Atrial standstill (AS) is an extremely rare arrhythmia, characterized by the absence of electrical and mechanical activity in the atria. Electrocardiographically, AS is characterized by bradycardia, the absence of P waves, and a junctional narrow complex escape rhythm. Different forms of the arrhythmia have been described, such as persistent versus transient AS \(^1\) and a total versus a partial form. About 50% of patients have Adams-Stokes attacks. In most reports, AS may be secondary to other clinical disorders such as Ebstein’s anomaly, Emery-Dreifuss muscular dystrophy (X-linked), Kugelberg-Welander syndrome (autosomal recessive), diabetes mellitus, or amyloidosis. Only a few cases of familial clustering of primary AS have been reported. The number of affected individuals in each family was small (two to three) and restricted to one generation, except in rare cases.

The genetic basis for familial AS is unknown. Identification of genetic factors for AS is hampered by the small number of affected individuals in each family. We identified a relatively large AS family with four affected individuals in two generations in whom structural heart disease or systemic disease was excluded. Because the primary characteristic of AS is inexcitability of the atrium, we consider AS an electrical disease and screened a number of genes encoding proteins relevant for action potential generation and conduction in this family. We identified a mutation in the cardiac sodium channel gene SCN5A, the first in patients with an atrial-specific arrhythmia. In addition, we identified relatively rare genotypes for two connexin40 (Cx40) polymorphisms in the AS patients. Our results suggest that coinheritance of these genetic variations leads to AS in this family.

**Materials and Methods**

**Patients**

Informed consent was obtained from participating family members according to guidelines of the local medical ethics committee. Relatives were asked about their health and specifically about possible signs or symptoms of AS such as palpitations or syncpe.
Standard 12-lead ECG recordings and blood for DNA extraction were obtained from all participating relatives. Individuals were considered affected by AS when they met the following criteria: absence of P wave on surface 12-lead ECG, bradycardia, and inability to stimulate the atria on invasive electrophysiological study (EPS).

**Genetic Studies**

DNA for genetic analyses was extracted from peripheral blood lymphocytes using standard protocols.

**Haplotype Analysis and Direct Sequencing of Candidate Genes**

Highly polymorphic microsatellite markers were selected close to each candidate gene (within 3 to 4 cM) and for each locus 3 to 5 markers were typed. PCR products for each marker were generated using fluorescently labeled primers and run on an ABI-377 prism (PE Applied Biosystems). These data were analyzed using Genescan and Genotyper software. For each locus, haplotypes were constructed from the combined marker data. Evidence for genetic linkage was calculated using the Linkage package, version 5.1.11 Probability values corresponding to logarithm of the odds (lod) scores were computed comparing lod score×4.6 against a χ² distribution with one degree of freedom (one-sided test). Bonferroni correction for multiple testing was applied wherever appropriate. The genetic risk factor for AS was modeled as a dominant gene with a penetrance of 50%. Small candidate genes were PCR-amplified from patient’s genomic DNA and sequenced directly using the BigDye terminator method (PE Applied Biosystems), and reactions were analyzed on an ABI-310 genetic analyzer (PE Applied Biosystems).

**SSCP Analysis of SCN5A and Mutation Detection**

The entire coding region of the cardiac sodium channel gene SCN5A was amplified by PCR and analyzed by single-stranded conformation polymorphism (SSCP) analysis and DNA sequencing as described previously.12 The D1275N mutation was confirmed by TaqI digest of exon 21 PCR product generated using primers as described.13

**Isolation of Upstream Sequences for the Human Cx40 Gene and Cx40 Polymorphism Genotyping**

Details of the isolation of the proximal Cx40 promoter, exon 1, and intron sequence downstream of exon 1 can be found in the online data supplement available at http://www.circrehsa.org. The sequence has been deposited in GenBank under accession No. AP246295.

Polymorphisms in the Cx40 upstream sequences of relatives and controls were genotyped by direct sequencing of PCR products generated with forward primer 5'-TGAGGACAAGGAGCACACAGGCAAG-3' and reverse primer 5'-CTCTTCTCTGGCCTACTTTCATATC-3'.

