Chapter 4.2

Titin gene mutations are common in families with both peripartum cardiomyopathy and dilated cardiomyopathy

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Published in European Heart Journal, 2014
ABSTRACT

Aims: Peripartum cardiomyopathy (PPCM) can be an initial manifestation of familial dilated cardiomyopathy (DCM). We aimed to identify mutations in families that could underlie their PPCM and DCM.

Methods and Results: We collected 18 families with PPCM and DCM cases from various countries. We studied the clinical characteristics of the PPCM patients and affected relatives, and applied a targeted next-generation sequencing (NGS) approach to detect mutations in 48 genes known to be involved in inherited cardiomyopathies. We identified 4 pathogenic mutations in 4/18 families (22%): 3 in TTN and 1 in BAG3. In addition, we identified 6 variants of unknown clinical significance that are likely to be pathogenic in 6 other families (33%): 4 in TTN, 1 in TNNC1, and 1 in MYH7. Measurements of passive force in single cardiomyocytes and titin isoform composition potentially support an upgrade of one of the variants of unknown clinical significance in TTN to a pathogenic mutation. Only 2/20 PPCM cases in these families showed recovery of left ventricular function.

Conclusion: Targeted NGS shows that potentially causal mutations in cardiomyopathy-related genes are common in families with both PPCM and DCM. This supports the earlier finding that PPCM can be part of familial DCM. Our cohort is particularly characterised by a high proportion of TTN mutations and a low recovery rate in PPCM cases.

Keywords: cardiomyopathy, peripartum cardiomyopathy, genetics, pregnancy, titin
INTRODUCTION

Peripartum cardiomyopathy (PPCM) is an idiopathic cardiomyopathy presenting with heart failure secondary to left ventricular systolic dysfunction towards the end of pregnancy or in the first months following delivery, where no other cause of heart failure is found. The left ventricle may not be dilated but the ejection fraction is nearly always reduced below 45%.1 According to this recent definition, the time frame is not strictly defined, in contrast to previous definitions.2–4 The severity of PPCM is highly variable, ranging from complete recovery to rapid progression to end-stage heart failure. PPCM affects 1:300 to approximately 1:3000 pregnancies, with geographic hot spots of high incidence such as in Haiti and Nigeria.4,5

The precise mechanisms that lead to PPCM are not fully known. Several risk factors and possible underlying pathological processes have received attention, such as abnormal autoimmune responses, apoptosis, and impaired cardiovascular microvasculature.5,6 Recent work into the pathogenesis of PPCM has shown involvement of a cascade with oxidative stress, the prolactin-cleaving protease cathepsin D, and the nursing hormone prolactin, which may lead to a target for a disease-specific therapy, namely pharmacological blockade of prolactin by bromocriptine.7–9 In addition, involvement of cardiac angiogenic imbalance may explain why PPCM is a disease seen in late pregnancy and why pre-eclampsia and multiple gestation are important risk factors.10 PPCM is probably caused by a complex interaction of more than one pathogenic mechanism. The large variation in incidence and clinical characteristics may reflect the involvement of specific mechanisms, or combinations thereof, in certain subgroups of PPCM.

We and others recently reported that PPCM can be an initial manifestation of familial dilated cardiomyopathy (DCM),11,12 indicating that, at least in a subset of cases, genetic predisposition plays a role in the pathophysiology of pregnancy-associated heart failure. Accordingly, Haghikia et al. reported a positive family history for cardiomyopathy in 16.5% (19/115) of PPCM cases from a German PPCM cohort.13 So far, eight cases with underlying mutations in DCM-related genes have been published11,12,14,15 and several other cases with familial occurrences of PPCM and DCM, as well as familial clustering of PPCM, have been reported.16–24 Here, we describe our extensive genetic analysis using next-generation sequencing (NGS) technology to identify potentially causal mutations in families with both PPCM and DCM from various parts of the world.
METHODS

Subjects and Clinical Evaluation

We collected a cohort of families with cases of both PPCM and DCM from various parts of the world (the Netherlands, Germany, and South Africa) and studied their clinical characteristics by reviewing medical reports. The local institutional review committees approved the study, and all participants gave their informed consent.

PPCM was diagnosed when a patient had an idiopathic cardiomyopathy presenting with heart failure secondary to left ventricular systolic dysfunction towards the end of pregnancy or in the first months following delivery, where no other cause of heart failure was found.1 DCM was diagnosed when a patient had both a reduced systolic function of the left ventricle (left ventricular systolic ejection fraction <0.45) and dilation of the left ventricle (left ventricular end-diastolic dimension ≥117% of the predicted value corrected for body surface area and age) and only after other identifiable causes like severe hypertension, coronary artery disease, and systemic disease had been excluded.25 If only one of the two criteria was fulfilled, the patient was labeled with “mild DCM”. If the family history suggested DCM in a relative but there were no medical reports to confirm this, the relative was labeled as having “possible DCM”. Familial PPCM/DCM was diagnosed when there were ≥2 affected family members, at least one with PPCM and one with DCM or sudden cardiac death (SCD) ≤35 years.

