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Genetic variants of uncertain significance: How to match scientific rigour and standard of proof in sudden cardiac death?

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ABSTRACT

In many SCD cases, in particular in pediatric age, autopsy can be completely negative and then a post-mortem genetic testing (molecular autopsy) is indicated. In NGS era finding new/rare variants is extremely frequent and, when only variants of unknown significance are found, molecular autopsy fails to find a cause of death. We describe the emblematic case of the sudden death of a 7-year-old girl. We performed a full-body micro-CT analysis, an accurate autopsy, a serum tryptase test and toxicological tests. Since the only macroscopic abnormality we found was a myocardial bridging (length: 1,1 cm, thickness: 0,5 cm) of the left anterior descending coronary artery, a molecular autopsy has been performed. NGS analysis on victim DNA detected rare variants in *DPP6*, *MYH7, SCN2B* and *NOTCH1* and segregation analysis was then achieved. On the basis of ACMG/AMP (clinical) guidelines, all the found variants were classified as of unknown significance. In other words, both the macroscopic and genetic anomalies we found were of uncertain significance and then the autopsy failed to find the cause of the death. Our case raises three main discussion points: (a) economical, ethical and legal limitations of genetic investigation; (b) risk that genetic testing does not succeed in finding a certain cause of the death; (c) absence of specific guidelines to face the problem of VUS in forensic cases.

1. Introduction

Sudden death (SD) is traditionally defined as the unexpected natural death of a healthy individual occurring within the first hour after the onset of symptoms or, if death is unwitnessed, within 24 h of the victim being seen in a healthy state. These events draw the interest of medical and legal communities that investigate, for different purposes, the predictability and the preventability of SDs, as well as the interest of the society, which has a primordial fear of unpredictable phenomena. In the general population, almost 85% of SDs has a cardiac origin, while in the young population (<35 years), this percentage is as low as 57% [1,2]. These deaths are defined as sudden cardiac death (SCD). Young individuals represent 2–10% of total SCD victims [3]. The incidence of SCD is 2–8 cases per 100,000 persons per year in those younger than 35 years. In details, it is 1–2 per 100,000 per year in those aged between 15 and 35 and is particularly low among children between 6 and 10 years of age [3–6]. However, the incidence of SCD is certainly underestimated: in many ambiguous cases, the identification of a macroscopic and/or microscopic abnormality at the autopsy, regardless of its functional significance, is considered sufficient to certify the cause of death [7]. In the general population, about the 80% of SCD is due to ischemic heart disease, while in the subpopulation of young adults, the main aetiology is represented by familial cardiomyopathies (especially hypertrophic cardiomyopathy) [8,9]. SCD often occurs at home and/or

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Table 1
Cardiomyopathies.

	HCM	DCM	ACM
Prevalence	1/500 [8,14,22–26]	Uncertain (commonest estimate: 1/2,500) [22,34–36].	1/2,000–1/5,000 [31,43]
Mortality	Annual cardiac mortality depends on the age: 6% in pediatric populations, 1–2% in adult populations, and 0.64% in the over 60 age group [22,23,27–29]	Annual incidence of ventricular arrhythmia or SCD: 1.6–6.9% (depending on the presence of fibrosis) [22]	Annual cardiac mortality: 0.9–2.0% [29,44,45]
Genetics	More than 50 (main: *MYBPC3, MYH7, TNNI3,* and *TNNT2*) [30–36]	More than 60 (main: *BAG3, LMNA,* and *TTN*) [22,30,31,37–42]	More than 10 (main: *DSC2, DSP, PKP2,* and *DSG2)* [14,30,31,34,43,46–49]
Diagnostic yield of comprehensive genetic analysis	Nearly 80% [22,24,27,30]	30–40% in sporadic cases and nearly 60% in familial cases [35,36,38]	Nearly 50% [22]

HCM – hypertrophic cardiomyopathy; DCM – dilated cardiomyopathy; ACM - arrhythmogenic cardiomyopathy.

during sleep, although factors such as intense physical exercise, epileptic seizures, febrile illness and emotional or acoustic stress are globally considered potential triggers [2,4,8,10,11]. Autopsy-negative SDs (the so-called sudden unexplained deaths, SUDs) are particularly common in young adult population: in up to 53% of these cases, neither macroscopic, microscopic nor toxicologic anomalies are found [2,5,12,13]. Cardiomyopathies (Table 1) cause SUDs mainly in children and athletes, in which at the autopsy the identification of their distinctive macroscopic and microscopic features and the differential diagnosis with benign conditions can be difficult [14]. On the other side, channelopathies (Table 2) are the leading cause of SUD.

Syndromes responsible for SUD are generally characterized by autosomal dominant inheritance, locus heterogeneity, incomplete penetrance and variable expressivity [10,14–16]. Currently, more than 100 genes have been associated with syndromes leading to SCD, but in cases at risk genetic analyses often are restricted to a limited number of genes - mainly because of economic reasons [17].

Regarding the variants found by the genetic testing, many of them are classified as variants of unknown significance (VUS), because there is not sufficient evidence to define their functional role in phenotype. In 2015, the ACMG/AMP published clinical guidelines on the genetic classification of variants based on several items such as *in silico* predictions, global population frequencies and functional *in vivo/in vitro* analysis [18]. In recent years, additional reports including modifications of these guidelines have been published, improving the potential pathogenic classification of rare variants [19–21]. At the present time, there are no forensic guidelines on the management and interpretation of cases in which VUS are found.

