Arrhythmogenic Right Ventricular Dysplasia/Cardiomyopathy
Pathogenic Desmosome Mutations in Index-Patients Predict Outcome of Family Screening: Dutch Arrhythmogenic Right Ventricular Dysplasia/Cardiomyopathy Genotype-Phenotype Follow-Up Study

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Background—Arrhythmogenic right ventricular dysplasia cardiomyopathy (ARVD/C) is an autosomal dominant inherited disease with incomplete penetrance and variable expression. Causative mutations in genes encoding desmosomal proteins are found in ~50% of ARVD/C index patients. Previous genotype-phenotype relation studies involved mainly overt ARVD/C index patients, so follow-up data on relatives are scarce.

Methods and Results—One hundred forty-nine ARVD/C index patients (111 male patients; age, 49 ± 13 years) according to 2010 Task Force criteria and 302 relatives from 93 families (282 asymptomatic; 135 male patients; age, 44 ± 13 years) were clinically and genetically characterized. DNA analysis comprised sequencing of plakophilin-2 (PKP2), desmocollin-2, desmoglein-2, desmoplakin, and plakoglobin and multiplex ligation-dependent probe amplification to identify large deletions in PKP2. Pathogenic mutations were found in 87 index patients (58%), mainly truncating PKP2 mutations, including cases with multiple mutations. Multiplex ligation-dependent probe amplification revealed 3 PKP2 exon deletions. ARVD/C was diagnosed in 31% of initially asymptomatic mutation-carrying relatives and 5% of initially asymptomatic relatives of index patients without mutation. Prolonged terminal activation duration was observed more than negative T waves in V1 to V3, especially in mutation-carrying relatives <20 years of age. In 45% of screened families, ≥1 affected relatives were identified (90% with mutations).

Conclusions—Pathogenic desmosomal gene mutations, mainly truncating PKP2 mutations, underlie ARVD/C in the majority (58%) of Dutch index patients and even 90% of familial cases. Additional multiplex ligation-dependent probe amplification analysis contributed to discovering pathogenic mutations underlying ARVD/C. Discovering pathogenic mutations in index patients enables those relatives who have a 6-fold increased risk of ARVD/C diagnosis to be identified. Prolonged terminal activation duration seems to be a first sign of ARVD/C in young asymptomatic relatives. (Circulation. 2011;123:2690-2700.)

Key Words: arrhythmogenic right ventricular dysplasia ■ cardiomyopathy ■ desmosome ■ follow-up studies ■ genetics
Arrhythmogenic right ventricular dysplasia/cardioomyopathy (ARVD/C) is histopathologically characterized by progressive fibrofatty replacement of myocardium, primarily of the right ventricle (RV). Although familial occurrence was recognized in the first report, only in the last decade has the genetic substrate been identified in genes encoding desmosomal proteins. Desmosomes are protein complexes in the intercalated disk, among others responsible for mechanical coupling of cardiac myocytes. Their impairment leads to both mechanical and electric uncoupling of cardiomyocytes, followed by cell death with fibrofatty replacement. Both uncoupling and altered architecture result in activation delay, which is the pivotal mechanism for reentry and thus ventricular tachycardia (VT).

One of the primary clinical challenges in ARVD/C is timely diagnosis of the concealed phase, when individuals are at risk for arrhythmias despite the absence of symptoms. Yet, previous studies on genotype-phenotype correlations involved mainly overt ARVD/C index patients. Follow-up data on their relatives are scarce. Hence, the proportion of relaters who develop signs of ARVD/C and/or (fatal) arrhythmias is unknown.

Analyzing multiple genes related to ARVD/C in all index patients is essential for both accurate diagnosis and appropriate family counseling and screening. More insight into the natural variability of the disease expression and phenotypic consequences of genetic findings of ARVD/C is required. We therefore sequenced all 5 desmosomal genes in 149 Dutch ARVD/C index patients. The 302 family members were screened for the pathogenic mutations identified in their respective index patients. All individuals were followed up for genotype-phenotype correlations to determine disease penetrance and expression, including arrhythmias and sudden death.

DNA Analysis
Genomic DNA was extracted from whole blood or paraffin-embedded tissues as described previously. Sufficient DNA was available in 149 patients (88%) for direct sequencing of PKP2, DSPG2, DSPC2, DSP, and JUP. In addition, multiplex ligation-dependent probe amplification analysis was performed to identify large deletions in PKP2 (SALSA multiplex ligation-dependent probe amplification kit P168 ARVC-PKP2, MRC Holland, Amsterdam, the Netherlands). Primer sequences and polymerase chain reaction conditions are available on request.