Targeted Next-Generation Sequencing of 48 Cardiomyopathy-Related Genes

Genomic deoxyribonucleic acid (DNA) was extracted from blood samples obtained from all the available PPCM patients and their affected relatives. Targeted NGS was performed in one or two affected relatives in the selected families (these individuals are marked with an arrow in Figures 1 and 2).

We developed a kit based on Agilent Sure Select Target Enrichment for mutation detection in 48 genes (all exonic and ± 20 bp of exon-flanking intronic sequences) known to be involved in inherited cardiomyopathies (ABCC9, ACTC1, ACTN2, ANKRD1, BAG3, CALR3, CRYAB, CSRP3/MLP, DES, DMD, DSC2, DSG2, DSP, EMD, GLA, JPH2, JUP, LAMA4, LAMP2, LMNA, MYBPC3, MYH6, MYH7, MYL2, MYL3, MYPN, MYOZ1, MYOZ2, PKP2, PLN, PRKAG2, PSEN1, PSEN2, RBM20, RYR2, SCN5A, SGCD, TAZ, TBX20, TCAP, TMEM43, TNNC1, TNNI3, TNNT2, TPM1, TTN, VCL, ZASP/LDB3).26 Samples
were prepared according to the manufacturer’s protocols and multiplexed to an amount still permitting a theoretical coverage of 100 reads per targeted sequence/per patient. All samples were sequenced using 151 bp paired-end reads on an Illumina MiSeq sequencer and analyzed using the MiSeq Reporter pipeline and Nextgene software.27 Eleven amplicons with low coverage were also analyzed by Sanger sequencing. Identified mutations were confirmed by Sanger sequencing. To study co-segregation, affected relatives were screened for carriership of the identified mutations by Sanger sequencing.

Sanger Sequencing STAT3 Gene

The STAT3 gene (all coding exons and flanking intronic sequences) was analysed by Sanger sequencing in PPCM patients of the collected families.

Classification of Identified Mutations

The criteria used to classify mutations were published recently.28 Briefly, we used a list of mutation-specific features based on in silico analysis using the mutation interpretation software Alamut (version 2.2.1). A score was given depending on the outcome of a prediction test for each feature (i.e. the PolyPhen-2 prediction tool). Then, depending on the total score and the presence/absence of the mutation in at least 300 ethnically matched control alleles (data obtained from the literature and/or available databases, e.g. http://evs.gs.washington.edu/EVS and http://www.nlgenome.nl, or from our own control alleles), we classified mutations as: pathogenic, not pathogenic, or as a variant of unknown clinical significance (VUS; VUS1, unlikely to be pathogenic; VUS2, uncertain; VUS3, likely to be pathogenic). Co-segregation data and/or functional analysis were needed to classify a mutation as pathogenic.

Functional Analysis of TTN mutation

Passive force was measured in single membrane-permeabilized cardiomyocytes mechanically isolated from the heart tissue.29,30 Titin isoform composition was analysed as described previously.30
Figure 1. Pedigrees of the Dutch families (NL1-11). Square symbols indicate men; circles, women; diamonds, unknown sex; and triangles, miscarriage. Blue symbols indicate a clinical diagnosis of PPCM; black symbols, (mild) DCM; grey symbols, possible DCM; orange symbols, sudden cardiac death (SCD). Diagonal lines through symbols indicate deceased; arrows indicate patients selected for targeted next-generation sequencing; and the number in a symbol indicates the number of individuals with this symbol (question mark if unknown).
RESULTS

Clinical Characteristics: Low Rate of Full Recovery in PPCM Cases of Familial PPCM/DCM

We collected 18 families with familial PPCM/DCM. These families originated from the Netherlands (n=11), Germany (n=6), and South Africa (n=1; black). Clinical data of the PPCM cases in these families are summarised in Table 1 and of all (likely) affected relatives in Supplemental Table S1. The pedigrees of all the families are shown in Figures 1 (NL1-11) and 2 (SA1 and GER1-6). In two families there were two cases of PPCM (NL1 and SA1). Eight families (NL1-7 and SA1) have been described previously.11,31

The median age at diagnosis in PPCM patients was 29 years (n=15; range 20-36 years), with mean parity 2 (n=13; range 1-4). PPCM diagnosis was postpartum in 12/14 patients. Only 2/20 PPCM patients showed a full recovery of left ventricular function, one of them even had an uneventful next
### Table 1. Clinical characteristics of confirmed PPCM cases

