CHAPTER 4

Next Generation Sequencing of a large gene panel in patients initially diagnosed with idiopathic ventricular fibrillation

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Abstract

Background: Idiopathic ventricular fibrillation (IVF) is a rare primary cardiac arrhythmia syndrome that is diagnosed in a resuscitated cardiac arrest victim, with documented ventricular fibrillation, in whom no underlying cause is identified after comprehensive clinical evaluation. In some patients causative genetic mutations are detected which facilitate patient treatment and follow-up. The feasibility of next generation sequencing (NGS) has increased with its greater availability and decreasing costs.

Objective: The aim of this study was to assess the diagnostic yield of NGS in IVF patients.

Methods: A total of 33 patients initially diagnosed with IVF were included (mean age 53±15; 42% male). In all included patients NGS of 33 genes + the *DPP6* haplotype were screened and normal in a previous stage. Genetic screening comprised NGS of a panel of 179 additional genes. Variants with a minor allele frequency of <0.05% were assessed for pathogenicity using existing mutation databases and *in silico* predictive algorithms.

Results: In one out of 33 patients, a likely pathogenic mutation was detected. The added yield of genetic testing with NGS of 179 additional genes is 3% in IVF patients. In 15% of the patients one or multiple variants of uncertain clinical significance were detected.

Conclusion: The added yield of genetic screening of extended NGS panels in patients initially diagnosed with IVF is minimal. Routine analysis of large diagnostic NGS panels is therefore not recommended.

Introduction

Idiopathic ventricular fibrillation (IVF) is a rare arrhythmia syndrome that is diagnosed in a resuscitated cardiac arrest victim, suffering from ventricular fibrillation, in whom no underlying cause is identified after comprehensive clinical evaluation.[1] The event is very distressful for the patient and the family members. Because no specific diagnosis is made, the implications for family members are unknown. Currently, there are no ways to clearly identify family members who are at risk for ventricular arrhythmias, except for genetic testing. Detection of a causative pathogenic mutation enables cascade family screening.

Genetic testing has contributed immensely to the detection of primary inherited arrhythmia syndromes. Because primary inherited arrhythmia syndromes, as well as inherited cardiomyopathies, are genetically heterogeneous, genetic testing has been expensive and time consuming in the past. The development of next generation sequencing (NGS) has enabled rapid, and more cost efficient screening of large gene panels. Therefore, the feasibility of genetic testing has increased in all inherited cardiac diseases such as cardiomyopathy and primary arrhythmia syndromes, including IVF.

The yield, defined as the identification of a pathogenic or likely pathogenic mutation, of genetic screening in IVF patients is modest, and is therefore not routinely recommended.[1] Previous studies have shown that the yield of genetic testing in IVF is 9%.[2,3] However, these studies have screened small gene panels of a maximum of 10 genes. The yield of screening larger gene panels in IVF patients is unknown. Targeted sequencing of large gene panels in sudden unexplained death victims <50 years with a structural normal heart show a yield of 21-32%.[4-6] Moreover, extensive genetic screening with whole exome or genome sequencing has identified several causative mutations in IVF patients, for example the Dutch *DPP6* risk haplotype, *CALM1*, a novel *RYR2* mutation that causes ventricular fibrillation in rest, and *IRX3* that is associated with short-coupled Torsade De Pointes.[7-10] The detection of these causative mutations has facilitated the identification and improved the clinical management of asymptomatic IVF family members.[11]

Although the feasibility of NGS has increased, extensive genetic screening also results in generation of 'genetic noise'. Less informative variants of uncertain clinical significance (VUS) are frequently detected and constitute a risk of a false positive genetic test result. No data are available regarding the number of VUS that are detected in IVF patients. These data are necessary to assess the risk-benefit ratio of genetic testing. In this study we used NGS to assess the diagnostic yield of genetic testing of an extended NGS panel and the number of detected VUS in IVF patients.

