A novel KCND3 gain-of-function mutation associated with early-onset of persistent lone atrial fibrillation

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Aims
Atrial fibrillation (AF) is the most common cardiac arrhythmia, and early-onset lone AF has been linked to mutations in genes encoding ion channels. Mutations in the pore forming subunit KV4.3 leading to an increase in the transient outward potassium current ($I_{to}$) have previously been associated with the Brugada Syndrome. Here we aim to determine if mutations in KV4.3 or in the auxiliary subunit K$^+$/Channel-Interacting Protein (KChIP) 2 are associated with early-onset lone AF.

Methods and results
Two hundred and nine unrelated early-onset lone AF patients (<40 years) were recruited. The entire coding sequence of KCND3 and KCNIP2 was bidirectionally sequenced. One novel non-synonymous mutation A545P was found in KCND3 and was neither present in the control group (n = 432 alleles) nor in any publicly available database. The proband had onset of persistent AF at the age of 22, and no mutations in genes previously associated with AF were found. Electrophysiological analysis of KV4.3-A545P expressed in CHO-K1 cells, revealed that peak-current density was increased and the onset of inactivation was slower compared with WT, resulting in a significant gain-of-function both in the absence and the presence of KChIP2.

Conclusion
Gain-of-function mutations in KV4.3 have previously been described in Brugada Syndrome, however, this is the first report of a KV4.3 gain-of-function mutation in early-onset lone AF. This association of KV4.3 gain-of-function and early-onset lone AF further supports the hypothesis that increased potassium current enhances AF susceptibility.

Keywords
Atrial fibrillation • KCND3 • KCNIP2 • KV4.3 • Transient outward potassium current • Brugada syndrome

1. Introduction
Atrial fibrillation (AF) is the most prevalent sustained cardiac arrhythmia, affecting almost seven million patients in the European Union and the USA.1–4 The estimated life-time risk of AF is almost 25% and it is the cause of significant morbidity and mortality.5,6 The pathogenesis and underlying mechanisms of AF have been the subjects of intense research.7 In most cases, AF arises relatively late in life and is secondary to hypertension, ischaemic, and/or structural heart disease.1,8 These disorders influence the electrical and structural remodelling of the atria and are thought to be central in the pathogenesis of AF.7 However, in 10–20% of AF cases, no association with underlying cardiovascular and systemic disorders is present and this is defined as lone AF.3

Fox et al.9 provided evidence for a familial predisposition for AF showing that development of AF is associated with parental AF. Further support comes from Oyen et al.10 who reported that an individual’s risk of developing lone AF at a young age increases drastically with both increasing number of relatives with lone AF, and decreasing age at onset of the disease in these relatives, indicating an underlying genetic component in early-onset lone AF. The importance of common genetic variants in the development of AF was demonstrated in recent genome-wide association studies.11 In line with this, rare mutations in genes encoding potassium channels (KCQ1, KCNH2,
2. Methods

2.1 Study subjects

Patients with lone AF (i.e., without clinical or echocardiographic findings of any known risk factors of AF such as other cardiovascular diseases, hypertension, metabolic or pulmonary diseases) and onset of AF before the age of 40 were included from eight hospitals in the Copenhagen region of Denmark.24 The control population consisted of healthy blood donors. Written informed consent was obtained from all included participants. The study was conducted in agreement with the principles outlined in the Declaration of Helsinki and approved by the Scientific Ethics Committee of Copenhagen and Frederiksberg (Protocol reference number KF 0131322).

2.2 Mutation screening

Genomic DNA was extracted from blood samples using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). Oligonucleotide primers for exons and splice junctions were designed using the known sequence of human KCND3 (NM_172198) and KCNIP2 (NM_173192) primers were designed with M13 tail sequences. DNA fragments amplified by Touchdown PCR were analysed using a high-resolution melting curve primer extension (PfuUltraII Polymerase, Stratagene, St Leon-Roth, Germany), and transformed into E. coli Blue cells. All constructs were verified by complete DNA sequencing of the cDNA insert (Macrogen, Inc., Republic of Korea).