<table>
<thead>
<tr>
<th>Family</th>
<th>Patient</th>
<th>Referred for</th>
<th>Diagnosis (age in yrs)</th>
<th>Timing at Diagnosis</th>
<th>Pregnancy</th>
<th>LVEF at Diagnosis</th>
<th>LVEF at Follow-Up</th>
<th>Cardiological remarks and outcome (age in yrs)</th>
<th>Pathology and other remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>NL1</td>
<td>II:6</td>
<td>HF</td>
<td>PPCM (29)</td>
<td>Just after delivery</td>
<td>P4</td>
<td>20%</td>
<td></td>
<td>D (31)</td>
<td>Myocyte hypertrophy</td>
</tr>
<tr>
<td>NL1</td>
<td>III:4</td>
<td>Cardiogenic</td>
<td>PPCM (27)</td>
<td>3 days after delivery</td>
<td>P1</td>
<td>20%</td>
<td></td>
<td>D MOF (27)</td>
<td></td>
</tr>
<tr>
<td>NL2</td>
<td>III:3</td>
<td>HF</td>
<td>PPCM (26)</td>
<td>Few days after delivery</td>
<td>P4</td>
<td>23%</td>
<td></td>
<td>LBBB, D asthma cardiale (26)</td>
<td>Dilated heart, myocyte hypertrophy, fibrosis</td>
</tr>
<tr>
<td>NL3</td>
<td>III:1</td>
<td>HF</td>
<td>PPCM (33)</td>
<td>37th week of pregnancy</td>
<td>P2 CS</td>
<td>25%</td>
<td>3 months 33%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NL4</td>
<td>III:2</td>
<td>HF</td>
<td>PPCM (30)</td>
<td>3 months after delivery</td>
<td>P1</td>
<td>21%</td>
<td>9 months no recovery</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NL5</td>
<td>III:1</td>
<td>HF</td>
<td>PPCM (33)</td>
<td>35th week of pregnancy</td>
<td>P1 AI CS</td>
<td>23%</td>
<td>6 months 44%, 7 years 42%</td>
<td>AF (30), PVCs, VTs (46), D HF (51)</td>
<td>Signs of acute myocarditis (EMB), suspicion of vasculitis</td>
</tr>
<tr>
<td>NL6</td>
<td>III:2</td>
<td>HF</td>
<td>PPCM (29)</td>
<td>2 months after delivery</td>
<td>P3</td>
<td>23%</td>
<td>6 months 23%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NL7</td>
<td>III:1</td>
<td>HF</td>
<td>PPCM (23)</td>
<td>Just after delivery</td>
<td>P1 CS</td>
<td>25%</td>
<td>8 years 10%</td>
<td>ICD/CRT (31), LVAD, VF, D cardiogenic shock (34)</td>
<td></td>
</tr>
<tr>
<td>NL8</td>
<td>III:3</td>
<td>HF</td>
<td>PPCM (35)</td>
<td>2 weeks after delivery</td>
<td>P2</td>
<td>18%</td>
<td></td>
<td>Thrombus LV apex, TIA, VT (35), PM, HTX (37), normal LVEF (51)</td>
<td></td>
</tr>
<tr>
<td>NL9</td>
<td>III:1</td>
<td>HF, respiratory insufficiency</td>
<td>PPCM (30)</td>
<td>Just after delivery</td>
<td>P1 CS, twin pregnancy</td>
<td>30%</td>
<td>6 months 55%, 3 years normal</td>
<td>New pregnancy, terminated (35)</td>
<td></td>
</tr>
<tr>
<td>NL10</td>
<td>III:6</td>
<td>Chest pain, coughing</td>
<td>PPCM (36)</td>
<td>3 weeks after delivery</td>
<td>P2 CS 29th week, HELLP</td>
<td>20-30%</td>
<td>6 months 55%, 2 years 45%, 3 years 50-55%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NL11</td>
<td>III:1</td>
<td>Dyspnea, tachycardia</td>
<td>PPCM (20)</td>
<td>Just after delivery</td>
<td>Poor</td>
<td></td>
<td>4 months 30-35%</td>
<td>Tachycardia</td>
<td></td>
</tr>
<tr>
<td>SA1</td>
<td>II:5</td>
<td>PPCM (23)</td>
<td>1 month after delivery</td>
<td>P2</td>
<td>22%</td>
<td></td>
<td>No recovery</td>
<td>No recovery</td>
<td></td>
</tr>
<tr>
<td>SA1</td>
<td>II:6</td>
<td>Screening, asymptomatic</td>
<td>PPCM (22)</td>
<td></td>
<td>P1</td>
<td>43%</td>
<td>24%</td>
<td>No recovery</td>
<td></td>
</tr>
<tr>
<td>GER1</td>
<td>II:1</td>
<td>PPCM</td>
<td>SB 27 weeks</td>
<td>20%</td>
<td></td>
<td>6 months 37%, 2 years normal</td>
<td>Full recovery with uneventful 2nd pregnancy 2 years later</td>
<td>ICD, HTX</td>
<td>Suspection of neurodermitis</td>
</tr>
<tr>
<td>GER2</td>
<td>II:1</td>
<td>PPCM</td>
<td></td>
<td>25%</td>
<td></td>
<td></td>
<td></td>
<td>ICD, HTX</td>
<td></td>
</tr>
</tbody>
</table>
AF indicates atrial fibrillation; AI, artificial insemination; AT, atrial tachycardia; (Bi)(L)VAD, (bi)(left) ventricular assist device; CRT, cardiac resynchronization therapy; CS, caesarean section; D, death; EMB, endomyocardial biopsy; HELPP, hemolysis, elevated liver enzymes, low platelet count; HF, heart failure; HTX, heart transplantation; ICD, implantable cardiac defibrillator; LBBB, left bundle branch block; LV, left ventricle; LVEF, left ventricular ejection fraction; MOF, multiple organ failure; P, pregnancy; PM, pacemaker; PPCM, peripartum cardiomyopathy; PVC, premature ventricular contraction; RV, right ventricle; SB, still birth; TIA, transient ischemic attack; VF, ventricular fibrillation; VT, ventricular tachycardia.

