5% of cases of the disease.
- The available clinical information on TPM1 carriers is relatively scarce in the literature. Therefore, genotype-phenotype correlation studies of populations carrying the same sarcomere HCM causal variant could be the ideal opportunity to better understand the clinical profile and associated prognosis.

WHAT DOES THIS STUDY ADD?

- This is the largest HCM population ever described with the same TPM1 variant, possibly constituting a founder effect in Portugal and Spain.
- TPM1 p.Arg21Leu is a pathogenic HCM variant with late onset/incomplete penetrance and a generally favorable prognosis.
- This study could be useful for the clinical interpretation of genetic findings in HCM and for the development of health policies in these regions.

FUNDING

No funding source was applied in this research.

CONFLICTS OF INTEREST

A. Lamounier Junior and M. Ortiz report personal fees from Health in Code SL, outside the submitted work. L. Monserrat Iglesias is shareholder of Health in Code SL.

APPENDIX. SUPPLEMENTARY DATA

Supplementary data associated with this article can be found in the online version available at https://doi.org/10.1016/j.rec.2021.01.001

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Supplementary Material

Appendix B

Other articles published in collaboration during this thesis.

Appendix B.

1. Other articles published in collaboration during this thesis.

1. Torrado M, Maneiro E, **Lamounier Junior A**, Fernández-Burriel M, Sánchez Giralt S, Martínez-Carapeto A, Cazón L, Santiago E, Ochoa JP, McKenna WJ, Santomé L, Monserrat L. Identification of an elusive spliceogenic MYBPC3 variant in an otherwise genotype-negative hypertrophic cardiomyopathy pedigree. Nature Sci Rep. 2022 May 4;12(1):7284. doi: 10.1038/s41598-022-11159-y. PMID: 35508642; PMCID: PMC9068804.

2. Dias GM, **Lamounier Júnior A**, Seifert M, Barájas-Martinez H, Barr D, Sternick EB, Medina-Acosta E, Campos de Carvalho AC, Cruz Filho FES. *MYH7* p.Glu903Gln Is a Pathogenic Variant Associated With Hypertrophic Cardiomyopathy. Circ Genom Precis Med. 2021 Oct;14(5):e003476. doi: 10.1161/CIRCGEN.121.003476. Epub 2021 Sep 24. PMID: 34555931.

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2. Communications presented at conferences and other scientific events throughout this thesis.

1. **A Lamounier Junior**, D Alonso Garcia, G Fernandez Ferro, I.J Cardenas Reyes, J Salazar-Mendiguchia Y Garcia, J.P Ochoa, M Nicolas

Cicerchia, M.L Pena-Pena, M Noel Brogger, S Garcia Hernandez, X Fernandez, M Ortiz, R Barriales-Villa, L Iglesias Monserrat, Survival analysis in arrhythmogenic/dilated cardiomyopathy caused by pathogenic DSP truncating variants, *European Heart Journal*, Volume 41, Issue Supplement_2, November 2020, ehaa946.2040, https://doi.org/10.1093/ehjci/ehaa946.2040

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Supplementary Material

Appendix C

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Appendix C. Centers and collaborators in this thesis

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Supplementary Material

Appendix D

Table chapter 5 - Excel

TPM1 genetic variants

Available only in electronic versions.

Supplementary Material

Appendix E

Table chapter 6 - Excel

TPM1 patients cohort

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Supplementary Material

Appendix F

Extended abstract in Spanish (minimum 3,000 words)