Nonsense, frameshift, and splice-site mutations affecting positions −2, −1, +1, and +2, as well as PKP2 exon deletions, were all labeled truncating and considered to be proven pathogenic unless identified as polymorphisms. To assess the possible pathogenic nature of missense mutations, we used the in silico predictive programs Sorting Intolerant From Tolerant (SIFT) and Polymorphism Phenotyping (PolyPhen), which use the following criteria: difference in physicochemical properties of amino acids in respective substitutions, evolutionary conservation of amino acids across several species, presence in an evolutionary conserved region, and localization in a predicted or proven functionally important domain. Missense mutations were considered to be most likely pathogenic when both programs predicted the genetic variants to affect protein function by a tolerance index score of ≤0.05 (SIFT) and the classification “probably damaging” (PolyPhen). Variants suspected of pathogenicity also had to be absent in 200 ethnically matched (ie, white Dutch) control subjects after direct sequencing. When available, data on segregation were taken into account. Family members were screened only for the pathogenic mutation found in their respective index patient. For genotype-phenotype analyses, proven pathogenic and most likely pathogenic variants were together labeled pathogenic, and comparisons of truncating and missense mutations were made. All variants present in the general population with a frequency of >1% were considered to be sequence polymorphisms. Sequence variants that did not fulfill our criteria for pathogenicity and were not polymorphisms were labeled unclassified variants (UVs).
In this article, “patients with mutations” and “mutation carriers” refer to patients carrying pathogenic mutations as identified in this study.

### Statistical Analysis

Continuous variables were compared by use of the Student t test. Categorical variables were analyzed by use of contingency tables and the Pearson \(\chi^2\) method. If the expected value was \(<5\), the Fisher exact test was used instead. To compensate for possible correlation of characteristics of relatives within families, mixed models with hierarchical structure of members within families were applied. When it was impossible to fit the data in these models owing to lack of variation within families, Pearson \(\chi^2\) tests were applied instead on a family level (ie, on the presence/absence of characteristics within separate families). Descriptive statistics are reported as mean±SD and estimates as estimated value±SE. Values of \(P<0.05\) were considered statistically significant. PASW statistics 17.0 software (SPSS, Chicago, IL) was used for calculations.

### Results

#### Arrhythmogenic Right Ventricular Dysplasia/Cardiomyopathy Index Patients

Initially, 169 ARVD/C patients fulfilled the 2010 TFC. Twenty patients were excluded because of incomplete DNA analyses. PKP2, DSG2, DSC2, DSP, and JUP were all screened in the remaining 149 patients (111 male patients; mean age at inclusion, 49±13 years). First presentation was at a median age of 37 years (range, 12 to 77 years), mostly with monomorphic VT (n=122, 82%). These VTs were sustained in 119 cases, and all but 2 had left bundle-branch block morphology (see Table 1). Presenting first symptoms were similar between men and women. Cardioverter-defibrillators were implanted in 95 patients (64%): in 57 directly after diagnosis and in 38 after 4±7 years of follow-up. During follow-up, 4 patients died of ventricular fibrillation (3 without implantable cardioverter-defibrillators, 1 with electrical storm) and 2 died of progressive heart failure. Three patients were indicated for heart transplantation; 1 received a transplantation.

#### DNA Analyses

All pathogenic mutations are summarized in Table 2, and UVs are summarized in Table II in the online-only Data Supplement. In total, 87 index patients (58%) carried at least 1 mutation (89 total: 70 truncating [68 PKP2] and 19 missense [12 PKP2]).

### Table 1. Clinical Characteristics of Arrhythmogenic Right Ventricular Dysplasia/Cardiomyopathy Index Patients Related to the Presence or Absence of Pathogenic Mutations

<table>
<thead>
<tr>
<th></th>
<th>Total (n=147)*</th>
<th>With Mutation (n=87)</th>
<th>No Mutation (n=60)</th>
<th>(P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>110 (75)</td>
<td>62 (71)</td>
<td>48 (80)</td>
<td>0.284</td>
</tr>
<tr>
<td>Age at onset, mean±SD, y</td>
<td>37±14</td>
<td>35±13</td>
<td>40±14</td>
<td>0.042</td>
</tr>
<tr>
<td>Follow-up, mean±SD, y</td>
<td>12±9</td>
<td>13±10</td>
<td>11±7</td>
<td>0.191</td>
</tr>
<tr>
<td>Reason for first evaluation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VT</td>
<td>122 (82)</td>
<td>72 (83)</td>
<td>47 (78)</td>
<td></td>
</tr>
<tr>
<td>Aborted sudden death</td>
<td>12 (8)</td>
<td>8 (9)</td>
<td>4 (7)</td>
<td></td>
</tr>
<tr>
<td>Prolonged syncope</td>
<td>2 (1)</td>
<td>1 (1)</td>
<td>1 (2)</td>
<td></td>
</tr>
<tr>
<td>Sudden death of a relative</td>
<td>2 (1)</td>
<td>0 (0)</td>
<td>2 (3)</td>
<td></td>
</tr>
<tr>
<td>Frequent PVCs</td>
<td>5 (3)</td>
<td>5 (6)</td>
<td>3 (5)</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>4 (3)</td>
<td>3 (1)</td>
<td>3 (5)</td>
<td></td>
</tr>
<tr>
<td>TFC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Epsilon wave†</td>
<td>22 (18)</td>
<td>14 (18)</td>
<td>8 (17)</td>
<td>0.153</td>
</tr>
<tr>
<td>Late potentials‡</td>
<td>44 (54)</td>
<td>22 (50)</td>
<td>22 (59)</td>
<td>0.395</td>
</tr>
<tr>
<td>Prolonged TAD†</td>
<td>78 (62)</td>
<td>44 (56)</td>
<td>34 (72)</td>
<td>0.734</td>
</tr>
<tr>
<td>Negative T waves</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(V_1-V_5)†</td>
<td>98 (78)</td>
<td>69 (88)</td>
<td>29 (62)</td>
<td>0.001</td>
</tr>
<tr>
<td>(V_1-V_6)†</td>
<td>8 (6)</td>
<td>5 (6)</td>
<td>3 (6)</td>
<td>1.000</td>
</tr>
<tr>
<td>(V_5-V_6)†</td>
<td>6 (5)</td>
<td>2 (3)</td>
<td>3 (6)</td>
<td>0.524</td>
</tr>
<tr>
<td>Ventricular fibrillation</td>
<td>12 (8)</td>
<td>8 (9)</td>
<td>4 (7)</td>
<td>0.281</td>
</tr>
<tr>
<td>LBBB VT with superior axis</td>
<td>67 (46)</td>
<td>39 (45)</td>
<td>28 (47)</td>
<td>0.943</td>
</tr>
<tr>
<td>LBBB VT</td>
<td>129 (88)</td>
<td>77 (89)</td>
<td>52 (87)</td>
<td>0.945</td>
</tr>
<tr>
<td>PVCs &gt;500/24 h</td>
<td>33 (22)</td>
<td>20 (23)</td>
<td>13 (22)</td>
<td>0.969</td>
</tr>
<tr>
<td>Structural major TFC</td>
<td>86 (59)</td>
<td>55 (63)</td>
<td>31 (52)</td>
<td>0.198</td>
</tr>
<tr>
<td>Structural minor TFC</td>
<td>25 (17)</td>
<td>15 (17)</td>
<td>10 (17)</td>
<td>0.963</td>
</tr>
</tbody>
</table>