Table 2. Potentially causal mutations identified in 10/18 families

<table>
<thead>
<tr>
<th>Family</th>
<th>Tested patient</th>
<th>Gene</th>
<th>Amino acid change</th>
<th>Nucleotide change</th>
<th>Classification</th>
<th>Co-segregation</th>
</tr>
</thead>
<tbody>
<tr>
<td>NL1</td>
<td>II:3</td>
<td>TTN</td>
<td>p.Arg27373*</td>
<td>c.82117C&gt;T</td>
<td>Pathogenic</td>
<td>Yes/Unknown</td>
</tr>
<tr>
<td>NL3</td>
<td>II:2</td>
<td>BAG3</td>
<td>p.Gln340*</td>
<td>c.1018C&gt;T</td>
<td>Pathogenic</td>
<td>Yes/Unknown</td>
</tr>
<tr>
<td>NL4</td>
<td>III:2</td>
<td>TNNC1</td>
<td>p.Gln50Arg</td>
<td>c.149T&gt;C</td>
<td>Pathogenic</td>
<td>Yes/Unknown</td>
</tr>
<tr>
<td>NL6</td>
<td>II:3</td>
<td>TTN</td>
<td>p.Asn28726Lysfs*3</td>
<td>c.86171_86174dupAAAG</td>
<td>VUS3</td>
<td>Yes/Unknown</td>
</tr>
<tr>
<td>NL9</td>
<td>III:3</td>
<td>TTN</td>
<td>p.Arg17599*</td>
<td>c.52795C&gt;T</td>
<td>Pathogenic</td>
<td>Yes/Unknown</td>
</tr>
<tr>
<td>NL10</td>
<td>II:6</td>
<td>TTN</td>
<td>p.Arg23956Thrs*9'</td>
<td>c.71867_71876delGAGTTCTGGA</td>
<td>VUS3</td>
<td>Yes/Unknown</td>
</tr>
<tr>
<td>NL11</td>
<td>III:1</td>
<td>TTN</td>
<td>p.Ser27317Lysfs*10</td>
<td>c.81949dupA</td>
<td>Pathogenic</td>
<td>Yes/Unknown</td>
</tr>
<tr>
<td>GER1</td>
<td>II:1</td>
<td>TTN</td>
<td>p.Trp18357*</td>
<td>c.55070G&gt;A</td>
<td>VUS3</td>
<td>Yes/Unknown</td>
</tr>
<tr>
<td>GER4</td>
<td>II:1</td>
<td>TTN</td>
<td>p.Lys15664Valfs*13</td>
<td>c.46990_46993delAAAG</td>
<td>VUS3</td>
<td>Yes/Unknown</td>
</tr>
<tr>
<td>GER5</td>
<td>II:1</td>
<td>MYH7</td>
<td>p.Arg1303Gly</td>
<td>c.3907C&gt;G</td>
<td>Pathogenic</td>
<td>Yes/Unknown</td>
</tr>
</tbody>
</table>

Nomenclature according to HGVS (Human Genome Variation Society) using the reference sequences: TTN (NM_001256850.1; Q8WZ42-1), BAG3 (NM_004281.3), TNNC1 (NM_003280.2), MYH7 (NM_000257.2). VUS indicates variant of unknown clinical significance (VUS3, likely to be pathogenic; VUS2, uncertain). † VUS2 p.Arg279Trp (c.835C>T) on same allele.
pregnancy (NL9 III:1, LVEF still normal 3 years after diagnosis; and GER1 II:1, full recovery with uneventful second pregnancy two years later). Another PPCM patient showed recovery of left ventricular function, but only under treatment with a beta-blocker and ACE inhibitor (NL10 III:6).

In addition to 20 confirmed PPCM patients in these families, five relatives show clinical characteristics suggestive for PPCM (NL4 II:2, GER1 I:1, GER3 I:1, GER4 I:1, GER5 I:1; Table S1). PPCM could not be confirmed because clinical data of these relatives was lacking. In addition, two relatives with DCM showed a decline of left ventricular function after delivery (NL2 IV:8 and SA1 II:3; Table S1).

**Targeted Next-Generation Sequencing: Potential Causal Mutations in Cardiomyopathy-Related Genes, in particular TTN, are Common in Familial PPCM/DCM**

Using our validated NGS approach, a mean coverage of 220x per individual patient was reached and, on average, 98.5% of all targeted nucleotides were covered at least 20x.