9. Extended abstract in Spanish

Antecedentes: Previamente en la literatura, se han asociado variantes genéticas en la alfa-tropomiosina cardíaca con el desarrollo de diferentes formas de cardiopatías hereditarias: miocardiopatía hipertrófica, miocardiopatía dilatada, miocardiopatía no compactada del ventrículo izquierdo y defectos cardíacos congénitos. No se ha asociado hasta la fecha ningúna variante genética en alfa-tropomiosina con la miocardiopatía arritmogénica, incluyendo la forma arritmogénica del ventrículo izquierdo. Esta proteína codificada por el gen *TPM1* es un homodímero con 284 aminoácidos, constituyendo una estructura espiral α-helicoidal de 33 kDa, que se asocia con los filamentos de actina para constituir los filamentos sarcoméricos delgados. La alfa-tropomiosina cardíaca desempeña un papel esencial en la contractilidad miocárdica, lo que puede explicarse mediante un modelo de tres estados que vincula el Ca^{2+} a la contracción muscular. El número de publicaciones con estudios funcionales sobre variantes en *TPM1* es mayor que las descripciones clínicas de portadores en este gen. Los estudios funcionales más frecuentes que evalúan el proceso regulador de la tropomiosina han llevado a cabo ensayos que miden los cambios de sensibilidad al Ca^{2+}. Se ha demostrado que las variantes en *TPM1* descritas previamente en pacientes con miocardiopatía hipertrófica, como p.Ala63Val, p.Lys70Thr, p.Val95Ala, p.Asp175Asn y p.Glu180Gly, aumentan la sensibilidad al Ca^{2+}. Por otro lado, los estudios funcionales sobre las variantes de *TPM1* identificadas en pacientes con miocardiopatía dilatada sugirieron que estas variantes tienen el efecto opuesto del mecanismo regulador de los filamentos delgados, disminuyendo la sensibilidad al Ca^{2+}. En la literatura se han descrito varios dominios en la proteína alfa-tropomiosina, más frecuentemente los dominios denominados regiones repetidas de unión a actina.

Solo las variantes de tipo missense en *TPM1* han sido consideradas claramente como causa de la enfermedad y no existe una correlación genotipo-fenotipo para una forma específica de miocardiopatía, correlacionada dominio particular de la proteína, tampoco con una posición específica en la repetición de la heptada. No se ha definido el papel causal de las variantes truncadas e intrónicas en *TPM1*. La secuenciación de miles de individuos de la población general ha sugerido que la haploinsuficiencia es un mecanismo tolerado en este gen.

El gen *TPM1* es uno de los genes más prevalentes en las miocardiopatías. No obstante, la mayoría de las descripciones de los portadores están restringidas a una sola familia o unos pocos casos índice por cada variante. La excepción a esta regla es la variante *TPM1* p.Asp175Asn identificada en varios pacientes finlandeses con miocardiopatía hipertrófica debido al hecho de que este es el único efecto fundador descrito en este gen. Los estudios de correlación genotipo-fenotipo de variantes fundadoras en poblaciones con miocardiopatías han contribuido a una mejor comprensión del curso clínico y pronóstico asociado con una misma variante, de acuerdo con diversas publicaciones. En nuestro centro, hemos planteado la hipótesis de que una variante de *TPM1* identificada en varios pacientes con miocardiopatía hipertrófica sería un efecto fundador en Galicia, y que esta población sería una oportunidad particular para un análisis de correlación genotipo-fenotipo con portadores de la misma variante. Este análisis se amplió para todas las variantes y pacientes con cardiopatías hereditarias identificados como portadores de variantes en el gen *TPM1*.

Objetivo: Nuestro objetivo principal es analizar la correlación genotipo-fenotipo en portadores de variantes en *TPM1*. Para llevar a cabo esta tarea hemos subdividido el objetivo principal en tres objetivos secundarios; (1.) Hemos realizado una correlación genotipo-fenotipo detallada de la variante *TPM1* p.Arg21Leu, que incluye los árboles

familiares, datos de cosegregación familiar, información clínica específica de cada portador y distribución geográfica de la familias portadoras. (2.) Todas las variantes genéticas identificadas en el gen *TPM1* en nuestro centro y en la literatura se enumeraron según su patogenicidad, fenotipo de portadores, características de la variante y dominio funcional. Finalmente, (3.) hemos analizado el fenotipo clínico en portadores de todas las variantes en el gen *TPM1* completo, incluida la edad de diagnóstico, los eventos cardiovasculares y el pronóstico. Nuestra hipótesis es que existe una correlación genotipo-fenotipo entre las variantes de *TPM1* y los datos clínicos de estos portadores que pueden ser útiles para la toma de decisiones médicas.