**Generation of D1275N Construct and cRNA Synthesis**

Details of the generation of D1275N construct and cRNA synthesis can be found in the online data supplement.

**Electrophysiological Characterization of SCN5A Mutant D1275N**

*Xenopus laevis* oocytes were isolated and injected with 2.5 to 10 ng wild-type or D1275N mutant cRNA, and two-electrode voltage-clamp measurements were performed 3 to 4 days after the day of injection.12 For expression in the presence of the β1-subunit of the sodium channel, β1, a 10-fold excess cRNA was co-injected with each α-subunit. Steady-state parameters for activation, fast inactivation, and recovery from inactivation were determined as previously described12 using 3 mol/L KCl in the microelectrodes. To ensure reliable voltage-clamp measurements, only oocytes displaying I<sub>max</sub> < 10 μA were analyzed. The pulse protocol to measure the slow component of inactivation was adapted from Vilim et al10 using a holding potential of −100 mV and an inactivating prepulse of 10 seconds. The rate of current decay after stepping to a test potential of −30 mV from V<sub>h</sub> = −100 mV was assessed by biexponential fits. All measurements were performed at room temperature, and the data were analyzed by Student’s t test for unpaired data except for the recovery from inactivation (Wilcoxon rank test for unpaired data).

**Functional Analysis of Cx40 Polymorphisms**

Details of construction of luciferase reporter gene constructs p(−44G,+71A) and p(−44A,+71G) and their transfection into A7r5 cells can be found in the online data supplement.

**Analysis of ECGs**

PR, QRS, and QTc intervals were determined from ECGs obtained from the patients and their relatives. ECGs were recorded in the supine position, and no medications were used. The data were statistically analyzed by Student’s t test for unpaired data.

**Results**

**Clinical Profile of the AS Family**

The proband (III-16, Figure 1), her father (II-10), and two of the father’s cousins (II-2 and II-4) were affected by AS. Their clinical histories were very similar (Table). In general, they first presented with symptoms of dizziness and syncope in their early twenties. By 30 to 40 years of age, symptoms had worsened such that more extensive clinical examinations were warranted. Surface ECGs showed a slow atrioventricular (AV) junctional rhythm in the absence of P waves in all affected relatives (Figure 2). On invasive EPS, the atria could not or could only partially be stimulated, and His-ventricular time was slightly prolonged. Initially, the proband had partial AS, which later developed into total AS with a reduced exercise tolerance. At ∼40 years of age, the proband as well as her father’s cousins, II-2 and II-4, required ventricular pacemaker implantation. All subjects were doing well at the start of our study. The proband’s father, although diagnosed with AS but not treated with either drugs or a pacemaker, died before this study at age 65 of cancer and multiple cerebrovascular accidents. ECGs were recorded in a further 40 members of this family, and no other individuals with AS were found nor did any other relatives indicate possible symptoms of AS. Individuals II-6, III-6, III-8, III-10, and IV-2 (all unaffected D1275N carriers, see below) were 68, 42, 43, 40, and 14 years old, respectively, at the time their ECGs were evaluated. The heart rate range from 24-hour Holter recordings in both unaffected D1275N carriers and rare Cx40 genotype carriers (II-8, III-1, III-2, III-15, IV-1, see below) did not differ from normal. With the exception of individual II-6 in the former group, who had atrial fibrillation/flutter with onset at age 68, two years after a myocardial infarction, no significant arrhythmias were observed.

**Genetic Analyses**

Candidate genes were selected either on the basis of their relevance for action potential generation and conduction or because of their atrial-specific expression pattern. Involvement of a number of candidate genes was excluded either because haplotype analysis showed no cosegregation of the
relevant chromosomal region with the disease (L-type calcium channel CACLN1A1 on chromosome 12, locus 19q13 previously linked to conduction disease,\textsuperscript{15} and the atrial-specific genes ANF on chromosome 1 and \(\alpha\)-myosin heavy chain \(\alpha\)-MHC on chromosome 14) or because no mutations were detected by direct sequencing of the proband’s DNA (Cx43, GenBank accession No. X52947; Cx45, GenBank accession No. AC005180; and MinK, GenBank accession No. M26685).