In 4/18 families (22%) pathogenic mutations in cardiomyopathy-related genes were identified (3 in TTN and 1 in BAG3). In addition, in 6 other families (33%) VUS3s were identified (4 in TTN, 1 in TNNC1, and 1 in MYH7). An overview of these mutations and VUS3s and the respective co-segregation analyses are shown in Table 2. All 7 TTN mutations/VUS3s were located in the titin A-band, for which over-representation of mutations in DCM patients was reported previously. No potential mutations were identified in 8 families (NL2, NL5, NL7, NL8, SA1, GER2, GER3, and GER6).

An overview of the 26 mutations that were not classified as potentially disease-causing (VUS1s and VUS2s) identified in the 18 families is shown in Supplemental Table S2.

**No STAT3 Mutations in PPCM Cases**

No STAT3 mutations were identified in 15 PPCM cases (DNA was available from 15/20 cases).

**Functional and Protein Analyses Support the Pathogenicity of a Likely Pathogenic TTN Mutation**

Heart tissue from PPCM patient GER4 II:1 with a VUS3 in TTN was available for functional and protein analyses. Passive force was measured in
single cardiomyocytes (n=4) at sarcomere lengths of 1.8 to 2.2 μm (see Figure 3). Our functional measurements of passive stiffness, which is largely based on titin composition in the heart, revealed a very low passive force development (1.0±0.3 kN/m²) at a sarcomere length of 2.2 μm in the PPCM sample compared to previously reported values in control hearts (~2.5 kN/m²). Analysis of titin isoform composition showed a shift towards the more compliant N2BA isoform evident from a higher N2BA/N2B ratio (0.72±0.02; mean of triplo) in the PPCM heart compared to the previously reported ratio (0.39±0.05) in control hearts.

**DISCUSSION**

This is the first report of a comprehensive genetic analysis in a large series of cases with familial occurrences of PPCM and DCM. We identified pathogenic mutations in cardiomyopathy-related genes in 4/18 families (22%) and VUSs that are likely to be pathogenic in 6 other families (33%). These data support the earlier finding that PPCM can be part of familial DCM.

Cascade genetic screening can identify relatives at risk in those families in which an underlying mutation has been identified. Our data also specifically show a low recovery rate in our cohort (only 10%) compared to reports in other groups not selected for familial cases (recovery rates of around 25 to 50%), indicating that the presence of an underlying mutation or positive family history for cardiomyopathy in a patient with PPCM may be a prognostic factor for a low recovery rate.

The targeted NGS approach that we have developed provides high-throughput, rapid and affordable molecular analysis for cardiomyopathies. As accurate annotation of mutations in cardiomyopathies is of the utmost importance, we were extremely careful in classifying these. Our study has

![Figure 3. Force measurements in heart tissue of GER4 II:1.](image)

Single cardiomyocyte of the PPCM heart sample (A). Passive force development was measured at sarcomere lengths of 1.8, 2.0 and 2.2 μm. (B)
several advantages: one is the inclusion of some large families, where co-segregation analysis added value to the classification of mutations. Another was the large number of genes we tested, including the large TTN gene, for which mutation analyses on a large scale were impossible before NGS became available, because exclusion of pathogenic mutations in 47 other candidate genes makes it more likely that the identified VUS3s have a pathogenic nature. Accordingly, the previously reported TNNC1 mutation is still the only potential genetic cause in family NL4. And although the pathogenicity of truncating TTN mutations is still under debate due to these types of mutations being found in apparently healthy controls (up to 3%) and the general population, the pathogenicity of TTN VUS3s identified in our families also becomes more likely after excluding pathogenic mutations in 47 other cardiomyopathy-related genes. Possible exclusion of mutations in other genes in patients carrying truncating TTN mutations was not explicitly addressed by Herman et al. As expected, we identified several mutations in the majority of patients, however, we focused on the pathogenic mutations and VUS3s. Other identified mutations (VUS1s and VUS2s; see Table S2) might be benign genetic variations, but some may also contribute to the development of disease in these families. Some of these VUSs might even be independently pathogenic, but additional testing is needed to confirm this (this might be the case for two VUS2s in TTN (p.Arg1408Cys (c.4222C>T) in GER2, and p.Glu2076Gly (c.6227A>G) in GER6). Other possibilities are that these VUSs may act as modifiers, or that they are risk factors with a low penetrance.