Métodos: Se trata de un estudio descriptivo con familias portadoras de variantes genéticas en *TPM1* (isoforma NP_001018005.1). Este estudio está de acuerdo con los principios de la Declaración de Helsinki, y forma parte de la línea de investigación registrada con el número 2012/139 en el Comité de Ética de la Investigación de Galicia, España. Desde marzo de 2008 hasta septiembre de 2021, se secuenció el gen *TPM1* en 21.671 casos índice consecutivos con diferentes cardiopatías hereditarias de diferentes hospitales de todo el mundo derivados a nuestro centro para diagnóstico genético molecular. Se estudiaron todos los exones y las regiones flanqueantes intrónicas de 213 genes relacionados con las miocardiopatías y la muerte súbita cardíaca. Un equipo multidisciplinar realizó análisis bioinformáticos y la interpretación clínica de los hallazgos genéticos. La clasificación de patogenicidad de las variantes fue realizada de acuerdo con las recomendaciones actuales del Colegio Norteamericano de Genética Médica. Se describieron variantes genéticas patogénicas/posiblemente patogénicas en cualquiera de los genes secuenciados, así como aquellas de significado clínico desconocido en los genes prioritarios. La información sobre la frecuencia alélica en la población general se consideró teniendo en cuenta la base de datos

gnomAD y la base de datos del programa TOPMed. El cribado genético familiar en cascada se realizó mediante el método de secuenciación de Sanger. Hemos diseñado los árboles genealógicos para evaluar la cosegregación familiar de *TPM1* p.Arg21Leu. El logaritmo de dos puntos de las probabilidades (LOD score) se calculó en todas las familias con esta variante específica utilizando el paquete PARAMLINK del software R. Especialmente para la variante *TPM1* p.Arg21Leu, hemos realizado un análisis más detallado. El análisis de correlación genotipo-fenotipo se realizó a partir de las historias clínicas de varios centros europeos, principalmente de España y Portugal. Se analizaron la patogenicidad y las características moleculares de cada variante, las características clínicas, las genealogías, la edad de diagnóstico y el pronóstico. Se obtuvo la puntuación de riesgo de muerte cardíaca súbita (calculadora ESC) para los portadores de esta variante con datos clínicos disponibles. Se consideró presentación precoz el diagnóstico antes de los 35 años. La probabilidad acumulada de ocurrencia de muerte cardiovascular (muerte súbita cardíaca, choque de desfibrilador apropiado, muerte por insuficiencia cardíaca, muerte relacionada con un accidente cerebrovascular y muerte cardíaca no especificada) y trasplante cardíaco fue estimada con el método de Kaplan-Meier y con la aplicación del log-rank (método de Mantel-Cox). La supervivencia se calculó desde el nacimiento para todos los portadores de *TPM1* colectivamente y por separado para los siguientes grupos: portadores de la variante p.Arg21Leu, portadores de miocardiopatía hipertrófica y portadores de miocardiopatía dilatada/ventrículo izquierdo no compactada. Los gráficos de edad de diagnóstico también se construyeron mediante el método de Kaplan-Meier para los mismos grupos. Se cartografió la distribución geográfica de la frecuencia de la variante *TPM1* p.Arg21Leu por provincia en los respectivos territorios.

Resultados: Se han identificado 83 portadores de *TPM1* p.Arg21Leu en 31 familias diferentes. El origen de estas familias se concentraba en las provincias de Galia, Extremadura y Norte de Portugal. Estos territorios comparten factores históricos y geopolíticos comunes que se remontan al siglo XI o incluso antes, sugiriendo la ocurrencia de un efecto fundador. *TPM1* p.Arg21Leu se identificó en 25/10.561 (0,23%) casos índice consecutivos con diferentes cardiopatías hereditarias secuenciadas en nuestro centro. Identificada en 25/4.099 (0,61%) probandos de miocardiopatía hipertrófica, y ausente en 6.462 casos índice con otros fenotipos cardíacos (0/3830 miocardiopatías arritmogénicas, dilatadas, no compactada del ventrículo izquierdo; 0/1590 canalopatías y 0/1042 otras cardiopatías hereditarias) ($p<0,0001$). Esta variante aparece en heterocigosis simple en 10/62.784 (0,015%) individuos de la población control del programa TOPMed y 5/120.158 (0,004%) individuos (rango de edad 55-65 años) de la población gnomAD (muestras no TOPMed). La cosegregación familiar se documentó en base a 28 árboles genealógicos. Quince portadores de 10 pedigríes tenían genotipos complejos, incluidos cuatro portadores homocigotos, y doce tenían una variante genética adicional. El porcentaje de portadores (18%) con una segunda variante genética es superior al descrito en la literatura (5%) para la miocardiopatía hipertrófica. No se describió muerte cardiovascular ni trasplante cardíaco en portadores confirmados con una variante genética adicional. Los portadores con una segunda variante genética tienen fenotipos leves o incluso no afectados, lo que podría explicarse por la presencia de dos variantes genéticas de penetrancia tardía/incompleta. Los portadores de *TPM1* p.Arg21Leu diagnosticados a edades más tempranas fueron predominantemente hombres y la práctica de deportes competitivos fue el único factor predisponente de hipertrofia del ventrículo izquierdo informado entre ellos. No se informó ninguna variante genética patogénica adicional (excepto una que tenía una variante *MYH7* de significado