VT indicates ventricular tachycardia; PVC, premature ventricular complex; TFC, Task Force criteria; TAD, terminal activation duration; and LBBB, left bundle-branch block. \(P\) values are the difference between mutation carriers and noncarriers.

*Two patients were diagnosed after autopsy; therefore, no clinical data were available.
†ECGs done while the patients were off drugs were available for 125 index patients: 78 with and 47 without mutation.
‡Late potentials were measured in 81 patients: 69 by signal-averaged ECG and 12 by mapping during electrophysiological studies.
Single mutations were identified in 84 index patients (56%): 76 PPK2 (64 truncating), 5 DSG2, 2 DSC2, and 1 DSP. In addition, 3 patients (2%) carried multiple mutations. Two had a truncating PPK2 mutation and a DSC2 or DSP missense mutation, and 1 carried a homozygous DSC2 mutation (see Table III in the online-only Data Supplement for details, including phenotypic characteristics).

We identified 52 UVs in 50 patients (36 different ones; Table II in the online-only Data Supplement). In 28 patients, the UV was carried in addition to a pathogenic mutation, and 2 patients carried 2 UVs. Table IV in the online-only Data Supplement shows the combinations of pathogenic mutations and UVs per patient. Of the 36 UVs, 29 were missense variants, 6 were silent variants, and 1 was a frameshift variant. The DSC2 frameshift variant p.Ala897fs was considered a UV because it is located at the far end of the gene and was found in 8 patients and in 3 of 200 controls (P = 0.06).24

### Table 2. Pathogenic Mutations

<table>
<thead>
<tr>
<th>Gene</th>
<th>DNA Change</th>
<th>Protein Change</th>
<th>Type</th>
<th>PolyPhen (PSIC)*</th>
<th>SIFT†</th>
<th>Index Patients, n</th>
<th>Yes</th>
<th>No</th>
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<tbody>
<tr>
<td>Proven pathogenic</td>
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<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>PKP2</td>
<td>Deletion exon 1–4</td>
<td>NA</td>
<td>Deletion</td>
<td>1</td>
<td>1</td>
<td>7</td>
<td></td>
<td></td>
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<tr>
<td>PKP2</td>
<td>Deletion exon 1–14</td>
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<td>Deletion</td>
<td>1</td>
<td>4</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>PKP2</td>
<td>Deletion exon 8</td>
<td>NA</td>
<td>Deletion</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c.148_151delACAG</td>
<td>p.Thr50SerfsX61</td>
<td>Frameshift</td>
<td>2</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c.235C&gt;T</td>
<td>p.Arg79X</td>
<td>Nonsense</td>
<td>9</td>
<td>3</td>
<td>15</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c.258T&gt;G</td>
<td>p.Tyr86X</td>
<td>Nonsense</td>
<td>1</td>
<td></td>
<td></td>
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<tr>
<td>c.397C&gt;T</td>
<td>p.Gln133X</td>
<td>Nonsense</td>
<td>9</td>
<td>3</td>
<td>16</td>
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<tr>
<td>c.917_918delCC</td>
<td>p.Pro318GlnfsX29</td>
<td>Frameshift</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>c.1211_1212insT</td>
<td>p.Val406SerfsX</td>
<td>Frameshift</td>
<td>11</td>
<td>6</td>
<td>15</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>c.1369_1372delCAAA</td>
<td>p.Gln457X</td>
<td>Nonsense</td>
<td>2</td>
<td>4</td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>c.1848C&gt;A</td>
<td>p.Tyr616X</td>
<td>Nonsense</td>
<td>4</td>
<td>6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c.2028G&gt;A</td>
<td>p.Tryp676X</td>
<td>Nonsense</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>c.2034G&gt;A</td>
<td>p.Tryp678X</td>
<td>Nonsense</td>
<td>1</td>
<td>2</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>c.2146G&gt;C</td>
<td>Splice site</td>
<td>7</td>
<td>2</td>
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<tr>
<td>c.2386T&gt;C</td>
<td>p.Cys796Arg</td>
<td>Missense</td>
<td>++ (3.410)</td>
<td>0.03</td>
<td>11</td>
<td>1</td>
<td>17</td>
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<tr>
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<td>p.Tryp807X</td>
<td>Nonsense</td>
<td>1</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>c.2489+1G&gt;A</td>
<td>Splice site</td>
<td>6</td>
<td>1</td>
<td>7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c.2489+4A&gt;C†</td>
<td>Splice site</td>
<td>4</td>
<td>9</td>
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<td></td>
</tr>
<tr>
<td>c.2509delA</td>
<td>p.Ser837ValfsX94</td>
<td>Frameshift</td>
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<td></td>
<td></td>
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<tr>
<td>c.2544G&gt;A</td>
<td>p.Tryp848X</td>
<td>Nonsense</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
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<tr>
<td>DSG2</td>
<td>c.943-1G&gt;A</td>
<td>Splice site</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DSC2</td>
<td>c.378+2T&gt;G</td>
<td>Splice site</td>
<td>1</td>
<td></td>
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<tr>
<td>DSC2</td>
<td>c.3337C&gt;T</td>
<td>p.Arg1113X</td>
<td>Nonsense</td>
<td>1</td>
<td>1</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