In this paper we want to discuss the main elements and issues that differentiate the forensic management of cases in which VUS are found, starting from the description of a SD pediatric case in which at molecular autopsy only VUS were found.

2. Case report

In the early morning, an obese (height: 128 cm, weight: 40 kg) 7-year-old girl was found dead when she was at home with her mother. The girl was last seen awake and in a healthy state by her parents about an hour earlier. The medical examiner did not find external signs of violence.

2.1. Anamnestic data

Her clinical history was negative, but in the last weeks, she had frequently been visited by a pediatrician to treat seasonal allergies. We were not authorized to obtain clinical information on her first-degree relatives.

2.2. Preautopsy imaging

A full-body CT-scan was performed to exclude abuse or trauma. It did not show any anomaly (Fig. 1).

2.3. Autopsy results

Prosecutor requested an autopsy to find the cause of the death and to exclude chiefly child abuse and pediatrician malpractice. It was performed the day after death. The heart (weight: 240 g, longitudinal diameter: 11 cm, transverse diameter: 10 cm, thickness of the anterior wall of the right ventricle: 0·3 cm, thickness of the left ventricle: 1·0 cm) did not reveal morphologic or structural anomalies of ventricular walls and valves. The coronary circulation was right-dominant and presented myocardial bridging (length: 1·1 cm, thickness: 0·5 cm) involving the middle segment of the left anterior descending coronary artery (Fig. 2). No other significant elements were found upon macroscopic observation, with the exception of pulmonary edema. The histopathological examination (Fig. 2) showed patchy interstitial fibrosis, myocyte disarray and waving of the myofibers in the tissue surrounding the tunneled artery. Several areas of interstitial and subendocardic fibrosis in the other samples of the left ventricular myocardium were also observed.

2.4. Postmortem tests

The low value of serum tryptase (6·12 μg/L) excluded a fatal anaphylaxis [62]. Toxicology tests were negative.

2.5. Genetic testing

Genomic DNA was extracted from postmortem whole blood of the proband with Chemagic MSM I (PerkinElmer, Waltham, MA, USA). Spectrophotometric measurements were performed to assess quality ratios of absorbance (260/280:260/230 minimum of 1.8:2.2). DNA concentration was determined by a Qubit fluorometer (Thermo Fisher Scientific, Waltham, MA, USA), and 3 μg of DNA was used for library preparation. Whole blood samples of the proband mother, father and sister were obtained to extract genomic DNA and thus to perform Sanger sequencing to analyze the segregation of each genetic variant identified by NGS.

For NGS analysis we used a custom resequencing panel which included 147 genes associated with cardiomyopathies and channelopathies, designed and optimized by Gendiag.exe SL and commercialized by Ferrer inCode as SudD inCode® (Table 3) [63–66]. The final size was 442,13 kb of encoding regions and UTR boundaries. We sequenced an isoform for each gene and only coding exons and 10 base pairs inside intronic regions. Exons were obtained from Ensembl site version 81 in GRCh38 and translated to hg19 [67]. Bait design was achieved using an in-house algorithm and submitted to Agilent SureSelect Design Web. The chip design was achieved using variable tailing bait and variable multiplicity bait to obtain homogeneous coverage and optimize sample load. The present bait design algorithm considers the GC content of the bait itself, as well as the target and the surrounding region of the

Table 2
Channelopathies.

	BrS	LQTS	SQTS	CPVT
Prevalence	0,5/1,000 worldwide (in Southeast Asia 2/1,000) [1,50–53]	1/2,000–1/5,000 [3,56]	Estimated 1/10,000 [50]	1/10,000 [3,57,60]
Mortality	SCD rate: 5,0% [54]	SCD rate in untreated patients: 0.33–0.90% [29]	Cardiac arrest risk by the age of 40 years: 40% [29]	Mortality in untreated patients: 30–50% [60]
Genetics	More than 15 (main: *SCN5A*) [3,50,55]	More than 50 (main: *KCNQ1* -in LQT1, 30–35% of the cases-, *KCNH2* -in LQT2, 25–30% of the cases-, and *SCN5A* -in LQT3, 5–10% of the cases-) [3,55,57,58]	More than 5 (main: *KCNQ1*, *KCNH2*, and *KCNJ2*) [3,50,59]	More than 5 (main: *RYR2* -autosomal dominant-) [3,50,57,61]
Diagnostic yield of comprehensive genetic analysis	30–35% [1,50]	Nearly 85% [50]	15–30% [50]	Nearly 60% [50]

BrS – Brugada syndrome; LQTS – Long QT syndrome; SQTS – Short QT syndrome; CPVT – Catecholaminergic polymorphic ventricular tachycardia.

targeted gene. This algorithm also considers multimapping of the bait regions and uses, when available, previous NGS results. Genomic DNA was fragmented by sonication using the Bioruptor (Diagenode). The 147 genes were enriched using the SureSelect Custom Target Enrichment System Kit (Agilent Technologies, Santa Clara, CA USA) according to the manufacturer's instructions for the "SureSelect Target Enrichment System for Illumina Paired-End Sequencing version B.1″ (SureSelect XT Custom library, Agilent Technologies, Inc.). The paired-end sequencing process was carried out on MiSeq System (Illumina) using 2 × 76 bp read length.