Methods

Cohort

Patients were derived from the FU-IVF cohort (follow-up of idiopathic ventricular fibrillation), a Dutch cohort of patients with an initial diagnosis of IVF. The exact details have been published elsewhere.[12] In summary, we enrolled all consecutive patients between 1986 and 2015 with an unexplained cardiac arrest with initial rhythm of ventricular fibrillation, in whom known cardiac, respiratory, metabolic, and toxicological causes were excluded at first presentation. Comprehensive clinical investigation was performed and accepted diagnostic criteria were used to exclude specific diseases.

In the FU-IVF cohort, NGS of a diagnostic panel of 34 cardiac arrhythmia genes was performed in 53 patients. If NGS of 34 genes revealed no abnormalities, patients were requested to participate in NGS of an extended panel of 179 genes.

The study was approved by the local ethics committee of the University Medical Center Utrecht. Informed consent was obtained in all participants. The study was performed according to the Principles of the Declaration of Helsinki.

NGS of 179 genes

Genomic DNA was obtained from peripheral blood. Mutation analysis was performed using targeted massively parallel sequence analysis/ NGS of the protein coding exons and accompanying splice-site sequences of 179 genes (diagnostic and candidate genes) associated with either cardiomyopathy, arrhythmias or congenital heart disease or selected based on known and expected disease pathway

or know protein function or localization. A detailed list of the 179 genes is shown in table 1, as well as the 34 genes that were analyzed in a previous stage. We used the Applied Biosystems 5500XL SOLID system that detects 95% of the nucleotide changes in the genes. The detection rate of NGS is ~70% to detect deletions, insertions, and indels of less than 5 nucleotide pairs. Deletions, insertions and indels of more than 5 nucleotide pairs are not reproducibly detected with this method but typically comprise <5% of the mutation spectrum.

The 179 genes were enriched from the genomic DNA by hybridization of fragmented genomic DNA with Agilent SureSelectXT Custom Probes. These oligonucleotide probes of 120 nucleotides were targeted for the 179 genes on the panel. After enrichment, the SOLID 5500 XL sequencing system was used for sequencing with an average read length of 50 nucleotides. These reads were mapped to the human reference genome and detected variations were analyzed for pathogenicity. Detected variants were checked for presence and allelic frequency in the Exome Aggregation Consortium (ExAC) database. Only variants with a minor allele frequency of <0.05% were further analyzed. We analyzed classification according to the ClinVar disease mutation database and *in silico* predictive algorithms (SIFT, PolyPhen2) to assess pathogenicity of the detected variants. Variants of uncertain clinical significance were considered not to be proven-pathogenic. Pathogenic and likely pathogenic mutations detected with NGS were confirmed by Sanger sequencing. All pathogenic and likely pathogenic mutations were used to calculate the diagnostic yield of NGS.

Data analysis

Patient characteristics were reported as percentages, counts, means ± standard deviations (SD) or medians with interquartile ranges (IQR), as appropriate. All data were analyzed using SPSS version 20.0 (IBM, Armonk, NY, USA).