Most likely pathogenic

<table>
<thead>
<tr>
<th>Gene</th>
<th>DNA Change</th>
<th>Protein Change</th>
<th>Type</th>
<th>PolyPhen (PSIC)*</th>
<th>SIFT†</th>
<th>Index Patients, n</th>
<th>Yes</th>
<th>No</th>
</tr>
</thead>
<tbody>
<tr>
<td>PKP2</td>
<td>c.2062T&gt;C</td>
<td>p.Ser688Pro</td>
<td>Missense</td>
<td>+</td>
<td>0.04</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>DSC2</td>
<td>c.608G&gt;A</td>
<td>p.Arg203His</td>
<td>Missense</td>
<td>++ (2.295)</td>
<td>0.00†</td>
<td>1</td>
<td>2</td>
<td></td>
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<tr>
<td>DSC2</td>
<td>c.942+3A&gt;6§</td>
<td>Splice site</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DSG2</td>
<td>c.2587G&gt;A</td>
<td>p.Gly863Arg</td>
<td>Missense</td>
<td>++ (2.492)</td>
<td>0.00†</td>
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<tr>
<td>DSG2</td>
<td>c.137G&gt;T</td>
<td>p.Arg46Gln</td>
<td>Missense</td>
<td>++ (2.013)</td>
<td>0.00†</td>
<td>2</td>
<td>4</td>
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<tr>
<td>DSG2</td>
<td>c.614C&gt;T</td>
<td>p.Pro205Leu</td>
<td>Missense</td>
<td>++ (3.054)</td>
<td>0.00†</td>
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<tr>
<td>DSG2</td>
<td>c.874C&gt;T</td>
<td>p.Arg292Cys</td>
<td>Missense</td>
<td>++ (2.759)</td>
<td>0.00†</td>
<td>1</td>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>

DSP | c.1982A>T | p.Asn661Ile | Missense | ++ (2.073) | 0.01† | 1 |

*Polyorphism Phenotyping (PolyPhen) prediction: +++, probably damaging; +, possibly damaging; --, benign. PSIC indicates Position-Specific Independent Counts.
†Sorting Intolerant From Tolerant (SIFT) prediction: Amino acids with scores <0.05 are predicted to be deleterious. For previous reports on pathogenicity, see Reference 31.
‡This substitution may have been predicted to affect function just because the sequences used were not diverse enough. There is low confidence in this prediction.
§The 4 splice-prediction programs used (SpliceSiteFinder, MaxEntScan, NNSPLICE, and GeneSplicer) indicated disruption of the splice donor site of DSC2 exon 7.
genic by both SIFT and PolyPhen. However, we found this variant in 2 of 149 patients and 3 of 300 controls \((P=0.67)\), and it has frequently been reported in other control populations.\(^{22,23,30,31}\) Therefore, we classified it as a UV.

**Genotype-Phenotype Correlation**

First, we compared the clinical characteristics of index patients with and without mutations independently of the number of variants (Table 1). Both groups showed a male predominance (71% and 80%), and age at the time of inclusion was similar (51±13 and 49±13 years).

Ventricular tachycardia and/or ventricular fibrillation was recorded in 134 of 149 index patients, showing similar frequencies among those with and without mutations. However, first arrhythmic events were documented at a significantly younger age in mutation carriers compared with noncarriers (median age, 35 versus 42 years; \(P=0.042\); Figure 1). The 3 patients carrying 2 mutations had their first VT at 14, 17, and 20 years of age. A comparison of criteria in mutation carriers and noncarriers demonstrated no significant differences except that negative T waves in V1 to V3 occurred more often in mutation carriers (88% versus 62%; \(P=0.001\)).

None of the criteria could be explained by the type of mutation (truncating versus missense) except for prolonged TAD, which was observed more often in patients with missense mutations (13 of 15 versus 31 of 63).

Patients carrying a single UV did not differ from patients without mutations on any criterion or on age of first event. However, fewer patients with a single UV had negative T waves in V1 to V3 than patients with 1 mutation (\(P=0.043\)), but they were similar in every other respect. Likewise, patients carrying a UV together with a mutation did not differ from either patients with a single mutation or those with 2 mutations (see Table V in the online-only Data Supplement for more details).