NGS analysis was submitted to GendiCall Pipeline (Gendicall Software from FerrerIncode), which cleans up and trims fastq files from sequencers and then Gendicall maps using either GEM or BWA mapper [68]. Subsequently, bam files were generated and sorted, and duplicates were removed with Picard (http://picard.sourceforge.net). Variant calls for SNVs and Small Indels were performed using Samtools (v.1.3.1) and internal Gendicall Caller. Annotation of called variants was based on several sources, and for population data, dbSNP [69], 1000 Genomes Project [70], Exome Variants Server (EVS) (Exome Variant Server, NHLBI GO Exome Sequencing Project (ESP), Seattle, WA 2014), Exome Aggregation Consortium (ExAC) Cambridge, MA [19] and Genome Aggregation Database (gnomAD; http://gnomad.broadinstitute.org) were used. The protein predictors consulted were PolyPhen2 [71], Sift [72], Provean [73] and Mutation Taster [74]. Finally, we also used the splicing predictors MaxEntScan [75], FSPLICE, GeneSplicer [76] and NNsplice [77]. Sanger sequencing was performed to sequence regions with coverage lower than 30X, as well as to validate the uncommon variants identified (allele frequency lower than 1% in general population). First, polymerase chain reaction (PCR) was performed, and the product was purified by ExoSAP-IT (USB Corporation, Cleveland, OH, USA) and directly sequenced by the dideoxy chain-termination method in an ABI Prism Big Dye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA). Sequencing was processed in a 3130XL Genetic Analyzer (Applied Biosystems) and analyzed by SeqScape Software v2.5 (Life Technologies), comparing the obtained results with the reference sequence from hg19. Protein numbering reflects the translation initiator methionine as + 1. Genetic variants were reported following the recommendations of the Human Genome Variation Society (HGVS). Regarding copy number variation (CNV), our approach was focused on capturing significant differences between expected normalized coverage and obtained normalized coverage for a given sample in a region of interest. Several samples were analyzed to corroborate similar levels of coverage between samples, as already published by our group [78,79]. Finally, each variant was classified according the current recommendations of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology (ACMG/AMP) [18]. All the investigators discussed data included in each item of the ACMG and reached a consensus on the final classification of all the variants.

We identified five rare genetic variants in heterozygous state (Table 4). Then, we performed Sanger sequencing on the DNA extracted from the mother, the father and the sister of the proband. The father carried both variants identified in the *DPP6* gene, whereas the mother carried the rare variants identified in *MYH7, SCN2B* and *NOTCH1*. The sister carried all the genetic variants (Fig. 3). Following ACMG/AMP criteria [18], all the variants were classified as VUS.

The mutated positions of all the variants proved to be conserved among different species (Fig. 4).

3. Discussion

In forensic field the assessment of the significance of a variant is linked to a complex web of medico-legal issues. In particular, attributing a precise likelihood to the causal relationship between the found variant and an event of legal interest is important to meet an adequate standard of proof.