The great majority of pathogenic mutations and VUS3s (7/10) were in the TTN gene, which encodes the giant sarcomeric protein titin. It was recently reported that truncating mutations in TTN account for a significant portion (approximately 25%) of the genetic etiology in familial DCM. The high yield of pathogenic mutations and VUS3s in TTN in our cohort of familial PPCM/DCM cases (39%; 7/18) suggests that TTN mutations are specifically related to PPCM. Changes in isoform expression and phosphorylation status of titin have been reported in acquired forms of heart failure (reviewed by Hildalgo and Granzier). We were able to measure functional properties and titin isoform composition in heart tissue from one of the PPCM patients with a VUS3 in TTN. The passive force was twice as low as the value previously reported in control groups, and was associated with a shift towards the more compliant N2BA titin isoform. The shift towards more compliant N2BA has been reported in human heart failure. Overall, our data from functional and protein analyses support the pathogenicity of this particular TTN mutation.
We still classify this mutation as VUS3, however extended experience with these functional analyses might drive us to re-classify this VUS3 towards a pathogenic mutation. Recent studies indicated that titin phosphorylation is indirectly altered by increased oxidative stress and, as such, may represent a likely pathomechanism in PPCM. Future studies will need to reveal the functional deficits induced by mutations in the TTN gene in relation to high oxidative stress, as present in PPCM.

There may be genetic factors specific for PPCM development, for example a factor tentatively underlying the geographical hotspot of incidence in Haiti, and a locus near the PTHLH gene reported by Horne et al. We only focused on the STAT3 gene as a possible specific genetic factor for PPCM. Because mice with cardiomyocyte-specific deletion of STAT3 develop PPCM, STAT3 might also be involved in human PPCM but there are no human genetic data supporting this yet. STAT3 mutations are so far only known to cause hyper-IgE syndrome. In contrast to the PPCM cases, some women in our PPCM/DCM families went through several pregnancies without developing PPCM. We therefore hypothesized that STAT3 mutations in the PPCM cases of these families contributed to the development of PPCM, in addition to an underlying cardiomyopathy-related mutation. However, we found no STAT3 pathogenic mutations or VUSs in these PPCM cases, which was consistent with previous findings. Exome sequencing of rare familial PPCM cases could lead to identifying novel genetic factors specific for PPCM. However, this approach is limited by the fact that familial PPCM cases with more than two affected relatives or with affected distant relatives are lacking. An alternative strategy could be to compare the data from exome sequencing on different PPCM cases in order to identify a shared genetic cause, but this might not lead to a result because the causal genetic factor may well be unique to each family.

**Limitations**

One limitation of our study is that it does not provide data on the frequency of familial disease in PPCM. Currently, we only have data from a German cohort reporting a positive family history for cardiomyopathy in 16.5% of PPCM cases, but we hope to gain more information via the Peripartum Cardiomyopathy Registry of EURObservational Research Programme (www.eorp.org). (unpublished data, 2013, manuscript submitted to European Journal of Heart Failure) Another limitation is that retrieving information on larger deletions/duplications from NGS data is not possible yet, although software to enable such analysis is being developed. We may therefore
have missed that type of mutation in our analyses. A further limitation is the difficulty of judging which TTN mutations are pathogenic, given the presence of truncating TTN mutations in the general population and reported truncating mutations that do not segregate with disease in DCM families.\textsuperscript{30, 36, 41}

In contrast to the latter observation, we were able to show co-segregation of truncating TTN mutations/VUS3s in five of our families (NL1, NL6, NL9, NL10 and NL11; Table 2), and we have data from functional and protein analyses supporting the pathogenicity of one likely pathogenic TTN mutation (GER4 II:1). Additional functional studies on TTN mutations and collection of large families carrying these mutations are needed. Moreover, although our findings suggest a specific role for TTN mutations in families with PPCM and DCM, we do realise that the number of families studied is currently too small to definitely conclude this. Finally, we were lacking some clinical data, especially of cases that showed clinical characteristics suggestive of PPCM.

**Conclusions and Practical Implications**

Potentially causal mutations in cardiomyopathy-related genes are common in families with both PPCM and DCM, in particular TTN mutations. The targeted next-generation sequencing approach we applied has been shown to be suitable for identifying such mutations. Functional studies as performed in the present study may provide a future tool to confirm pathogenicity of TTN mutations. Our results provide more support for the earlier finding that PPCM can be a manifestation of familial DCM. Cascade genetic screening can identify relatives at risk in those families in which an underlying mutation has been identified. Moreover, the presence of an underlying mutation or a positive family history for cardiomyopathy in a PPCM patient may be a prognostic factor for low recovery rate.

**ACKNOWLEDGEMENTS**

We thank all the patients who participated in this study; the Study Group on PPCM of the Heart Failure Association of the European Society of Cardiology; Birgit Sikkema-Raddatz for her help in validating and implementing the targeted enrichment kit; Ludolf Boven, Eddy de Boer and Lennart Johansson for technical assistance; Wies Lommen for assistance with functional analyses; Nicolaas de Jonge, cardiologist, for cardiac evaluation of family NL10; Wilma van der Roest, genetic counselor, for counseling some of the Dutch families; and Jackie Senior for editing this manuscript. Rowida Almomani was supported by the Netherlands Heart Foundation (grant 2010B164).
REFERENCES