Table 1: Overview of the genes for NGS
NGS of 179 additional genes

ABCC9	*CBL*	*FKRP*	*LBD3*	*MYOZ2*	*PPP3CA*	*TGFB2*
ABRA	*CBS*	*FKTN*	*LEFTY2*	*MYOZ3*	*PPP3B*	*TGFBR1*
ACADVL	*CFC1*	*FHL1*	*MIB1*	*MYPN*	*PPP3R1*	*TGFBR2*
ACTA1	*CFL2*	*FHL2*	*LIMS1*	*MYZAP*	*PRKAG2*	*TMOD1*
ACTA2	*CHRM2*	*FLNC*	*LIMS2*	*NEB*	*PRKCE*	*TMPO*
ACTC1	*CMYA5*	*FOXH1*	*LMCD1*	*NEBL*	*PTPN11*	*TNNC1*
ACTN1	*COL3A1*	*GAA*	*MAP2K1*	*NEURL2*	*RAF1*	*TNNI3*
ACTN2	*COL5A1*	*GATA4*	*MAP2K2*	*NEXN*	*RBM20*	*TNNT1*
ACVR2B	*COL5A2*	*GATD1*	*MIR208*	*NKX2-5*	*RYR1*	*TNNT2*
AKAP13	*COX15*	*GDF1*	*MYBPC3*	*NODAL*	*SDHA*	*TPM1*
ALMS1	*CRELD1*	*GJA1*	*MYH11*	*NPPA*	*SGCD*	*TPM2*
ANKRD1	*CRYAB*	*GJA5*	*MYH6*	*NOTCH1*	*SHOC2*	*TPM3*
ANKRD2	*CSRP3*	*GJC1*	*MYH7*	*NRAP*	*SLC25A4*	*TRIM54*
ATP2A2	*CTNNA3*	*GLA*	*MYH7B*	*NRAS*	*SLC2A10*	*TRIM55*
BAG3	*DICER1*	*GLRX3*	*MYL2*	*NRG1*	*SLC8A1*	*TRIM63*
BCAR1	*DMD*	*HDAC1*	*MYL3*	*OBSCN*	*SMAD3*	*TTN*
BMP10	*DNAJB6*	*HDAC2*	*MYL5*	*OBSL1*	*SMYD1*	*TTR*
BRAF	*DNM1L*	*HRAS*	*MYL7*	*PAK1*	*SMYD2*	*TXNRD2*
CALR3	*DTNA*	*ILK*	*MYLK*	*PALLD*	*SOS1*	*UNC45B*
CAPN1	*ELN*	*ITGB1BP2*	*MYLK2*	*PARVB*	*SYNE1*	*VCL*
CAPN2	*EMD*	*JAG1*	*MYLK3*	*PDE5A*	*SYNE2*	*XIRP1*
CAPN3	*EYA4*	*JPH2*	*MYO6*	*PDLIM1*	*SYNM*	*XIRP2*
CAPNS1	*FBN1*	*KBTBD13*	*MYOM1*	*PDLIM3*	*TAZ*	*ZIC3*
CAPZA1	*FBN2*	*KRAS*	*MYOM2*	*PDLIM5*	*TBX20*	
CAPZA2	*FBXL22*	*LAMP2*	*MYOT*	*PDLIM7*	*TBX5*	
CAPZB	*FERMT2*	*LAMA4*	*MYOZ1*	*POLR2M*	*TCAP*	
NGS of 34 genes						
AKAP9	*CASQ2*	*DSP*	*KCNQ1*	*KCNJ2*	*PLN*	*SCN5A*
ANK2	*CAV3*	*DPP6*	*KCNE1*	*KCNJ5*	*RYR2*	*SNTA1*
CACNA1C	*DES*	*GPD1L*	*KCNE2*	*KSNJ8*	*SCN1B*	*TGFB3*
CACNA2D1	*DSC2*	*HCN4*	*KCNE3*	*LMNA*	*SCN3B*	*TMEM43*
CACNB2	*DSG2*	*JUP*	*KCNH2*	*PKP2*	*SCN4B*	

Results

Baseline characteristics

We included a total of 33 patients (14 males (42%); median age 53 years (IQR 39-64 years)). Of the original 53 patients in whom diagnostic NGS of 34 genes was performed, 5 had a pathogenic mutation detected in NGS of 34 genes (1 patient with a *KCNQ1* mutation, 1 patient with a *MYL2* mutation, and 3 patients with the *DPP6* haplotype associated with short-coupled Torsade de Pointes in Dutch patients[11]) , 4 declined participation, 5 were lost to follow-up, and 6 patients had a VUS that needed further investigation to determine pathogenicity (1 patients with an ANK2 VUS, 1 patients with a KCNQ1 VUS, 1 patient with a TMEM43 VUS, 1 patient with a KCNH2 and KCNE1 VUS, and 2 patients with the same JUP VUS). Therefore, these 20 patients were not included in this study.