2.3 Molecular biology

Plasmids containing cDNAs encoding human K\textsubscript{V}4.3 (NM_004980.4) and KChIP2.1 (NM_173192) for expression in mammalian cells have been described previously.26 It should be noted that we used K\textsubscript{V}4.3 isoform 2 in our experiments that lacks 19 amino acids in its C-terminus compared with isoform 1. Hence, the mutation is denoted A545P in our study. Radicke et al.27 have shown similar mRNA levels for both isoforms in all regions of non-failing hearts and Xie et al.28 reported that the two isoforms are kinetically similar. Site-directed mutagenesis introducing the mutation c.1690G>C, nomenclature based on isoform 1, (NM_004980.4) into K\textsubscript{V}4.3 cDNA was performed by mutated oligonucleotide extension (PfuUltrall Polymerase, Stratagene, La Jolla, CA, USA) from a plasmid template harbouring K\textsubscript{V}4.3 wild-type cDNA, digested with DpnI (Fermentas, St Leon-Roth, Germany), and transformed into E. coli XL1 Blue cells. All constructs were verified by complete DNA sequencing of the cDNA insert (Macrogen, Inc., Republic of Korea).

Table 1: Clinical characteristics of the lone AF population (n = 209)

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median age of onset, years (IQR)</td>
<td>31.5 (26–36)</td>
</tr>
<tr>
<td>Male gender, %</td>
<td>82</td>
</tr>
<tr>
<td>Height, cm</td>
<td>183 ± 9</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>89 ± 17</td>
</tr>
<tr>
<td>BMI, kg/m\textsuperscript{2}</td>
<td>26.7 ± 4.6</td>
</tr>
<tr>
<td>Blood pressure, mmHg</td>
<td></td>
</tr>
<tr>
<td>Systolic</td>
<td>131 ± 13</td>
</tr>
<tr>
<td>Diastolic</td>
<td>78 ± 9</td>
</tr>
<tr>
<td>AF type</td>
<td></td>
</tr>
<tr>
<td>Paroxysmal, %</td>
<td>55.9</td>
</tr>
<tr>
<td>Persistent, %</td>
<td>35.9</td>
</tr>
<tr>
<td>Permanent, %</td>
<td>8.2</td>
</tr>
<tr>
<td>Family history of AF</td>
<td></td>
</tr>
<tr>
<td>First-degree relatives with AF, %</td>
<td>31</td>
</tr>
</tbody>
</table>

All numbers are reported as mean ± standard deviation unless otherwise noted. IQR, interquartile range.
Statistical analysis was performed using an ANOVA test followed by a Student–Newman–Keuls test or Student’s t-test, as appropriate, using SigmaStat software.

2.6 Computer simulation

Computer simulations were performed using the Courtemanche et al. atrial cell model (CRN). The model code was obtained from the CellML repository (http://www.cellml.org/). All simulations were performed in Matlab (MathWorks, Natick, MA, USA) using the ode15s solver. To simulate the effects of the A545P mutation, the maximum conductance ($g_{to}$) and the time constant of inactivation ($t_{oi}$) for the $I_{to}$ component of the CRN model was modified as follows: $g_{to}$ was increased by a factor of 1.75 and $t_{oi}$ was increased by a factor of 1.15. These factors were based on the whole-cell voltage-clamp measurements of WT + KChIP2 and A545P + KChIP2 channels. The simulations were performed at basic cycle lengths (BCLs) of 500, 400, 300, and 250 ms. At each BCL, the action potential duration at 90% repolarization (APD$_{90}$) was calculated for the 20th beat.

**Figure 1** (A) DNA sequence analysis of proband. (B) ECG from proband after DC conversion, paper speed 25 mm/s, 1 mV/mm. (C) Pedigree of the family with the novel KCND3 A545P mutation. Squares: male, circles: female family members, respectively. Arrow indicates the proband. Solid black symbols indicate the presence of AF, open symbols: unaffected members, (+/−): presence of the heterozygous mutation for persons with DNA samples available for testing.
3. Results

3.1 Mutation screening

Direct DNA sequencing of KCND3 and KCNIP2 in 209 index patients (Table 1) revealed two non-synonymous mutations in KCND3. The variant KCND3 c.456A>G resulting in a substitution of threonine to alanine, T486A, was present in two patients, but has previously been described in control populations19 and was also present with a minor allele frequency of 0.11% in the European American population in the NHLBI GO Exome Sequencing Project [Exome Variant Server (EVS), NHLBI GO Exome Sequencing Project (ESP), Seattle, WA, USA (http://evs.gs.washington.edu/EVS/, 20 September 2012 accessed)].