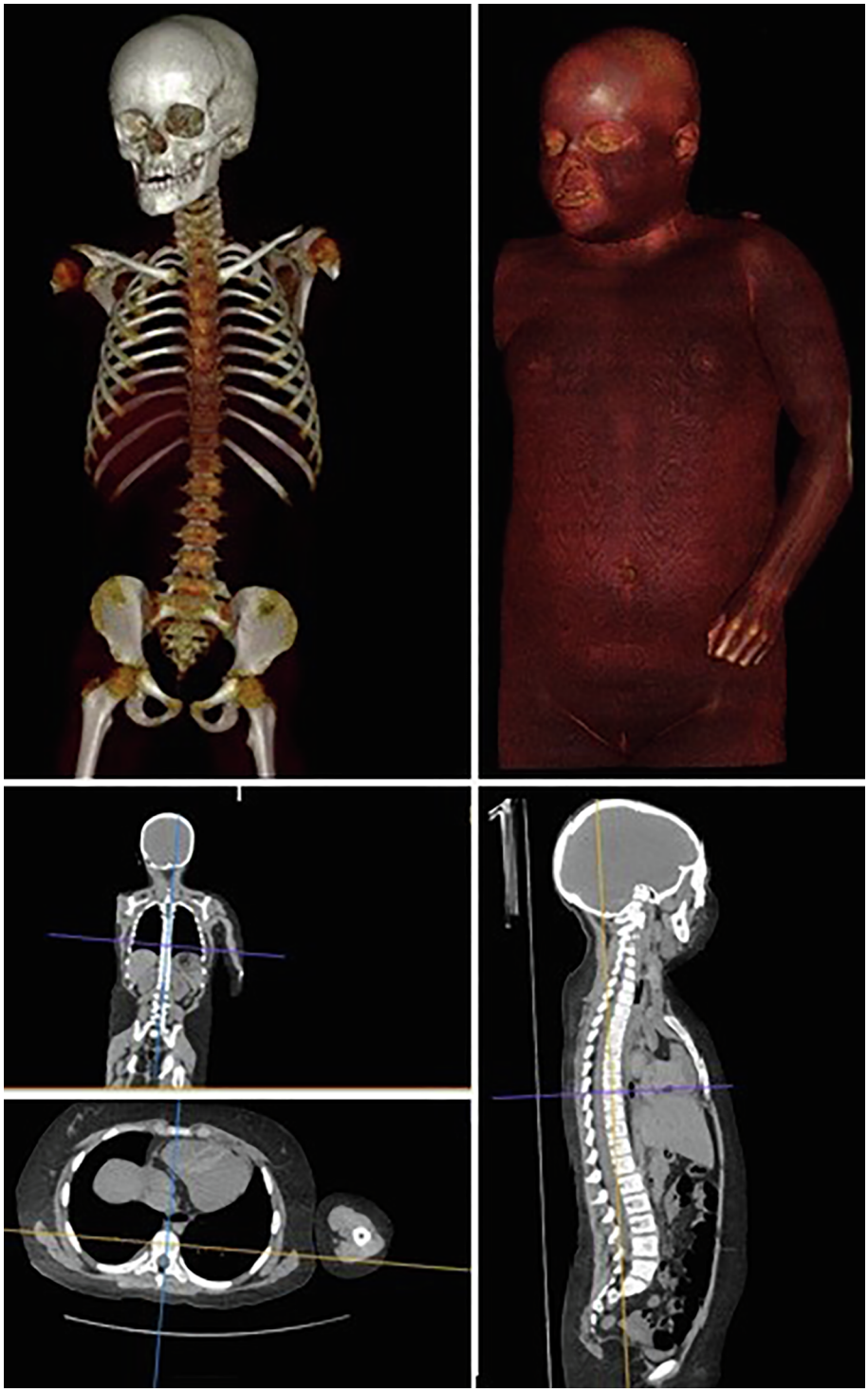

Fig. 1. negative micro-CT.

Regarding the interpretation/management of cases in which VUS are found, an important aspect is represented by the legal responsibility of the medical examiner: autopsy is requested by public authorities to obtain certain answers, and an undefined cause of death could jeopardize an entire investigation and raise unwarranted suspicions (in our case, that of a child abuse) in public opinion and among the investigators. Furthermore, a forensic pathologist/forensic geneticist who underestimates the pathogenicity of a variant could be considered legally responsible for the possible cardiac events or SCD of the victim's relatives [80]. On the other hand, the overestimation of these data can wrongfully lead to dramatic life changes (e.g., the ineligibility for sports and particular jobs) and to highly invasive interventions (like the implantation of an ICD). In cases in which SD is suspected to have a cardiac origin, the pathologist task is further complicated by the fact that the carrier of the VUS is dead, and so he cannot opt for watchful waiting.

These are not niche issues: finding VUS is extremely common in forensic practice, since in the majority of cases, the assignment of a certain or likely significance to the found variants is impossible because of the lack of supporting data [18].

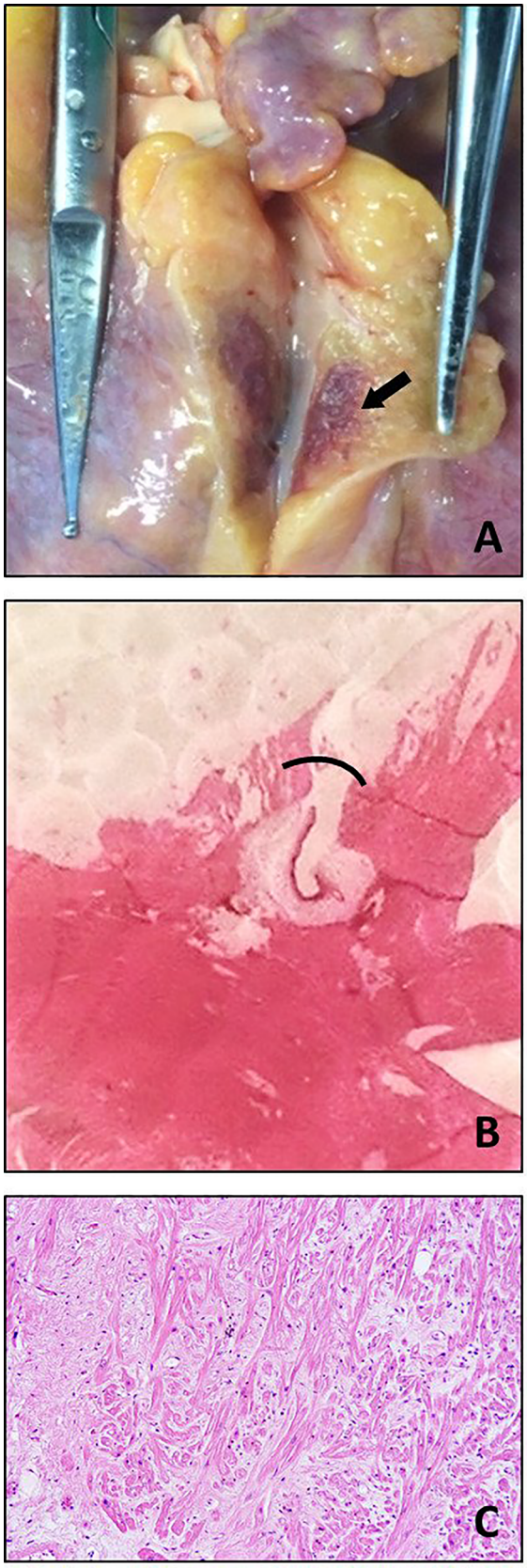

Fig. 2. A. Macroscopic picture of the myocardial bridge (black arrow) involving the middle segment of the left anterior descending coronary artery; B. Histological section of the left anterior descending coronary artery (black arch on the resected tunneled tract); C. Focal myocardial disarray in the surrounding myocardium.

Table 3
List of the genes included in the custom resequencing panel.