The median age at time of the index event was 43 years (IQR 31-53). The index event occurred in rest in 67% (22/33). Only 1 patient had a positive family history for sudden cardiac death (3%). Symptoms before the index event were present in 36% (12/33). Nine patients (27%) received appropriate ICD therapy in a median follow-up of 7.8 years (IQR 2.6-15.0).

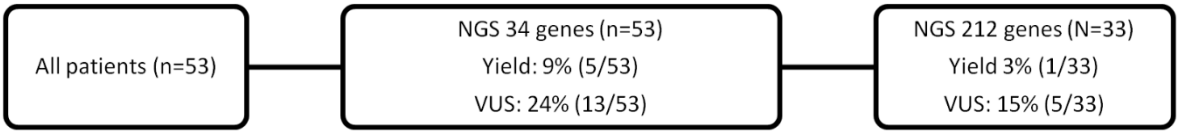

Figure 1: Overview of the yield of the different stages of genetic testing

NGS of 179 genes

NGS in 33 patients identified 11 variants in 9 patients. In 2 patients, 2 variants were detected. After *in silico* analysis of the 11 variants, 1 was labeled as likely pathogenic, 7 were labeled as VUS, 2 were labeled as incidental finding (2 patients with a heterozygous pathogenic NEB mutation) and 1 was

labeled as a reading artefact (1 patient with a OBSL1 mutation, however, in only 34% of the reads this mutation was detected).

The yield of likely pathogenic and pathogenic mutations was therefore 3%, the percentage of VUS was 21% (2 patients revealed 2 VUS, in a total of 15% of patients a VUS was detected). Figure 1 shows an overview of the yield of NGS of 34 and NGS of 179 genes. Table 2 shows an overview of the detected mutations.

The likely pathogenic mutation was confirmed by Sanger sequencing. The *TTN* mutation was a *TTN* nonsense truncating mutation, associated with dilated cardiomyopathy .[14,15] This specific mutation however, has not been previously reported in a patient. However, it is a nonsense mutation in exon 155 of the transcript NM_133432.3, that is expressed in all relevant *TTN* transcripts and is therefore considered likely pathogenic. SIFT labeled this *TTN* mutation as deleterious, Polyphen 2 labeled this mutation as probably damaging.