clínico desconocido) en este grupo. El análisis de supervivencia de *TPM1* p.Arg21Leu muestra que menos del 5% de nuestra población tuvo una muerte cardiovascular o un trasplante de corazón a la edad de 30 años y un 10% a los 50 años para ambos sexos. El 25% de las mujeres y el 44% de los hombres habían sufrido un evento cardiovascular importante con 70 años. No se observó diferencia estadística entre géneros (p = 0,24). La incidencia anual de eventos cardiovasculares fue del 0,25% en el análisis de supervivencia, lo que sugiere un mejor pronóstico para *TPM1* p.Arg21Leu que el esperado para la población general con miocardiopatía hipertrófica (≅0,5 %/año). Se informaron tres muertes cardiovasculares (una muerte cardíaca súbita, una muerte por insuficiencia cardíaca y una muerte cardíaca no especificada) y un trasplante de corazón (en total, 4/83; 5%) en portadores de *TPM1* p.Arg21Leu. Durante un tiempo medio de seguimiento de 4,9 (±6,7) años, no se informó adicionalmente casos de muerte cardíaca súbita o trasplante cardíaco. El escore de muerte súbita cardiaca fue predominantemente de bajo riesgo, seguido por aproximadamente el 20% de los casos con puntuaciones intermedias. Se informaron otros doce eventos cardiovasculares en familiares de primer/segundo grado sin pruebas genéticas; cinco muertes cardíacas súbitas, dos muertes por insuficiencia cardíaca, dos muertes relacionadas con accidentes cerebrovasculares y tres muertes cardíacas no especificadas. Se informaron cinco portadores con eventos cardiovasculares menores que no se incluyeron en las curvas de supervivencia; dos miectomías, un reemplazo de válvula mitral por obstrucción del tracto de salida del ventrículo izquierdo debido al movimiento anterior sistólico y dos accidentes cerebrovasculares no fatales. El riesgo de muerte cardíaca súbita ESC se calculó para todos los portadores con hipertrofia del ventrículo izquierdo (n = 47). La mayoría (79%; 37/47) tenían una puntuación de riesgo baja (<4% de riesgo a 5 años), el 17% (8/47) tenían

valores intermedios (4-6% de riesgo a 5 años) y solo el 4 % (2/47) tenían riesgo alto (>6 % de riesgo a los 5 años).

Doscientas y cincuenta y siete variantes de *TPM1* se enumeraron en total a partir de este estudio, la literatura y otras bases de datos. Se caracterizaron diecinueve variantes patogénicas, 24 probablemente patogénicas, 58 variantes de significado incierto - potencialmente relevante incierto y 156 de significado incierto. Los datos disponibles sobre las variantes de *TPM1* de la literatura y este estudio fueron sistematizados, lo que permitió cambios en la patogenicidad de 87 variantes previamente descritas en la base de datos ClinVar. El número de variantes identificadas en cada residuo de la heptada proteica es diferente, en orden descendente en las siguientes posiciones: *a, d, g, f, b, e* y *c*, con respectivamente, 22, 22, 18, 13, 13, 10 y 5 variantes en cada posición. No se identificó ninguna correlación entre genotipo y fenotipo por posición de heptada. Solo la posición de heptada *e* parece tener predominantemente variantes de miocardiopatía dilatada que variantes de miocardiopatía hipertrófica (p = 0,009). No obstante, el número de variantes con información disponible sobre el fenotipo aún es bajo para determinar realmente la correlación entre la posición de la heptada y el fenotipo. La sobrerrepresentación de las variantes de TPM1 por dominio funcional muestra diferentes regiones de puntos críticos para la miocardiopatía hipertrófica frente a la miocardiopatía no compactada del ventrículo izquierdo/dilatada. El enriquecimiento de la miocardiopatía hipertrófica por regiones versus controles internos (con otros fenotipos cardíacos heredados) señaló que las regiones N-terminal de la unión superpuesta cabeza-cola, el sitio de unión de leiomodina y el sitio de unión de actina repiten 1 como sobrerrepresentados. En el extremo opuesto de la molécula ocurre lo mismo con la porción C-terminal de la unión superpuesta cabeza-cola y la repetición 7 del sitio de unión de actina, también asociadas a hipertrófica. Finalmente, la