ABCC9, ACTA2, ACTC1, ACTN2, AKAP9, ANK2, ANKRD1, BAG3, BRAF, CACNA1C, CACNA2D1, CACNA1G, CACNA1H, CACNA1I, CACNB2, CALM1, CALM2, CALM3, CALR3, CASQ2, CAV3, CBL, COL3A1, CRYAB, CSRP3, CTNNA3, GJA1, CTF1, DES, DMD, DMPK, DPP6, DSC2, DSG2, DSP, DTNA, ECE1, EMD, EN1, EYA1, EYA4, FBN1, FBN2, FHL2, FKTN, FLNA, FLNC, GAA, GJA5, GLA, GPD1L, HCN1, HCN2, HCN4, HRAS, JPH2, JUP, KCNA5, KCND3, KCNE1, KCNE2, KCNE3, KCNE4, KCNE5, KCNH2, KCNJ2, KCNJ5, KCNJ8, KCNQ1, KRAS, LAMA4, LAMP2, LDB3, LMNA, MAP2K1, MAP2K2, MYBPC3, MYH11, MYH6, MYH7, MYL2, MYL3, MYLK2, MYOZ2, MYPN, NEBL, NEXN, NF1, NOS1AP, NOTCH1, NPPA, NRAS, NUP155, PDLIM3, PHOX2A, PHOX2B, PITX2, PKP2, PLN, PRKAG2, PTPN11, RAF1, RANGRF, RBM20, RET, RIT1, RYR2, SCN1B, SCN2B, SCN3B, SCN4B, SCN5A, SCN10A, SDHA, SGCD, SHOC2, SLC22A5, SLC6A4, SLC8A1, SLMAP, SLN, SMAD3, SNTA1, SOS1, SOS2, SPRED1, TAZ, TCAP, TGFB2, TGFB3, TGFBR1, TGFBR2, TGFBR3, TLX3, TMEM43, TMPO, TNNC1, TNNI3, TNNT2, TP63, TPM1, TRDN, TRIM63, TRPM4, TTN, TTR, VCL

We decided to start the discussion of this topic presenting a case of SCD in the young because in this subpopulation autopsies are generally more challenging (in particular because of the frequency of SUDs) and bear a particular social and legal burden [81].

The described case is an example of possible medicolegal issues in SCD-VUS cases. The victim's body did not show external signs of abuse, and the reported circumstances of the death were compatible with SD/SCD. We decided to perform a CT-scan before the autopsy to exclude traumatic causes of death, such as strangulation [8,82]. After obtaining the (negative) result, we performed the autopsy, which only revealed myocardial bridging (MB) in the context of a macroscopically normal heart with a weight near the upper limit of the normal (240 g – note that, according to Scholz et al., the normal heart weight of a 40 Kg female child ranges from 105 to 245 g) [83].

We were uncertain about the significance of the deep MB and then, before considering the autopsy negative, we consulted the scientific literature on the clinical significance of MB.

MB is a common (40–80% of the autopsies) congenital anomaly, has a mean length of 14·64 ± 9·03 mm and a mean thickness of 1·23 ± 1·32 mm, and in 67–98% of cases (like in this case) affects the middle trait of the left anterior descending coronary artery [84,85]. It has been associated with major cardiac events, such as acute myocardial infarction and severe arrhythmias [86].

Hostiuc et al. found an association between hemodynamically significant (width ≥ 2 mm) MB and interstitial fibrosis [86,87]. In general, the recurrence of this anomaly in HCM cases is well assessed [88]. Regarding the pediatric age, Yetman et al. described MB in children with HCM as a possible cause of myocardial ischemia and Gori et al. reported a case affected by both these conditions in which SCD occurred at rest [87,89]. In the light of these data, Thiene stated that "myocardial bridge is part of the phenotypic spectrum of HCM and can account in selected cases for ischemia" [90]. Despite this evidence, its clinical and prognostic significance appears to be highly controversial: a recent metanalysis confirmed that MB may cause myocardial ischemia but excluded a statistically significant relationship between MB and cardiovascular death [91]. Moreover, 2017 AECVP guidelines classify this anomaly as an uncertain cause of SCD [6].

In addition to the MB, in this case we found myocyte disarray and interstitial fibrosis, that, as stated by 2017 AECVP guidelines, are suggestive of HCM even in the absence of myocyte hypertrophy [6]. It should be considered that HCM is an extremely insidious syndrome: in young patients the phenotypic expression is usually absent or mild, and in 2017 the HCMNet Study found that the carriers of pathogenic variants tend to show altered cardiac function even if the ventricular thickness is normal [92]. This phenomenon has been reconducted to two main mechanisms: haploinsufficiency and the so-called "poison polypeptide effect" (the abnormal sarcomeric proteins could impair the global function of the heart) [26].

In particular, in our case at microscopic examination of the heart we

Table 4
Report of rare genetic variants identified in the index case.

Gene	Variant	dbSNP	ExAC NFE count[1]	ExAC total count[2]	gnomAD	PPH2	SIFT	Provean	Mutation Taster	ClinVar	ACMG score
MYH7	c.2585C > T p.(Ala862Val)	rs149576470	3·295e-05	1·499e-05	8/282828 (0.002829%)	B	T	N	DC	UC	VUS
SCN2B	c.93G > T p.(Glu31Asp)	rs767173208	8·237e-06	0	5/ 251,370 (0.001989%)	B	T	N	DC	UC	VUS
NOTCH1	c.6938G > A p.(Arg2313Gln)	rs371069660	0·0001364	0	24/278680 (0.008612%)	PD	T	N	DC	UC	VUS
DPP6	c.2140G > A p.(Ala714Thr)	rs188276022	0·001543	0·002353	375/244858 (0.15%)	B	T	N	P	UC	VUS
DPP6	c.2259 + 9C > A	rs373530414	0	<0·001[3]	19/227850 (0.0083%)	–	–	–	–	UC	VUS

DC: Disease Causing; P: Polymorphism; N: Neutral; T: Tolerated; B: Benign; PD: Probably Damaging; PPH2: PolyPhen2; UC: Uncertain Significance; VUS: Variant Unknown Significance.
[1] Allele count from ExAC (European Non Finnish).
[2] Allele count from ExAC (All populations).
[3] Data obtained from NHLBI Exome Sequencing Project.

found diffuse fibrosis, a feature that is particularly interesting if the victim age and her (negative) medical history are considered.