Haplotype analysis for SCN5A revealed a haplotype shared by all living affected relatives (\(n=3\)) as well as some unaffected relatives (\(n=5\), not shown). A multipoint lod score (10 lod for linkage) of 1.59 was calculated, which is approximately equivalent to \(P=0.03\) after correction for the fact that 9 candidate genes were tested for linkage. Heterozygosity for a G→A substitution in the first nucleotide of codon 1275 was found (Figure 3A), resulting in the substitution of Asp by Asn (D1275N). This mutation was not present in 360 chromosomes from unrelated persons. DNA from all relatives was tested for the presence of the D1275N mutation by TaqI digest (a TaqI site is abolished). Figure 3B demonstrates the presence of the D1275N mutation in all carriers of the shared haplotype by the absence of a TaqI restriction enzyme site in one of the two alleles in the relatives who inherited the mutation (also indicated in Figure 1).

Direct sequencing of the coding region of the gene for the atrial-specific gap junction protein Cx40 (GenBank accession No. AF151979) in the proband’s DNA revealed no anomalies. However, screening of the 5′ untranslated exon and the proximal promoter region of the Cx40 gene revealed two

### Clinical Characteristics of Family Members Affected by AS

<table>
<thead>
<tr>
<th>Age at First Symptoms or Doctor’s Visit, yr</th>
<th>Atrial Potentials</th>
<th>Atrial Stimulation Threshold, mA</th>
<th>His-Ventricular Time, ms</th>
<th>QRS</th>
<th>Arrhythmias</th>
<th>Echocardiography</th>
</tr>
</thead>
<tbody>
<tr>
<td>III-16 (proband)</td>
<td>22</td>
<td>Low; later absent</td>
<td>Right: &gt;-10</td>
<td>60</td>
<td>Normal axis, 100 ms</td>
<td>Before total AS: atrial tachycardia and bradycardia; ventricular tachycardia</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Mitral valve prolapse of posterior leaflet; LA=42 mm</td>
</tr>
<tr>
<td>II-10</td>
<td>22</td>
<td>Mostly absent</td>
<td>Right: 1.5; left: no capture</td>
<td>60</td>
<td>Left axis, 100 ms</td>
<td>History of atrial arrhythmias and overdrive suppression</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>LA enlargement; LA=53 mm</td>
</tr>
<tr>
<td>II-2</td>
<td>33</td>
<td>Absent</td>
<td>No capture</td>
<td>NA</td>
<td>Normal axis, intermittent RBBB</td>
<td>Later sinus arrest, syncope, and atrial asystole</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>NA</td>
</tr>
<tr>
<td>II-4</td>
<td>Late teens</td>
<td>Absent</td>
<td>No capture</td>
<td>NA</td>
<td>Right axis, 100 ms</td>
<td></td>
</tr>
</tbody>
</table>

RBBB indicates right bundle-branch block; LA, left atrium; and NA, not available.
changes. One was a homozygous G→A change (Figure 4, left) at 44 nucleotides upstream of the transcription start site (TSS), and the other was a homozygous A→G change (Figure 4, right) in exon 1 at 71 nucleotides downstream of the TSS. Affected relatives II-2 and II-4 also had the same genotype as the proband at positions −44 and +71 (Figure 1). Genotyping of all other relatives revealed that homozygosity for these base changes did not occur exclusively in the affected relatives but also in 5 unaffected relatives (Figure 1). In DNA from 60 unrelated individuals, the frequency of the genotypes for each polymorphism was as follows: −44 bp: GG 34, AG 22, and AA 4; +71 bp: AA 32, AG 24, and GG

![Figure 2](https://i.imgur.com/2.png)

**Figure 2.** Selected ECGs from AS family. The ECGs of the proband (III-16), her father (II-10), and his cousin (II-4) show absence of atrial activity and narrow QRS complex escape rhythm (III-16 and II-10) or ventricular escape rhythm (II-4). The narrow QRS complex escape rhythm presumably originates from the AV node or upper specialized conduction system. For comparison, a normal sinus rhythm ECG of the sister of the proband (III-15) is shown.