repetición 6 del sitio de unión a actina también está sobrerrepresentada debido a la presencia de varias variantes de miocardiopatía hipertrófica en esta región. El enriquecimiento de fenotipos para las variantes de miocardiopatía dilatada frente a los controles internos mostró que la región media está sobrerrepresentada. Aquí, hemos incluido la miocardiopatía dilatada y la miocardiopatía no compactada del ventrículo izquierdo como un solo grupo en comparación con otros fenotipos cardíacos hereditarios. La región media corresponde a los sitios de unión a actina con las repeticiones 3 y 4, por lo que estos períodos también se destacan en la sobrerrepresentación del dominio por fenotipo. Se identificaron veinticuatro variantes de *TPM1* que podrían producir una proteína de tropomiosina truncada: 19 variantes intrónicas ubicadas dentro de la zona de empalme de consenso (posiciones flanqueantes -/+ 10) y cinco de tipo frameshift o missense. No se observó evidencia de cosegregación familiar ni sobrerrepresentación para las variantes intrónicas. Nuestros datos no pueden establecer un papel patogénico para las variantes intrónicas en este gen.

Una nueva cohorte con 380 pacientes portadores de variantes en *TPM1* no publicados, incluida la población p.Arg21Leu de la variante fundadora. Hemos identificado 308 individuos de diferentes 115 familias con miocardiopatía hipertrófica, totalizando 273 portadores (256 con datos clínicos disponibles). Entre los notificados con diagnóstico de miocardiopatía hipertrófica, 198 (77%) eran portadores afectados. Solo un único portador (0,45%) se reportó como miocardiopatía restrictiva por importante disfunción diastólica; sin embargo, tenía segmentos miocárdicos hipertrofiados. Cincuenta y cuatro (21%) individuos reportados como familiares eran portadores no afectados. Se informaron cuatro portadores (1,55%) con fenotipo de muerte súbita cardíaca inexplicable que se enumeran en el grupo de miocardiopatía hipertrófica. Además, en 35 familiares de primer y segundo grado con

genotipos desconocidos en familias con miocardiopatía hipertrófica fue reportado como muertes cardíacas súbitas inexplicables. Se informaron antecedentes familiares positivos de defectos cardíacos en el 9,5% de las familias (11/115) con hipertrófica y sólo cinco portadores (1,9%; 5/257) con un defecto cardíaco asociado. Entre estas 115 familias de miocardiopatía hipertrófica, se identificaron treinta y una variantes de *TPM1*, todas variantes de tipo missense. Tres variantes fueron particularmente más frecuentes; p.Arg21Leu (67 portadoras), p.Met281Val (53 portadoras) y p.Asp175Asn (36 portadoras). Treinta y nueve portadores fueron diagnosticados con una variante genética adicional (solo dos tenían tres o más [5,1%] variantes genéticas) en otros genes relevantes de miocardiopatía y muerte súbita cardíaca. No se identificó ningún portador homocigoto, excepto los cuatro descritos previamente con *TPM1* p.Arg21Leu. Entre los pacientes con miocardiopatía hipertrófica podemos observar menos del 5% de mortalidad a los 20 años, y este porcentaje sube al 12,5% en los varones a los 40 años, y al 6% en las mujeres portadoras. A los 60 años, la tasa de mortalidad es de hasta un 30% para los hombres portadores y un 23% para las mujeres portadoras. La mayoría de los eventos cardiovasculares se informaron entre los 50 y 60 años en ambos sexos, con otro aumento significativo de la mortalidad después de los 70 años en los hombres. Se observó una diferencia estadística significativa entre hombres y mujeres portadores (valor de p = 0,039). Se informaron dieciocho eventos cardiovasculares entre los portadores de *TPM1* de nuestra cohorte de miocardiopatía hipertrófica. En estos portadores se informaron tres eventos relacionados con la insuficiencia cardíaca y doce eventos relacionados con la arritmia. En este grupo también se informaron otras dos muertes cardíacas no especificadas y una muerte relacionada con un accidente cerebrovascular. Quince eventos cardiovasculares menores en portadores se enumeran en este mismo grupo. Hemos identificado 72