In HCM cases the association between myocardial bridging and fibrosis is well-known, and, according to some authors, in young patients affected by HCM and with a negative medical history, myocardial disarray and ischemia-caused fibrosis (possibly due to vascular abnormalities like myocardial bridging) could cause electrical instability and thus SCD [26]. Moreover, in HCM fibrosis can be considered an independent risk factor of SCD [93].

Since, as said, there is not a solid scientific basis for the interpretation of the deep MB we found as the cause of death and, at the same time, a certain diagnosis of HCM could not be made on the basis of our macroscopic/microscopic findings, we decided to perform a molecular autopsy.

We found variants in *DPP6, MYH7, SCN2B,* and *NOTCH1*. *MYH7* gene is very often implicated in HCM cases and, in particular, the specific variant that we found has been proposed by some authors to be disease-causing [94]. However, the application of ACMG/AMP guidelines lead to the interpretation of all the found variants as VUS.

Overall, the main issue of the described case is that on one side there are histopathological features suggestive of HCM together with a morphological abnormality of unknown significance but considered by some authors part of the phenotypic spectrum of HCM (myocardial bridging), while on the other side there are genetic abnormalities of unknown significance, one of which affects a gene typically involved in HCM and has already been described in an HCM case. These findings should be communicated to family members for clinical evaluation, since in adult carriers of pathogenic variants it is more likely to find an explicit phenotype of HCM (and thus defining the variant significance could be easier after thorough evaluation) [92].

However, from a forensic point of view, the evidentiary value that should be given to these findings is highly debatable. In this specific case, for example, in our opinion the presence of HCM and its role as cause of (arrhythmic) death remain the most plausible hypothesis.

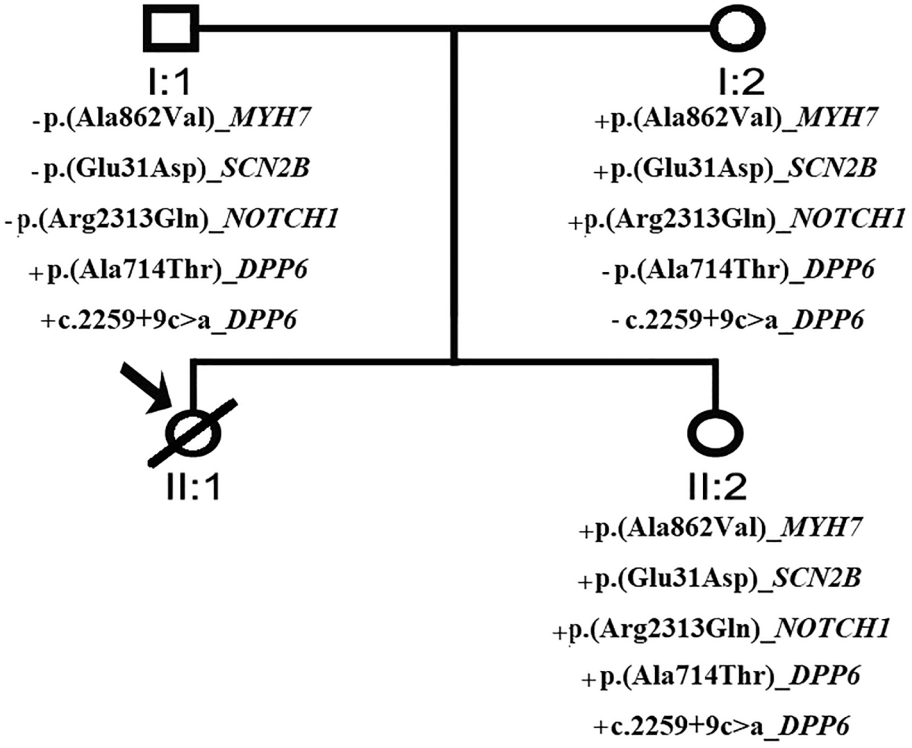

Fig. 3. pedigree.