![Figure 3](https://i.imgur.com/3.png)

**Figure 3.** Identification of SCN5A mutation D1275N. A, Sequence electropherograms around SCN5A amino acid residue 1275 in control and proband. The arrow indicates the homozygous G nucleotide in the control or the heterozygous A/G nucleotides in the proband. B, TaqI restriction digest of individuals with the shared SCN5A haplotype. The lower band indicates wild-type chromosome; upper band, presence of D1275N mutation. Part of the pedigree from Figure 1 is shown. For explanation of pedigree symbols, see Figure 1. Lane M indicates marker (specific sizes for two marker bands are given). Individual III-7 was included as a control to confirm complete TaqI digestion of the PCR products.
The functional consequences of the D1275N mutation were studied by voltage-clamp measurements in the absence and presence of hβ1. Figure 5A illustrates the inward sodium current obtained for both wild-type and mutant channels. Peak current amplitude did not differ between wild-type and mutant channels (e.g., $I_{\text{max}}$ wild-type $5.95 \pm 0.83 \mu A$ [$n=13$] and $I_{\text{max}}$ D1275N $5.02 \pm 0.82 \mu A$ [$n=9$] for 2.5 ng cRNA injected). In the absence of hβ1, a depolarizing shift (+3.8 mV, $P<0.05$) in the voltage dependence of activation was found (Figure 5B, left panel), which did not occur in the presence of hβ1 (Figure 5B, right panel). No differences in both fast and slow inactivation (Figures 5B and 5C) parameters were found either in the absence or presence of hβ1. In addition, there was no difference in time constants of current decay between wild-type and mutant channels in the absence or presence of hβ1 (see Table in the online data supplement). Paired pulse analysis showed that mutant channels recovered slightly faster (1.28-fold, $P<0.01$) from inactivation than wild-type channels (Figure 5D, left panel). In the presence of hβ1, mutant channels also tended to recover faster than wild-type channels albeit not significantly due to the large spread in the data (Figure 5D, right panel).

The tendency of the D1275N mutation to shift the voltage dependence of activation to more depolarized voltages could lead to decreased excitability in myocardial cells.

**Functional Analysis of Cx40 Gene Polymorphisms**

The effect of Cx40 polymorphisms in the proximal promoter region/untranslated exon 1 of the gene on luciferase gene expression was determined in the Cx40-expressing cell line A7r5. Whereas the activity of the rat promoter plasmid p(−175,+85) was comparable to that of the common haplotype plasmid p(−44G,+71A), a 65% reduction in luciferase activity of the plasmid with the rare haplotype p(−44A,+71G) compared with that of the more common haplotype p(−44G,+71A) was found (Figure 6). These results show that the combined effect of the rare Cx40 genotypes cause a reduction in Cx40 gene expression in vitro.

**Genotype-Phenotype Analysis**

The rare Cx40 genotypes for both polymorphisms (i.e., AA at −44 bp and GG at +71 bp) always occurred together in the same individuals (see also Figure 1). Thus, with respect to the Cx40 polymorphisms, the family could be divided in two groups: one with both rare Cx40 genotypes (−44 bp/+71 bp AAGG, abbreviated as “rare Cx40 genotype”) and one with neither (−44 bp/+71 bp GGAA or AGAG). To determine whether relatives with the D1275N mutation with or without the rare Cx40 genotype showed ECG alterations compared with relatives with neither the D1275N mutation nor the rare Cx40 genotype, PR, QRS, and QTc intervals were determined from all available ECG recordings in this family. Figure 7 illustrates each individual’s PR interval value. The PR interval could not be determined in two Cx40 D1275N genotyped AS patients inasmuch as their first ECG showed total AS nor in one D1275N carrier without the rare Cx40 genotype (II-6) due to atrial arrhythmia. Although there is some overlap in the data, the PR interval was moderately prolonged ($P<0.05$) in D1275N carriers compared with noncarriers, in the absence of hβ1.

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of the rare Cx40 genotype. However, in non-D1275N carriers, the PR interval in rare Cx40 genotype carriers did not differ from those without the rare Cx40 genotype (Figure 7). QRS duration did not differ between any of the groups. There was a small increase in the QTc interval within the normal range (393 ms versus 424 ms, $P<0.05$) between noncarriers and D1275N mutation carriers, in the absence of the rare Cx40 genotype (not shown).