individuos de 29 familias con fenotipo de miocardiopatía dilatada o no compactada del ventrículo izquierdo, totalizando 59 portadores confirmados. Se describieron veinticinco probandos (42,4%) con miocardiopatía dilatada, tres (5,1%) probandos con fenotipo superpuesto dilatado/no compactada del ventrículo izquierdo y un solo (1,7%) con miocardiopatía no compactada sin dilatación ventricular. Se identificaron diecinueve (32,2 %) familiares afectados y 11 (18,6 %) familiares no afectados que portaban variantes de *TPM1*. Cinco personas fueron reportadas con muerte por insuficiencia cardíaca, sólo una era portadora genotipada y nueve fueron reportadas con muerte cardíaca súbita/arrítmica sin pruebas genéticas. Se informaron antecedentes familiares positivos de defectos cardíacos en nueve probandos (15,2%). Cuatro portadores (6,8%) fueron identificados con un defecto cardíaco asociado al fenotipo principal. Entre estas 29 familias, se identificaron veintitrés variantes de *TPM1*, todas variantes de tipo missense (ver tabla 6.4). Cinco variantes fueron recurrentes en diferentes familias; p.Asp84Asn, p.Ala109Val, p.Leu113Val, p.Ala120Val y p.Lys128Gln. Se informaron siete familias de miocardiopatía dilatada/no compactada del ventrículo izquierdo con una variante genética adicional en genes relevantes. Entre los portadores con fenotipo de dilatación/ventrículo izquierdo no compactado, podemos observar un 5% de mortalidad a la edad de 15 años, con eventos cardiovasculares registrados desde la primera infancia. Esta proporción se mantiene prácticamente igual hasta los 35 años. En este punto, la mortalidad observada aumentó un 20%, y este valor se mantiene hasta la sexta-séptima década de la vida. Entre los 59 portadores de este grupo, observamos dos muertes por insuficiencia cardíaca a las edades de 7 y 72 años, respectivamente, y dos trasplantes de corazón. Se informaron otras 14 muertes cardíacas súbitas en familiares de primer y segundo grado en este grupo.

Conclusiones: El gran número de portadores de *TPM1* p.Arg21Leu revela que se trata de una variante patogénica asociada a enfermedad de presentación tardía, penetrancia incompleta y evolución clínica generalmente favorable. La identificación de portadores no afectados con esta variante a edades avanzadas, tanto en nuestras genealogías como en la población general, sugiere una penetrancia incompleta. El análisis de supervivencia para esta población portadora de esta misma variante muestra un mejor pronóstico que en cohortes no seleccionadas. No obstante, los datos agrupados de las tres variantes más prevalentes (p.Arg21Leu, p.Asp175Asn y p.Met281Val) junto con todas las variantes de miocardiopatía hipertrófica identificadas en todo el gen *TPM1* en general mostraron manifestaciones de inicio tardío y un pronóstico similar al descrito en la literatura para cohortes no seleccionadas de miocardiopatía hipertrófica. Se observa una posible edad más temprana de diagnóstico en familias con miocardiopatía dilatada/no compactada del ventrículo izquierdo, especialmente en los casos de la primera infancia. En este grupo también se observó penetrancia incompleta. Las cardiopatías congénitas no se identificaron como casos aislados de malformación cardíaca, sino sólo en asociación con miocardiopatía. Se identificaron diferentes tipos de defectos cardíacos, incluidas anomalías auriculares, del tabique auricular y de la válvula aórtica. Los defectos cardíacos hereditarios fueron más frecuentes en el grupo de miocardiopatía no compactada del ventrículo izquierdo/dilatado. Los resultados genéticos de familias con enfermedades cardíacas hereditarias asociadas con variantes de *TPM1* pueden ser útiles para el proceso de toma de decisiones clínicas. El asesoramiento genético también puede ser más asertivo para estas familias según los datos aquí proporcionados. Los datos clínicos de nuestra cohorte también ayudarán a la interpretación clínica de los hallazgos genéticos y a la elaboración de políticas públicas de salud para estos pacientes en el contexto de la medicina de precisión y personalizada.