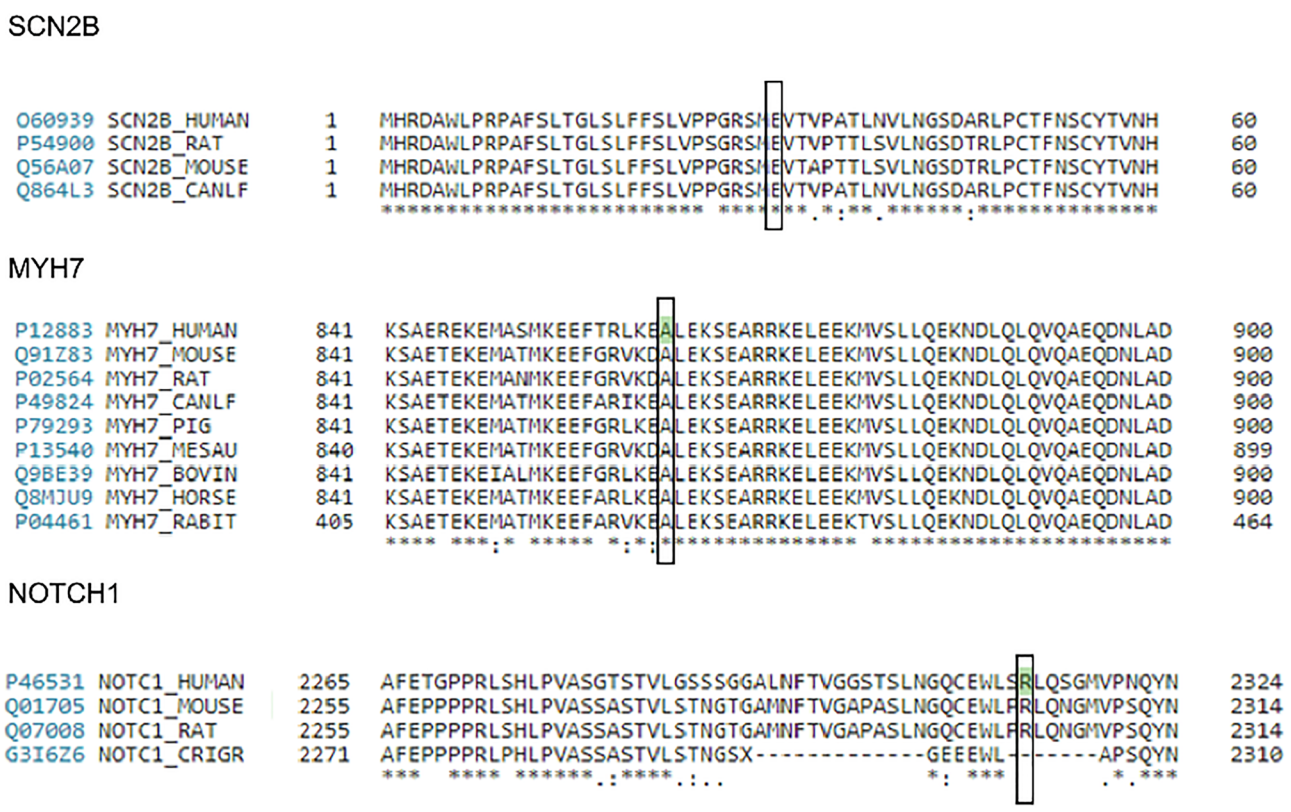

Fig. 4. Conservation between different species diagram.

However, currently there is not a standardized way to critically evaluate and express this hypothesis and its likelihood (or the ratio of the likelihood of finding this evidence – in particular these genetic variants - in HCM and the likelihood of finding it in other contexts) in a standardized form that could be unequivocal, practical and useful from both public health and judiciary perspectives.

Our forensic investigation hence did not find a certain cause of the death and raises three main discussion points:

(a) economical, ethical and legal limitations of genetic investigation
(b) risk that genetic testing does not succeed in finding a certain cause of the death
(c) absence of specific guidelines to face the problem of VUS in forensic cases

(a) ESC guidelines recommend analysis of potentially disease-causing genes in all sudden death victims in whom a specific inheritable channelopathy or cardiomyopathy is suspected [29]. In most of the western countries medical examiners investigations are bound by the local laws and by the decisions of prosecutors. More generally, they are functions of several economical, legal and ethical factors (e.g. the additional cost/time requested by genetic testing, the reluctance among prosecutors to accept a thesis on the cause of the death only based on genetic data). Moreover, a SCD case has a relevant cost, also in terms of human resources, since it implies the involvement of multiple health professionals (e.g. pathologists, cardiologists, geneticists, genetic counselors, bioinformaticians) and the use of expensive and complex technologies. Anyway, a broader perspective is needed to perform a correct cost-benefit analysis: hundreds of thousands of dollars per family can be saved by the healthcare systems indicating molecular autopsies in selected cases [95–98].

Lastly, there is the relevant issue of the access to clinical information of the victim relatives: in many countries, the possibility to collect the clinical history of the victim family depends on the decisions of prosecutors. However, lack of this information is certainly a limitation, but it usually does not jeopardize the investigation, since segregation of a variant is rarely considered a strong and highly reliable evidence of pathogenicity [18].

In our opinion, the most cost-effective strategy would be to perform them in referral centers where NGS is possible. It is important to remark that an NGS analysis can be completed in few days - thus it does not require too much time for a criminal investigation [99]. Regarding the access to information, a clear explanation of judicial and public health benefits and a standardized workflow would certainly sensitize prosecutors on the importance of this issue.

(b) As above mentioned, more than 100 gens have been associated with diseases leading to SCD but current guidelines only recommend the analysis of main prevalent in each disease: BrS (*SCN5A*), LQTS (*KCNQ1, KCNH2*, and *SCN5A*), SQTS (*KCNQ1, KCNH2*, and *KCNJ2*), CPVT (*RyR2*), HCM (*MYH7, MYBPC3*, *TNNI3,* and *TNNT2*), DCM (*LMNA* and *BAG3* –not *TTN,* despite it has recently been reported as the main prevalent-), and in ACM (*PKP2, DSC2, DSG2,* and *DSP*) [29]. The sensitivity of genetic testing depends on the specific disease: diagnostic rate ranges from approximately 30% in BrS to 80–85% in LQTS or HCM. These data indicate that there still is a consistent share of SCDs in which no cause is found. It should be taken into account that the custom gene strategy can only identify variants in the genes of which the association with the targeted disease has already been assessed. For this reason, when a panel of genes usually associated with channelopathies and cardiomyopathies fails to find variants that can explain the proband phenotype, as occurred in the case presented, a wider analysis provided by WES (whole exome sequencing) and WGS (whole genome sequencing) can be considered [100]. At the present time, the economic cost of resequencing panel, WES and WGS is similar. However, clear insight into the diagnostic sensitivity of WES and WGS remains still a matter of argue due to few studies published in this area. It is accepted that the genetic diagnostic rate reached by WES was higher than that achieved by commercially available genetic panels [101] but WES is