Figure 5. A. Macroscopic current traces generated by wild-type or D1275N channels in the absence or presence of h$j1$ as indicated. Sodium currents were stimulated by test potentials from $-70$ to $+40$ mV in 5-mV increments from a holding potential of $-100$ mV. B. Comparison of the voltage dependence of activation and inactivation of wild-type (WT) and D1275N sodium channels in the absence (left) and presence (right) of h$j1$. Data were fitted with a Boltzmann equation to estimate the potential for half-maximal activation or inactivation ($V_{1/2}$) and slope factor ($k$) (see Table in the online data supplement). $V_{1/2}$ activation for D1275N channels in the absence of h$j1$ was shifted $+3.8$ mV compared with wild-type channels ($P<0.05$). No other differences were found. C. Slow component of inactivation. No difference was found between wild-type and D1275N channels either in the absence (left) or presence (right) of h$j1$. Data for $V_{1/2}$, slope factor, and steady-state level of slow inactivation can be found in the online Table, which is available in the data supplement. D. Recovery from inactivation, measured by stepping from $V_h$ $-100$ mV to a test potential of $-30$ mV, in the absence (left) and presence (right) of h$j1$. Tau WT: 32.1 $\pm$ 1.65 ms and D1275N: 25.1 $\pm$ 1.95 ms ($P<0.01$); tau WT+h$j1$: 20.12 $\pm$ 3.19 ms and D1275N+h$j1$: 13.95 $\pm$ 0.74 ms.
The genetic basis for familial AS cannot easily be unraveled because of the extremely low prevalence of the disease. This Dutch family, with four affected relatives, is one of the largest reported to date. We used a candidate gene approach to test the involvement of specific genes in AS. On the basis of haplotype analysis, genes encoding proteins involved in action potential configuration (L-type calcium channel, MinK), cardiac conduction (locus 19q13, Cx43, Cx45), or genes with an atrial-specific expression pattern (ANF, α-MHC) could be excluded. This left the genes for the cardiac sodium channel SCN5A and the atrial-specific gap junction protein Cx40 to be further analyzed.

Cardiac Sodium Channel Gene Mutation in AS

Haplotype analysis indicated that the AS patients, as well as five other unaffected relatives, shared a haplotype for SCN5A. The lod score in support of linkage was 1.59, which hinted at a role for the cardiac sodium channel in AS. The SCN5A-D1275N mutation detected in this family is a novel mutation, and no other mutations in the transmembrane segment S3 of domain III (DIIIS3) have been described. Closely occurring mutations are associated with Brugada syndrome, LQTS-3, and progressive conduction disease.16–18

Mutation D1275N results in the loss of one of two negatively charged residues in DIIIS3, near the cytoplasmic end of S3. The aspartate residue 1275 in SCN5A is highly conserved in voltage-gated sodium channels,19 as well as in potassium and calcium channels, and is thought to be part of the voltage-sensing structure of these ion channels. Mutation of the equivalent aspartate residue in mouse brain K.1.1 abolishes channel activation.20 Compared with wild-type channels, D1275N-SCN5A mutant channels changed by a shift of the activation curve to more positive voltages, potentially resulting in reduced excitability of D1275N-SCN5A–expressing myocytes. Although this effect is apparently lost in the presence of hβ1 in our experiments (using a 1:10 ratio of αβ), the precise magnitude of the effect of the D1275N mutation in vivo is uncertain, as the exact stoichiometry of the α and β sodium channel subunits in the heart is disputable.18 However, because a moderate prolongation of the PR interval (Figure 7) was found in D1275N carriers compared with noncarriers, this suggests that the depolarizing shift in the activation V1/2 is relevant in vivo. The prolonged PR interval indicates slowing of conduction anywhere between sinus node and ventricle (ie, atrium, AV node, specialized ventricular conduction system). Because QRS duration did not differ, an effect on ventricular conduction itself is unlikely.

Mutations in the sodium channel gene have been linked to conduction disease,21 as well as to the long-QT syndrome22 and Brugada syndrome23 (for a review, see Reference 18), all associated with (discrete) conduction defects. Although these syndromes appear to affect the ventricle primarily, AS has occasionally been noted in patients with SCN5A mutations.21,24,25 These conduction abnormalities in the atrium may underlie the increased prevalence of supraventricular arrhythmias in Brugada syndrome.26 However, the D1275N
mutation is the first SCN5A mutation associated with an atrial-specific arrhythmia.