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<table>
<thead>
<tr>
<th>Family</th>
<th>Patient M/F</th>
<th>Referred for</th>
<th>Diagnosis (age in yrs)</th>
<th>Timing at diagnosis</th>
<th>Pregnancy</th>
<th>LVEF at Diagnosis</th>
<th>LVEF at Follow-Up</th>
<th>Cardiological remarks and outcome (age in yrs)</th>
<th>Pathology and other remarks</th>
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<tr>
<td>NL1</td>
<td>II:1 M</td>
<td>Screening</td>
<td>DCM (83)</td>
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<td>PVCs, VTs (70)</td>
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<td>II:3 F</td>
<td>Dyspnea</td>
<td>DCM (61)</td>
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<td>23%</td>
<td>1 year 41%, 12 years 20-25%</td>
<td>AVB1, PVCs, VTs, ICD (61)</td>
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</tr>
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<td>NL1</td>
<td>III:6 F</td>
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<td>PPCM (29)</td>
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<td>P4</td>
<td></td>
<td>20%</td>
<td>Myocyte hypertrophy</td>
<td>D (31)</td>
</tr>
<tr>
<td>NL1</td>
<td>III:4 F</td>
<td>Cardiogenic shock</td>
<td>PPCM (27)</td>
<td>3 days after delivery</td>
<td>P1</td>
<td></td>
<td>24%</td>
<td>PVCs, VTs (48), ICD (58), D HF (59)</td>
<td>D MOF (27)</td>
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<tr>
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<tr>
<td>NL1</td>
<td>III:8 M</td>
<td>Died</td>
<td>SIDS</td>
<td></td>
<td></td>
<td></td>
<td>25%</td>
<td>9 years 18%</td>
<td>D HF (60)</td>
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<tr>
<td>NL2</td>
<td>I:2 F</td>
<td>Possible DCM</td>
<td></td>
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<td>DCM</td>
<td></td>
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<tr>
<td>NL2</td>
<td>III:2 F</td>
<td>HF</td>
<td>DCM (41)</td>
<td></td>
<td></td>
<td></td>
<td>35-40%</td>
<td>8 years 45%</td>
<td>D (63)</td>
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<tr>
<td>NL2</td>
<td>III:3 F</td>
<td>HF</td>
<td>PPCM (26)</td>
<td>Few days after delivery</td>
<td>P4</td>
<td></td>
<td>45-50%</td>
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<td>Mild DCM (25)</td>
<td></td>
<td></td>
<td></td>
<td>37-49%</td>
<td>7 years 40-45%</td>
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<td>40%</td>
<td>5 years 35-40%</td>
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<td>Screening</td>
<td>DCM (20)</td>
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<td>30-40%</td>
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<td></td>
<td></td>
<td>43%</td>
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<td>IV:8 F</td>
<td>Screening</td>
<td>DCM (35)</td>
<td>10 weeks after P5, but 4 years earlier already abnormal contraction LV with preserved LVEF</td>
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<td>37th week of pregnancy P2 CS</td>
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<td>9 months no recovery</td>
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<td>7 years stable</td>
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</table>