**Family History of Sudden Cardiac Death**

Twenty-five index patients reported 30 relatives with sudden cardiac death (SCD). In 18 families of index patients with mutations, SCD had occurred in 19 relatives (16 male subjects). Autopsy was performed in 6 cases and revealed ARVD/C in all of them. In 7 families of index patients without mutations, SCD had occurred in 11 relatives (8 male relatives). No autopsies had been performed. Mean ages at SCD were similar in families with and without mutations (28 years [range, 15 to 49 years] versus 32 years [range, 21 to 43 years]; \(P=0.108\)). However, all 7 relatives with SCD at <20 years of age (23%) belonged to families with PKP2 mutations (6 truncating; Table 3).

**Family Members**

Both genotypic and phenotypic data were available for analysis of 302 members from 93 different families (58 with and 35 without mutations).

**Relatives Symptomatic at First Presentation**

Twenty relatives from 18 families presented with cardiac symptoms before family screening was performed (13 men; mean age, 42 years [range, 20 to 71 years]). In 10 relatives, monomorphic left bundle-branch block VT was the first symptom. In addition, 4 family members had ventricular fibrillation (1 was successfully resuscitated), 3 had prolonged syncope, 2 had palpitations and 1 had atrial fibrillation. Eighteen relatives fulfilled the 2010 TFC, and 18 carried pathogenic mutations (all PKP2, 16 truncating; Table 2 and Figure 2).

**Family Screening of Asymptomatic Relatives**

The remaining 282 asymptomatic relatives (123 male subjects, 44%) were evaluated in the course of family screening. Mean age at first clinical examination was 39±18 years, and follow-up 4±4 years, which was similar for male and female subjects. In 119 members (42%) from 56 families, pathogenic
mutations were identified: in 111 PKP2 (93%; 78% truncating), 5 DSG2, and 3 DSC2 (Table 2 and Figure 2).

Table 4 shows the results of family screening in the 282 initially asymptomatic family members. Most criteria were perceived either exclusively or considerably more often in mutation carriers. Because of the low presence of characteristics and low variation in families, mixed models could not be applied to these data. To recognize the possible dependence of relatives and their phenotypes among families, statistical analyses were performed at the family level (ie, families were scored positive

Table 3. Relatives <20 Years of Age With Sudden Death or Arrhythmogenic Right Ventricular Dysplasia/Cardiomyopathy Signs

<table>
<thead>
<tr>
<th>Age, y</th>
<th>Sex</th>
<th>PKP2 Mutation in Family†</th>
<th>PKP2 Mutation‡</th>
<th>Autopsy</th>
<th>TFC Identified</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>Male</td>
<td>c.235C&gt;T</td>
<td></td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>Male</td>
<td>c.2489+1G&gt;A‡</td>
<td></td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>Male</td>
<td>c.2489+1G&gt;A‡</td>
<td></td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>Male</td>
<td>c.2386T&gt;C</td>
<td>Yes (ARVD/C)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>Male</td>
<td>c.397C&gt;T</td>
<td></td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>Male</td>
<td>c.1211-1212insT</td>
<td>Yes (ARVD/C)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>Female</td>
<td>c.2421C&gt;A</td>
<td>Yes (ARVD/C)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Family screening* 14 Male Deletion exons 1–4 Prolonged TAD

17 Male  c.1211-1212insT Prolonged TAD plus 500 PVCs/24 h
18 Male  c.2489+4A>C Prolonged TAD
18 Male  c.2386T>C Prolonged TAD
19 Female c.148_151delACAG Prolonged TAD
15 Male  c.1369_1372delICAAA Negative T waves in V1–V2
19 Female c.2386T>C Negative T waves in V1–V2

TFC indicates Task Force criteria; ARVD/C, arrhythmogenic right ventricular dysplasia/cardio-myopathy; TAD, terminal activation duration; and PVC, premature ventricular complex.
*Age after follow-up.
†All mutations in PKP2.
‡From the same family.

Mutations were found in none of the other genes. All mutations were truncating, except missense c.2386T>C.

Figure 2. Schematic representation of relatives demonstrating the distribution of arrhythmogenic right ventricular dysplasia/cardio-myopathy (ARVD/C) signs and symptoms as well as mutations. Numbers in parentheses indicate the number of different families.
for separate criteria if present in ≥1 relative; Table 4). Strikingly, prolonged TAD was observed in more cases than negative T waves in V1 to V3. This difference varied with age (Figure 3). Relatives <20 years of age had no negative T waves in V1 to V3, but prolonged TAD was already observed in 5 of 7 young relatives with signs of ARVD/C (Table 3). VTs were recorded in 9 initially asymptomatic relatives (3%) from 8 families. All carried PKP2 mutations (7 truncating; Table 4). Five women (mean age, 51 years [range, 34 to 70 years]) had hemodynamically well-tolerated nonsustained left bundle-branch block VT during their first exercise tests. The other 4 (1 female) had VT after a mean follow-up of 4 years (2 to 8 years at a mean age of 43 years [range, 34 to 56 years]). Two left bundle-branch block VTs were sustained with rates of 220 and 240 bpm, and resulted in syncope.