generally considered to have a lower coverage than that of the re-sequencing panels. LaDuca et al. showed that these two techniques can achieve similar coverages [102], at least for clinical diagnosis due to threshold currently established at 30x. In addition, Lionel et al. recently suggested that WGS could have a higher diagnostic yield than WES and genetic panels, if proper technical coverage is obtained [103]. Lastly, Rueda et al., focusing on SUD in the young, have shown that the diagnostic yield did not improve when the genetic analysis escalated from gene panels to WES [104].

Because of these controversial aspects, in our opinion the best approach is a combination of strategies: a massive parallel sequencing of target disease-related genes and candidate genes, complemented with a posterior confirmation and validation of the identified potentially pathogenic variants by the Sanger method. In case of negative results after comprehensive genetic analysis and familial segregation analysis (including genotype-phenotype correlation), WES is next step to consider. Concerning WGS, nowadays it is not a good approach for diagnosis despite remains a main tool in genetic research. Despite all these NGS approaches, Sanger will still remain the gold standard to confirm genetic variations identified using NGS (if the coverage is lower than 30x) as well as in family segregation of potentially disease-related variants [100].

(c) Concerning the role of the guidelines, there are no unanimous indications for the molecular autopsy or for the medico-legal interpretation of its results. In 2017 the Association for European Cardiovascular Pathology (AECVP) recommended interpreting a probable or uncertain cause always in light of the entire body of the evidences, considering also the clinical history of the victim and the circumstances of the death [6]. More recently, in 2019, a group of European authors (including representatives of the AECVP) published some recommendations on the same matter, focusing on the importance of a multidisciplinary management of SCD cases [105]. Regarding genetic aspects, the authors did not give explicit recommendation/indication criteria but observed that "if there is a clear indication for genetic testing, for instance, in case of inherited cardiac disease, such as cardiomyopathy, a panel of genes related to the condition could be used. However, the indication for genetic testing is more debated when the phenotype is unclear (the autopsy is normal, unexplained SCD, SADS)". Furthermore, they specified that when VUS are found phenotypic information of the deceased and segregation analysis are needed. Finally, they stated that coroners often do not indicate a molecular autopsy because they think it would require much too money and time and it would have no impact on the judicial procedure.

The reported opinion of some medical examiners proves to be certainly wrong not only in the cases like the one here described but also, for example, in the cases of athletes SDs [106].

In these cases, the molecular autopsy may give information of extreme evidentiary value, since it can help to identify arrhythmogenic syndromes that could have been diagnosed by the physicians (e.g. cardiologists, sports medicine experts) who followed the athlete before the death [60]. In other words, when a suspected medical malpractice is claimed and autopsy is inconclusive, how is it possible to discuss the possibility of a missed diagnosis if the exact cardiac disease/cause of the death remains uncertain? This consideration is valid not only when the death has been caused by channelopathies, but also, for example, in the frequent scenario of athlete HCM difficult to be differentiated from an insidious sport-induced ventricular remodeling [92].

Regarding genetic aspects, more explicit and complete indications were given in 2015 by the ACMG/AMP clinical guidelines, that recommended considering different parameters (among which the phenotypic information and segregation analysis) to orientate the attribution of one of the five possible significances (benign/likely benign/of unknown significance/likely pathogenic/pathogenic) to the variant [17]. However, each of these criteria has strong limitations, especially when used in forensic context, and is neither sufficient nor necessary to define the pathogenicity of a variant. Moreover, these guidelines advise against the use of VUS in the decision-making [17].

4. Conclusion

In the present forensic scenario, molecular autopsies frequently identify genetic variants which are classified as VUS. In these cases, the autopsy should be declared uncertain or inconclusive. However, it is easy to imagine that investigating authorities would be disappointed by this line of action, because in the legal field this attitude would lead to a dead-end investigation.

On the other hand, the arbitrary attribution of significance to a VUS is not acceptable, because legal goals cannot justify the absence of scientific rigour.

For these reasons, in many SCD cases, pathologists and forensic geneticists seem to be condemned to operate beneath a sword of Damocles.

Sharing data about variants significance indicators in public databases and regularly revisiting variant classifications can help to contain the incidence of VUS in SCD cases are, but in forensic field these measures are not sufficient [107,108].

In facts, despite the difficulty of defining a common line of action that takes into account the huge legal, economic and cultural heterogeneity of different countries, forensic community would benefit from clear, explicit and detailed indications for molecular autopsy and guidelines on management/interpretation of rare/new genetic variants in SCD cases that take into account the peculiarities, the limitations and the goals of forensic investigations. These innovations could help, for example, to standardize the decision-making process (public authorities are usually more inclined to authorize international consensus-based methodologies) and to communicate the medico-legal significance of results, avoiding allegations caused by misunderstanding or suspicion of malpractice.

In conclusion, in our opinion, since forensic investigations serve the public interest, when in a SD case obtaining a diagnosis is not otherwise possible, performing a molecular autopsy is needed both as a last attempt to obtain evidence for judicial purposes and as a public health intervention.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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