**Cardiological remarks and outcome (age in yrs):**
- **NL4 II:5:** F, II:5, F, Heart murmur, 16 months, Mild DCM, 7 years stable, 9 months no recovery, Thrombus LV apex, DCM (16).
- **NL4 II:6:** F, II:6, F, Heart murmur, 16 months, Mild DCM, 7 years stable, 9 months no recovery, Thrombus LV apex, DCM (16).
- **NL5 II:1:** M, II:1, M, Dyspnea, 16 months, DCM, 7 years stable, 9 months no recovery, Thrombus LV apex, DCM (16).
- **NL5 II:2:** F, II:2, F, Heart murmur, 16 months, Mild DCM, 7 years stable, 9 months no recovery, Thrombus LV apex, DCM (16).
- **NL6 II:3:** F, II:3, F, Heart murmur, 16 months, Mild DCM, 7 years stable, 9 months no recovery, Thrombus LV apex, DCM (16).
- **NL6 II:4:** F, II:4, F, Heart murmur, 16 months, Mild DCM, 7 years stable, 9 months no recovery, Thrombus LV apex, DCM (16).
- **NL6 II:5:** F, II:5, F, Heart murmur, 16 months, Mild DCM, 7 years stable, 9 months no recovery, Thrombus LV apex, DCM (16).
- **NL6 II:6:** F, II:6, F, Heart murmur, 16 months, Mild DCM, 7 years stable, 9 months no recovery, Thrombus LV apex, DCM (16).
- **NL7 III:1:** F, III:1, F, Heart murmur, 16 months, Mild DCM, 7 years stable, 9 months no recovery, Thrombus LV apex, DCM (16).
- **NL7 III:2:** F, III:2, F, Heart murmur, 16 months, Mild DCM, 7 years stable, 9 months no recovery, Thrombus LV apex, DCM (16).
- **NL7 III:3:** F, III:3, F, Heart murmur, 16 months, Mild DCM, 7 years stable, 9 months no recovery, Thrombus LV apex, DCM (16).
- **NL7 III:4:** F, III:4, F, Heart murmur, 16 months, Mild DCM, 7 years stable, 9 months no recovery, Thrombus LV apex, DCM (16).
- **NL7 III:5:** F, III:5, F, Heart murmur, 16 months, Mild DCM, 7 years stable, 9 months no recovery, Thrombus LV apex, DCM (16).
- **NL7 III:6:** F, III:6, F, Heart murmur, 16 months, Mild DCM, 7 years stable, 9 months no recovery, Thrombus LV apex, DCM (16).
- **NL8 III:1:** F, III:1, F, Heart murmur, 16 months, Mild DCM, 7 years stable, 9 months no recovery, Thrombus LV apex, DCM (16).
- **NL8 III:2:** F, III:2, F, Heart murmur, 16 months, Mild DCM, 7 years stable, 9 months no recovery, Thrombus LV apex, DCM (16).
- **NL8 III:3:** F, III:3, F, Heart murmur, 16 months, Mild DCM, 7 years stable, 9 months no recovery, Thrombus LV apex, DCM (16).
- **NL8 III:4:** F, III:4, F, Heart murmur, 16 months, Mild DCM, 7 years stable, 9 months no recovery, Thrombus LV apex, DCM (16).
- **NL8 III:5:** F, III:5, F, Heart murmur, 16 months, Mild DCM, 7 years stable, 9 months no recovery, Thrombus LV apex, DCM (16).
- **NL8 III:6:** F, III:6, F, Heart murmur, 16 months, Mild DCM, 7 years stable, 9 months no recovery, Thrombus LV apex, DCM (16).
- **NL9 III:1:** F, III:1, F, Heart murmur, 16 months, Mild DCM, 7 years stable, 9 months no recovery, Thrombus LV apex, DCM (16).
- **NL9 III:2:** F, III:2, F, Heart murmur, 16 months, Mild DCM, 7 years stable, 9 months no recovery, Thrombus LV apex, DCM (16).
- **NL9 III:3:** F, III:3, F, Heart murmur, 16 months, Mild DCM, 7 years stable, 9 months no recovery, Thrombus LV apex, DCM (16).
- **NL9 III:4:** F, III:4, F, Heart murmur, 16 months, Mild DCM, 7 years stable, 9 months no recovery, Thrombus LV apex, DCM (16).
- **NL9 III:5:** F, III:5, F, Heart murmur, 16 months, Mild DCM, 7 years stable, 9 months no recovery, Thrombus LV apex, DCM (16).
- **NL9 III:6:** F, III:6, F, Heart murmur, 16 months, Mild DCM, 7 years stable, 9 months no recovery, Thrombus LV apex, DCM (16).
**Confirmed PPCM cases are displayed in bold; affected cases with clinical characteristics suggestive for PPCM are displayed in bold and italic.** 

AF indicates atrial fibrillation; AI, artificial insemination; AVB, atrioventricular block; (Bi)(L)VAD, (bi)(left) ventricular assist device; CRT, cardiac resynchronization therapy; CS, caesarean section; CVA, cerebral vascular accident; D, death; DCM, dilated cardiomyopathy; DIC, diffuse intravascular coagulation; EMB, endomyocardial biopsy; F, female; HELLP, hemolysis, elevated liver enzymes, low platelet count; HF, heart failure; HTX, heart transplantation; ICD, implantable cardiac defibrillator; IV, intravenous; LA, left anterior; LBBB, left bundle branch block; LE, lung embolism; LV, left ventricle; LVEF, left ventricular ejection fraction; M, male; MI, myocardial infarction; MOF, multiple organ failure; P, pregnancy; PAC, premature atrial contraction; PAD, peripheral arterial disease; PM, pacemaker; PPCM, peripartum cardiomyopathy; PTCA, percutaneous transluminal coronary angioplasty; PVC, premature ventricular contraction; SB, still birth; SCD, sudden cardiac death; SIDS, sudden infant death syndrome; TIA, transient ischemic attack; VF, ventricular fibrillation; VT, ventricular tachycardia.
<table>
<thead>
<tr>
<th>Family</th>
<th>Tested patient</th>
<th>Gene</th>
<th>Amino acid change</th>
<th>Nucleotide change</th>
<th>Classification</th>
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<td>III:2</td>
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</table>

Nomenclature according to HGVS (Human Genome Variation Society) using the reference sequences: *TTN* (NM_001256850.1; Q8W242-1), LAMA4 (NM_001105206.1), PRKAG2 (NM_016203.3), PKP2 (NM_004572.3), DMD (NM_004006.2), RYR2 (NM_001035.2), MYBPC3 (NM_000256.3), TMEM43 (NM_024334.2), RBM20 (NM_001134363.1), MYPN (NM_032578.2).

VUS indicates variant of unknown clinical significance (VUS1, unlikely to be pathogenic; VUS2, uncertain).

‡ II:2 and III:1 were both analyzed; only shared mutations were investigated further (analyzed in silico)

† pathogenic mutation on same allele (p.Arg23956Thrfs*9 (c.71867_71876delGAGTTCTGGA))