| Table 4. Clinical Characteristics of Asymptomatic Arrhythmogenic Right Ventricular Dysplasia/Cardiomyopathy Relatives Related to the Presence or Absence of Pathogenic Mutations |
|------------------|------------------|------------------|
|                   | Total (n=282)    | With Mutation (n=119) | No Mutation (Index Patient With Mutation; n=67) | No Mutation (Index Patient No Mutation; n=96) | P* |
|                   | n | %  | n | %  | n | %  | n | %  |     |
| Male              | 123 | 44 | 45 | 38 | 32 | 48 | 47 | 49 | 0.096 |
| Age at first evaluation, mean±SD, y | 39±18 | 39±18 | 40±18 | 38±17 | 0.419 |
| Follow-up, mean±SD, y | 4±4 | 4±3 | 4±3 | 3±3 | 0.131 |
| ARVD/C diagnosis | 42 (27) | 15 | 37 (23) | 31 | 0 | 0 | 5 (4) | 5 | 0.004 |
| Epsilon wave     | 4 (4) | 1 | 4 (4) | 3 | 0 | 0 | 0 | 0 | 0.292 |
| Late potentials† | 8 (8) | 10 | 6 (6) | 21 | 0 | 0 | 2 (2) | 2 | 0.047 |
| Prolonged TAD    | 34 (26) | 12 | 26 (21) | 22 | 0 | 0 | 8 (5) | 8 | 0.030 |
| Negative T waves |                  |                  |                  |                  |                  |
| V1–V3            | 20 (18) | 7 | 19 (17) | 16 | 0 | 0 | 1 | 1 | 0.002 |
| V1–V2            | 7 (6) | 2 | 6 (5) | 5 | 0 | 0 | 1 | 1 | 0.410 |
| V3–V6            | 3 (2) | 1 | 1 | 0 | 0 | 0 | 3 (2) | 3 | 0.130 |
| VT with LBBB morphology |          |                  |                  |                  |
| With superior axis | 2 (2) | 1 | 2 (2) | 2 | 0 | 0 | 0 | 0 | 0.532 |
| With inferior axis | 9 (9) | 3 | 9 (9) | 8 | 0 | 0 | 0 | 0 | 0.023 |
| >500 PVCs/24 h   | 34 (19) | 12 | 23 (14) | 19 | 0 | 0 | 9 (5) | 9 | 0.304 |
| Structural abnormalities |          |                  |                  |                  |
| Major            | 12 (11) | 4 | 10 (9) | 8 | 0 | 0 | 2 (2) | 2 | 0.315 |
| Minor            | 10 (7) | 4 | 10 (7) | 8 | 0 | 0 | 0 | 0 | 0.045 |

ARVD/C indicates arrhythmogenic right ventricular dysplasia/cardiomypathy; TAD, terminal activation duration; VT, ventricular tachycardia; LBBB, left bundle-branch block; PVC, premature ventricular complex.*Difference between families of relatives with mutation vs relatives of index patients without mutation. Numbers in parentheses indicate the numbers of different families.†Late potentials were measured in 77 patients: 29 with and 48 without mutation.

Figure 3. Presence of prolonged terminal activation duration (TAD) and negative T waves in V1 to V3 per age group in relatives with mutation vs relatives from index patients without mutation. Prolonged TAD was already present in many young relatives, with similar frequencies among all age groups (P=0.269). On the contrary, negative T waves were present more frequently with increasing age, almost exclusively in mutation carriers. Numbers in parentheses indicate the numbers of relatives per age group.
Altogether, 57 family members (20%) from 35 families showed signs of ARVD/C because they fulfilled ≥1 criteria besides those concerning family history. This group comprised more women than men (39 versus 18; P=0.04), and their mean age after follow-up was 47±16 years. Forty-eight (84%) carried pathogenic mutations (all PKP2, 41 truncating), and the remaining 9 belonged to families with no identified mutation. Fourteen relatives with an unremarkable first clinical evaluation developed signs of ARVD/C after a mean follow-up of 4.2 years (range, 1 to 10 years). For family members up to 50 years of age, a higher age was associated with an increased prevalence of ARVD/C signs, in contrast to family members ≥50 years of age (Figure 4).

Forty-two initially asymptomatic family members from 27 families were diagnosed with ARVD/C: at first evaluation in 34 patients and after a mean follow-up of 5.0 years (range, 2 to 10 years) in 8 patients. This group had a mean age at diagnosis of 39±15 years and comprised 29 females (69%).

**Familial Risk of Arrhythmogenic Right Ventricular Dysplasia/Cardiomyopathy**

In initially asymptomatic relatives, ARVD/C was diagnosed in 5% (5 of 96) when their index patient had no identified mutation and 20% (37 of 119+67) when their index patient had a mutation; all 37 also carried mutations (Figure 2). Thus, identifying a mutation in an index patient implies a relative risk for ARVD/C diagnosis in their asymptomatic relatives of 3.8±1.6. This risk increases to as much as 6.0±2.4 for mutation-carrying relatives compared with relatives of index patients without a mutation (37 of 119 versus 5 of 96).

When the symptomatic and asymptomatic mutation-carrying relatives were combined, 54 of 137 (39%) had an ARVD/C diagnosis compared with 6 of 98 relatives of index patients without mutations (6%).