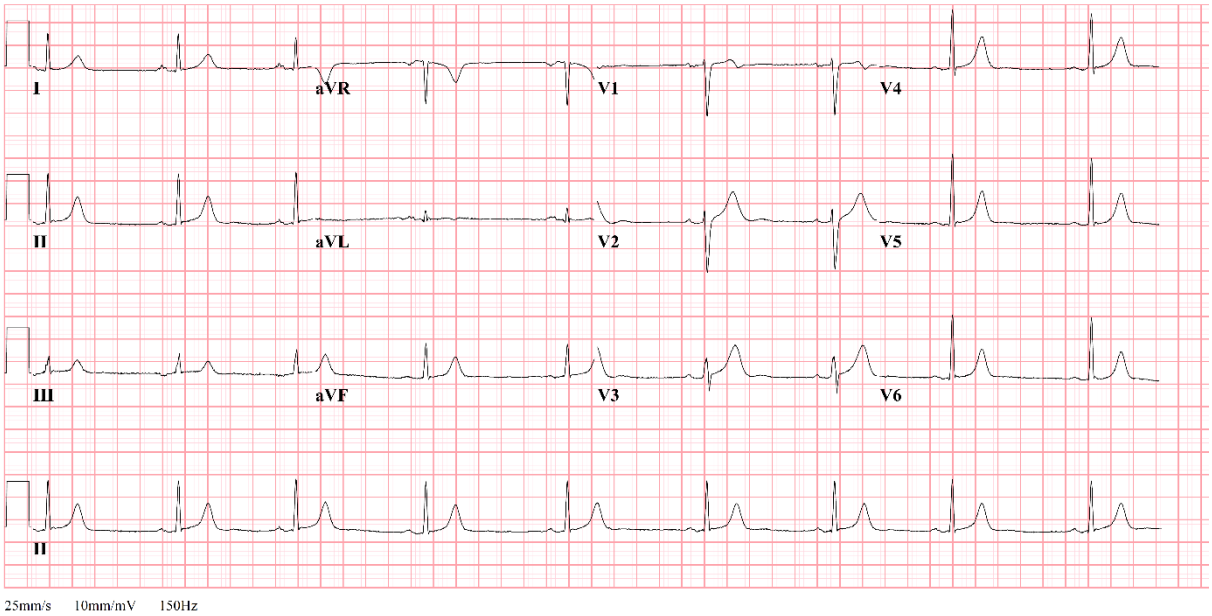

Figure 2. ECG of the patient with the TTN mutation at time of the index event

Phenotype-association in the pathogenic mutation

Although the patient with the *TTN* mutation had a deleterious *TTN* nonsense truncating mutation, no evident signs of dilated cardiomyopathy were detected. She experienced the index event during pregnancy in 2004. The ECG was normal (figure 2). The left ventricle showed a high-normal end-diastolic left ventricular internal diameter at echocardiography (5.5 cm), however MRI showed normal dimensions of the left ventricle (figure 3). After the pregnancy, electrocardiographic and echocardiography parameters remained stable. No family screening was performed: her parents were deceased and she had no siblings.