### Cx40 Polymorphisms

Although the mode of transmission of AS is unknown, some previous reports of familial AS could be suggestive of a recessive mode of transmission, where consanguinity may have played a role. Within the family presented (Figure 1), there was no evidence of consanguinity. Most of the relatives have, however, stayed in the same, although not a geographically isolated, region. Whether this has contributed to an increased frequency of the rare Cx40 genotype is unknown.

The rare genotypes for two Cx40 polymorphisms detected in this family are located in the immediate upstream promoter region (−44 bp) and untranslated region (+71 bp) of the Cx40 gene. The homologous positions of these polymorphisms in the rat Cx40 gene fall within its basic promoter region. Functional analysis of the rare Cx40 haplotype (−44 bp A; +71 bp G) showed a more than 50% reduction in luciferase gene expression compared with the more common Cx40 haplotype (−44 bp G; +71 bp A; Figure 6). Preliminary data with the same luciferase reporter constructs in rat neonatal myocytes suggest a similar reduction of luciferase expression also in this cell type. This suggests a reduction in Cx40 gene expression in rare Cx40 genotype carriers. Whether the amount of Cx40 protein is reduced in vivo depends, among other factors, on its half-life. Although unknown for Cx40, relatively short half-lives of 2 to 4 hours have been reported for other connexins. Assuming a short half-life, the reduced Cx40 expression could result in a reduction of the total amount of Cx40 protein in vivo. Despite these effects, conduction parameters were not different between carriers of the rare Cx40 genotype alone and noncarriers. In addition, in heterozygous Cx40 knockout mice, where the absence of one Cx40 allele is expected to result in only 50% of the Cx40 protein of the wild-type animal, no changes in cardiac conduction velocities or any other cardiac electrophysiological parameters were reported. This is consistent with the lack of effect of the rare Cx40 genotype on ECG parameters.

### Coinheritance of SCN5A Mutation and Cx40 Polymorphisms as Substrate for AS

AS was observed exclusively in individuals in which both the D1275N mutation and the rare Cx40 genotype were inherited. By themselves, the effect of each genetic variation is neither sufficient to explain the phenotype of total inexcitability of AS, nor does it appear pathogenic. Although the Na+ channel mutation D1275N does have functional consequences as shown by both the in vitro channel gating changes and the in vivo prolongation of the PR interval, no evidence of AS or other arrhythmias was found in D1275N carriers who did not have the rare Cx40 genotype. All unaffected D1275N carriers (excluding IV-2 due to his young age) were symptom-free at the age at which the AS patients became affected and long thereafter. Similarly, carriers of the rare Cx40 genotype had no arrhythmias despite the fact that these polymorphisms proved relevant for Cx40 expression. Because Cx40 expression in the heart is confined to the atrium and the ventricular conduction system and because cardiac conduction results from normal functioning of sodium channels and connexins, we propose that the combined effect of relatively benign functional changes of these proteins leads to an additive and progressive pathological response, specific for the atrium and the specialized ventricular conduction system (note the increased His-ventricular time in the two affected patients in whom it was measured).

We hypothesize that AS is a primary SCN5A-related disorder, which displays complete penetrance in a Cx40 background that occurs in 7% in the population. In both LQT-3 and Brugada syndrome, SCN5A mutations tend to have low penetrance.

The likelihood of chance concurrence of the rare Cx40 genotypes and a sodium channel mutation is low. This could explain the extremely rare prevalence of this arrhythmia and the absence of families with large numbers of affected persons. For confirmation of our proposed mechanism, an analysis would be required of several additional families with the same condition. Because of the small number of known families, such studies are at present not feasible. However, both functional changes, ie, the reduced excitability and reduced electrical coupling, are in support of our hypothesis that together they are the initiating factors of progressive AS in this family.

### Acknowledgments

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### References


A Cardiac Sodium Channel Mutation Cosegregates With a Rare Connexin40 Genotype in Familial Atrial Standstill


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