Of the 93 families in which ≥2 members, including the index patient, were examined, 42 had familial ARVD/C (45%; 38 with mutations, all PKP2, 33 truncating). Thus, PKP2 mutations were identified in 90% of familial ARVD/C cases.

**Discussion**

Here, we report the results of follow-up of a large number of ARVD/C families, including comprehensive DNA analysis. In 87 of 149 ARVD/C index patients (58%), pathogenic mutations were identified, predominantly truncating PKP2. ARVD/C was diagnosed in 60 of 302 family members (18 symptomatic, 42 asymptomatic), of whom 54 (90%) carried pathogenic mutations (all PKP2, 47 truncating). Identifying a pathogenic mutation in an index patient predicts outcome in relatives. Compared with relatives of index patients without mutations, mutation-carrying relatives have a 6-fold risk of ARVD/C diagnosis, markedly enhanced risk of ventricular arrhythmias in analyzed relatives, and earlier onset of ARVD/C signs and symptoms (for concurrent numbers, see Familial Risk of Arrhythmogenic Right Ventricular Dysplasia/Cardiomyopathy, Table 4, and Figure 4, respectively). In young relatives <20 years of age, sudden death and signs of ARVD/C occurred exclusively in PKP2 mutation carriers. Prolonged TAD, a marker of activation delay, appeared to be an early ARVD/C sign.

**Pathogenicity of DNA Variants**

The large majority of DNA variations were truncating PKP2 mutations. Notably, in this first study applying multiplex ligation-dependent probe amplification on a large scale in ARVD/C patients, large PKP2 deletions were identified in 3 cases. This mutational yield of 2%, which is comparable to the sequencing of DSG2 or DSC2, underscores the importance of performing this additional analysis. Notably, because this has not been tested, we cannot exclude the possibility that large deletions might be present in other desmosomal genes in patients that are as yet without a pathogenic mutation.

The fact that a significant proportion of genetic variants remains unclassified represents a gap in risk assessment for index patients, and UVs are noninformative for family screening. However, establishing pathogenic effects of nonsense variants is difficult and requires well-validated functional assays, which are not widely available and are highly complex to perform. Linkage and segregational studies can be helpful to establish pathogenicity. Yet, low frequency of variants, small family size, and age-dependent penetrance hamper the use of these methods.

Previous studies on mutations in DSC2, DSG2, and DSP used different criteria to define the pathogenicity of DNA variants. The largest studies used criteria of absence in control subjects and/or occurrence in a functionally important domain, alteration of conserved amino acids, or cosegrega-
tion with disease in a family. Thus, in contrast to our study, none of these studies used all the possible predictive strategies together. Consequently, the variants p.Thr335Ala and p.Val392Ile in DSG2 and p.Gln90Arg in DSP were previously classified as pathogenic, but with our definition, they were classified as UVs.21,22

Index Patients
In this study of white Dutch patients, PKP2 was by far the most important contributor to mutation yield (found in 52% of patients, 90% of pathogenic mutations). Previous studies reported PKP2 mutations in 19% to 45% of ARVD/C patients and mutations in other desmosomal genes in 1% to 12%.21,22,31,32 These differences can be due to various causes, such as the presence of founder mutations, use of different definitions for pathogenicity, regional differences in other genetic and nongenetic causes, and strictness in applying the TFC.6,19 New genetic techniques, such as high-density genotyping array with haplotype sharing or exome sequencing, might elucidate new genes involved in ARVD/C.33 This will improve the distinction between familial and sporadic ARVD/C cases and ameliorate risk stratification for relatives.

Our study suggests that UVs as defined here do not result in a more severe phenotype or earlier onset of ARVD/C. However, most UVs were found only in single patients; therefore, comparisons on the level of individual variants were not possible. Because the pathogenic influence of UVs is unknown, relatives were not tested for these variants. Consequently, it cannot be ruled out that, within families, carrying a specific UV is a risk factor for disease development.

Index patients were included only if they fulfilled the 2010 TFC. Consequently, this obviously affected population cannot be regarded as representative of the variable disease expression. Therefore, analysis of asymptomatic family members was crucial.

Family Members
The large majority (84%) of initially asymptomatic family members showing signs of ARVD/C carried desmosomal gene mutations (90% truncating, all PKP2). Overall, we found no differences between carriers of truncating and missense mutations. In 4 families with ≥2 members diagnosed with ARVD/C and in whom no mutations were identified, a genetic cause is highly suspected.

Negative T waves in V1 to V3 have always been considered the most sensitive ECG abnormality in ARVD/C. However, in family members, we observed the new criterion of prolonged TAD more often and at younger age (Figure 3). In 4 of 7 family members <20 years of age with signs of ARVD/C, prolonged TAD was the only clinical abnormality observed. Longer periods of follow-up are needed to demonstrate the disease progression after prolonged TAD is found and whether it is a good predictive marker of arrhythmias and SCD.

Disease Penetration
Because ARVD/C is a progressive disease with age-related penetrance, it seems counterintuitive that the percentage of family members showing any signs of ARVD/C was lower in those ≥50 years of age than in their younger counterparts. Because individuals ≥50 years of age are also at higher risk of coronary artery disease, those already seeing a cardiologist may not have been referred for family screening.