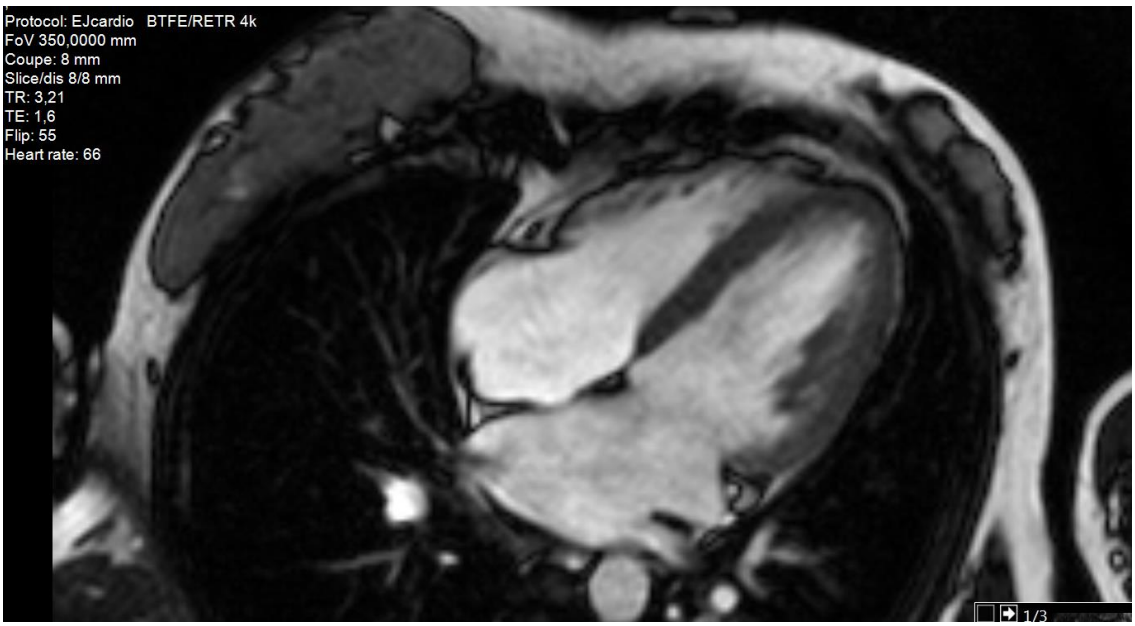

Figure 3. MRI of the patient with the TTN mutation

Table 2: Overview of the detected mutations					
Patient	Gene	Mutation	Description	Phenotype Correlation	FU duration†
Likely pathogenic					
1	*TTN*	c.52198G>T p.E17400*	ExAC: Not reported Clinvar: Pathogenic SIFT: deleterious PPH2: probably damaging	Mild dilatation left ventricle during pregnancy, index event during pregnancy. Normal dimensions LV on MRI.	12 years
VUS					
2	*DSP*	c.242G>A p.C81Y	ExAC: MAF 0.02% ClinVar: Pathogenic	No signs of structural cardiac pathology	12 years
3	*TTN*	c.21775T>C p.S7259P	ExAC: 2x reported ClinVar: VUS PPH2: Possibly damaging	No signs of structural cardiac pathology	5 years
	VCL	c.1237G>A p.A413T	NFE: MAF 0.037% SIFT: deleterious PPH2: benign	No signs of structural cardiac pathology	
4	*PKP2*	c.643G>A p.D215N	ExAC: Not reported	No signs of structural cardiac pathology	19 years
5	*RyR1*	c.2987C>T p.A996V	ExAC: Not reported	No signs of structural cardiac pathology	18 years
6	*CACNA1C*	c.6637G>A p.D2213N	ExAC: NFE MAF 0.016%, pathogenic[19] SIFT: tolerated PPH2: probably damaging	No signs of electric cardiac pathology, except clinical presentation with IVF	11 years
	MYH7	c.3301G>A p.G1101S	ExAC: 8x reported GoNL: 1x reported Published as VUS[20-22] CLinVar: VUS PPH2: Possibly damaging	No signs of structural cardiac pathology	
Incidental findings					
7	*NEB*	c.25288C>T p.R8430*			
8	*NEB*	c.1266T>A p.Y422*			

Abbreviations: ARVD/C: Arrhythmogenic Right Ventricular Dysplasia/ cardiomyopathy, CACNA1C: Calcium Voltage-Gated Channel Subunit Alpha1 C, DCM: Dilated Cardiomyopathy, DSP: desmoplakin, ECG: Electrocardiogram, HCM: Hypertrophic cardiomyopathy, IVF: idiopathic ventricular fibrillation, LV: left ventricle, MRI: Magnetic Resonance Imaging, MYH7: Myosin heavy chain 7, NEB: Nebulin gene; associated with recessive nemaline myopathy, NFE Non-Finnish Europeans, PKP2: plakophilin 2, TNNT2: troponin-T, TTN: Titin, RyR1: Ryanodine 1, VCL: vinculin,
†Since index event

Discussion

Main results

This study is one of the first to evaluate the added yield of extended NGS in IVF patients. The added yield of an extended NGS panel of 179 genes is only 3% in IVF patients in whom NGS of 34 genes failed to identify a pathogenic mutation. In 15% of the patients one or multiple VUS were detected

with extended NGS of 179 genes. Therefore, NGS of large gene panels has limited value for diagnostic purposes. Limited data are available regarding the yield of genetic testing in IVF patients as it is not routinely recommended in the 2013 European Heart Rhythm Society Association/ Heart Rhythm Society/ Asia Pacific Heart Rhythm Society expert consensus statement of inherited primary arrhythmia syndromes.[1]

Yield of genetic testing in IVF

In our study, 1likely pathogenic mutation associated with cardiomyopathy was detected. The limited success rate is a result of the study-design in which patients were included after NGS of 34 genes that was performed in an earlier stage (in the FU-IVF study). In the FU-IVF study, five pathogenic mutations were detected with NGS of 34 genes (figure 1). In an earlier stage of the study, Sanger sequencing was performed based on phenotype and 7 pathogenic mutations were identified. Therefore, the total yield of genetic testing in the FU-IVF cohort was 15% (12 mutations in 74 patients who were genetically tested), which is quite high considering the absence of a phenotype in IVF.