Cohorts of ARVD/C index patients universally demonstrate a male predominance. Interestingly, among the asymptomatic family members in this study, women were affected more often than men. Apparently, men with ARVD/C experience arrhythmias and die suddenly at a younger age and thus are more likely to be the index patient. The underlying mechanism is not yet known, but strenuous exercise by men and the prevention of programmed cell death in cardiac myocytes in women due to estradiol are believed to play a role.34

At first examination, 43 of 282 asymptomatic family members (15%) already showed any sign of disease, including nonsustained VT in 5 patients and ≥500 premature ventricular complexes in 24 hours in 30 patients. Because relatives with these arrhythmias were asymptomatic, the age of onset is unknown. Besides, only 4 relatives (all mutation carriers) went on to develop VT during follow-up. Therefore, it was not possible to calculate the annual risk of arrhythmias or to identify predictive risk factors other than carrying a pathogenic mutation. Longer periods of follow-up of large series of relatives who initially have no signs of disease are needed to provide this insight. Large multicenter studies are required to achieve this.

Contrary to our study, all previous genotype-phenotype analyses in ARVD/C families separately addressed mutations in different desmosomal genes and involved only a few families. We found that 66 of 137 mutation-carrying family members (48%) showed some sign of ARVD/C, with ARVD/C diagnosis made according to 2010 TFC in 39%. No direct comparison with other studies can be made, because different sets of diagnostic criteria were used. However, similar numbers of affected relatives with PKP2 mutations were reported by Dalal et al35 and Syrris et al20 (49% and 47%, respectively). Other family studies have also reported high percentages of relatives diagnosed with ARVD/C: 58% in DSG2, 75% in DSC2, and 54% in DSP mutation carriers. However, these studies comprised only 8, 2, and 4 families, respectively.8,30,36

Study Limitations
Sequence alterations were divided into pathogenic or UVs according to arbitrarily defined criteria. However, in the absence of any data on pathogenicity, conclusions regarding the utility of genetic testing are speculative.

All index patients were offered family screening, regardless of their mutational status. However, relatives of 93 of 149 fully genotyped index patients (62%) underwent both clinical and genetic screening. Although there is no such indication, this might potentially create a bias in the population of relatives studied. Not all relatives underwent all the diagnostic tests, as indicated in the Methods section. All family members included in this study underwent at least 2-dimensional echocardiography to screen for RV structural abnormalities. Additional magnetic resonance imaging was
performed in only a minority of cases, so minor abnormalities might have been missed. Late potentials were measured in 25% of relatives, but activation delay was measured by means of prolonged TAD in all relatives.

Thirty-six relatives of mutation-carrying index patients were not clinically evaluated because they appeared to be noncarriers; thus, they could not be included in this study. Consequently, of families with pathogenic mutations, more relatives with mutations were included than without mutations (119 versus 67).

Conclusions
In this large follow-up study in Dutch ARVD/C families, pathogenic desmosomal gene mutations were found in the majority of ARVD/C index patients (58%); these were mainly truncating PKP2 mutations, with multiple mutations in 2% of cases. Performing multiplex ligation-dependent probe amplification also is important, because its mutational yield is comparable to the sequencing of DSG2 or DSPC2. Mutation carriers presented at a younger age than noncarriers; this age was even lower when multiple mutations were identified.

In total, 20% of initially asymptomatic relatives who underwent family screening showed some sign of ARVD/C (84% mutation carriers), mainly ECG abnormalities. Mutation-carrying relatives had an earlier onset of signs and symptoms, a markedly increased risk of arrhythmias, and a 6-fold increased risk of ARVD/C diagnosis. Familial cases were identified in 45% of the families screened. Prolonged TAD seems to be an early marker of RV abnormalities, especially in mutation-carrying relatives <20 years of age.

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Disclosures
None.

References
DNA analysis comprised sequencing of the desmosomal genes PKP2, DSC2, DSG2, DSP, and JUP and multiplex ligation-dependent probe amplification to identify large deletions in PKP2. Pathogenic mutations were identified in 87 of 149 ARVD/C index patients (58%), mainly truncating PKP2 mutations with multiple mutations in 2% of cases. Performing multiplex ligation-dependent probe amplification also appeared to be important; its 2% mutational yield was comparable to the sequencing of DSG2 or DSC2. Identification of mutations in index patients had major consequences for the diagnostic evaluation of patients and clinical impact in practice.

Arrhythmogenic right ventricular dysplasia/cardiomyopathy (ARVD/C) usually shows an autosomal dominant inheritance pattern, with incomplete penetrance and variable clinical expression. Classically, index patients present between the second and fourth decades of life with right ventricular tachycardia. However, sudden death can occur at adolescence, whereas sudden death can occur at adolescence, whereas sudden death can occur at adolescence, whereas sudden death can occur at adolescence. Previous genotype-phenotype studies involved four families with arrhythmogenic right ventricular cardiomyopathy. Penetration of mutations in plakophilin-2 among families with arrhythmogenic right ventricular dysplasia/cardiomyopathy. Eur Heart J. 2011;79:459–467.


CLINICAL PERSPECTIVE

Arrhythmogenic right ventricular dysplasia/cardiomyopathy (ARVD/C) usually shows an autosomal dominant inheritance pattern, with incomplete penetrance and variable clinical expression. Classically, index patients present between the second and fourth decades of life with right ventricular tachycardia. However, sudden death can occur at adolescence, whereas mutation carriers may remain without signs and symptoms until old age. Previous genotype-phenotype studies involved