The limited available data show a yield that is comparable to the yield of 3% in our study, although in all previous studies a limited number of genes were tested.[2,3] The Cardiac Arrest Survivors with Preserved Ejection Fraction Registry (CASPER) evaluated a total of 200 patients with an unexplained cardiac arrest. Comprehensive clinical evaluation revealed an underlying diagnosis in 34% at time of the index event and in 7% a diagnosis emerged during follow-up. The number of 'true' IVF patients was 119. In 2 (2%) of these 119 patients a pathogenic mutation was detected and in 2 (2%) a VUS. Genetic testing was based on phenotype and based on the clinically supported panel. Possible genes that were selected were: *KCNQ1, KCNH2, SCN5A, KCNE1*, and *KCNE2* for long QT-sydrome; *SCN5A* for Brugada syndrome; *PKP2* and *DSP* for arrhythmogenic right ventricular cardiomyopathy*; RYR2* for catecholaminergic polymorphic ventricular tachycardia.

The modest yield in our study is possibly explained by the following reasons: First, IVF could have a monogenetic origin, with new causative mutations in genes that are currently unknown. This hypothesis is supported by the detection of several causative mutations with whole exome or genome sequencing.[7-10] Possibly, more monogenetic causative mutations will be detected in IVF patients in the future. Second, IVF could have an oligogenetic origin in which several variants in a patient accumulatively contribute to enhanced arrhythmogenicity. This hypothesis is in accordance with our finding of the relatively high level of VUS that we detect in IVF patients. Possibly, one VUS does not enhance the arrhythmogenicity, but several VUS combined might do. Clearly, this is solely a hypothesis, based on our study we cannot conclude that several VUS in one patient increase the arrhythmogenicity. To support such a claim, burden testing in large patient groups is needed. Third, non-genetic factors could increase the arrhythmogenicity, especially in patients who already have a genetic susceptibility for ventricular arrhythmias. Last, combinations of the abovementioned theories could result in increased arrhythmogenicity and hereby cause IVF.

Detected pathogenic mutation

The *TTN* mutation was not associated to the phenotype because the patient did not show any signs of dilated cardiomyopathy. Although *TTN* truncating mutations are more frequently reported in patients with dilated cardiomyopathy, they are also reported with low frequencies in the general population. Herman et. al reported a frequency of 3% of truncating mutations in the general population and Akinirade et al. reported a frequency of 0.36%.[14,17] However, as the patient with the *TTN* mutation experienced ventricular fibrillation during pregnancy, a modulating effect of the *TTN* mutation cannot be excluded as *TTN* mutations are also associated with peripartum cardiomyopathy.[18] We therefore labeled this mutation as likely pathogenic.

Clinical implications

Future research in larger IVF patient cohorts is necessary to further assess the yield of NGS. Research studies, using whole exome or genome sequencing may identify new gene mutations associated with IVF. We recommend diagnostic genetic testing in IVF patients to exclude concealed primary arrhythmia syndromes, with a basic arrhythmia panel.[19] The detection of a pathogenic mutation in patients facilitates the detection of the underlying disease. Especially in IVF patients, who may develop a specific disease in the years after the index event. [12,20] However, we believe genetic testing for diagnostic purposes in IVF patients should be restricted to a basic panel of the most common disease-causing genes.[19] Routine screening of large diagnostic NGS panels and even whole exome sequence analysis could result in large numbers of VUS, potentially hampering patient care.

Limitations

Our study has several limitations. Firstly, the number of patients was limited because IVF is a rare disease and many patients were lost to follow-up, declined participation to this study, or had VUS that needed further investigation. Secondly, the stepwise approach of NGS of 34 genes with extension to NGS of 179 genes decreased the a-priori chance of detecting a mutation as the most common genetic causes of ventricular fibrillation were investigated in a previous stage.

Conclusion

The yield of diagnostic NGS of 179 genes in patients with IVF is 3%. More VUS were detected than (likely) pathogenic mutations. The yield of NGS in IVF patients is minimal, therefore NGS of large gene panels is not recommended for diagnostic purposes. However, routine analysis of a basic arrhythmia panel is recommended in IVF patients, as genetic testing is the only way to identify concealed primary arrhythmia syndromes or mutations associated with IVF. Detection of these mutations enables cascade family screening. Future research is needed to further establish the role of diagnostic NGS in IVF patients.

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