The mutation KCND3 c.1633G>C resulting in an alanine to proline substitution, A545P (isoform 1) or A545P (isoform 2), respectively (Figure 1A), was present in one patient who was heterozygous for the mutation. In the following, the mutation is labelled A545P. A545P was not present in our control population (n=216), and has not previously been reported in the NCBI dbSNP database or in the NHLBI GO Exome Sequencing Project (n=6502). The amino-acid residue resides in the C-terminus of the channel protein and is highly conserved across species suggesting a functional importance. No non-synonymous mutations were found in KCNIP2.

3.2 Clinical data

The proband carrying the A545P mutation had onset of persistent AF at the age of 22. His ECG showed a QTc interval of 382 ms and was without indications of BrS (Figure 1B). The ECG also exhibited high narrow T-waves, which could indicate repolarization abnormalities but could also just be a normal variation as tall and narrow T-waves are occasionally seen in young men. There were no symptoms suggesting ventricular arrhythmias, such as palpitations, dizziness, syncope, or shortness of breath. The echocardiography was without signs of structural changes or cardiomyopathy (left-atrial diameter 3.86 cm). To rule out BrS, we performed a flecainide test. The test was negative with no signs of BrS type 1, 2, or 3 patterns. There was no family history of either sudden cardiac death or AF. It has not been possible to include family members in the study for genetic testing (Figure 1C). The patient’s AF was successfully treated by DC conversion.

3.3 A545P causes a gain-of-function of Kv4.3

The functional effects of A545P on Kv4.3 were investigated by transiently expressing WT or A545P in CHO-K1 cells and whole-cell currents were recorded under voltage-clamp.

Representative recordings from WT and A545P are shown in Figure 2A. A545P had an increased peak-current density compared with WT as demonstrated on the current–voltage relationship (Figure 2B). Furthermore, A545P had significantly higher time-constants of decay (τ) indicating that the inactivation was slowed by the mutation (Figure 2C).

Steady-steady inactivation was addressed by a two-pulse protocol with increasing interpulse intervals. For WT, τ1/2 was 244.43 ± 1.47 mV with slope factor of −5.89 ± 0.14 (n=8). For A545P, τ1/2 was 244.54 ± 2.19 mV with a slope factor of −7.08 ± 0.41 (n=8), which were not significantly different (Figure 3A and B). Recovery from inactivation was addressed by a two-pulse protocol with increasing interpulse intervals. For WT, τ = 74.10 ± 7.76 ms.
**Figure 3** Steady-state inactivation and recovery from inactivation for Kv4.3 WT and A545P. (A) Representative currents using the steady-state inactivation protocol. (B) Peak currents at the +20 mV step were normalized and plotted as a function of the voltage of the preconditioning step. A Boltzmann sigmoidal function was fitted to the data points. (C) Recovery was addressed using a two-pulse protocol. Representative recordings are shown. (D) Peak currents were normalized to the first pulse and plotted as a function of interval time. An exponential equation was fitted to the data.

**Figure 4** Current–voltage relationship for Kv4.3 WT and A545P co-expressed with KChIP2. (A) Representative recordings. (B) Current–voltage relationship. (C) A mono-exponential equation was fitted to the 100 first milliseconds of the decay phase to obtain time constants (τ).
n = 8) and for A545P, $\tau = 65.78 \pm 9.09$ ms (n = 10), which were not significantly different (Figure 3C and D).

In the heart, KV4.3 co-assembles with KChIP2 to constitute $I_{to}$. This prompted us to characterize A545P in the presence of KChIP2. In the presence of KChIP2, A545P exhibited a similar gain-of-function compared with WT on peak-current density as well as a slowing of current decay (Figure 4A and B). Steady-state inactivation was addressed, WT + KChIP2 had $V_1 = -40.34 \pm 1.47$ mV, a slope factor of $-3.87 \pm 0.14$, n = 13, whereas A545P + KChIP2 had a $V_2 = -40.62 \pm 0.85$ mV and a slope factor of $-3.99 \pm 0.17$, n = 10 (Figure 5A). For both parameters, A545P + KChIP2 did not differ significantly from WT + KChIP2. Recovery from inactivation for WT + KChIP2 had a $\tau$ of $16.32 \pm 1.86$ ms, n = 11 and A545P + KChIP2 had a $\tau$ of $18.09 \pm 1.98$ ms, n = 12 (Figure 5B) which were not significantly different.

As the A545P mutation affected multiple kinetic parameters, we tested how KV4.3 + KChIP2 currents were affected by different action potential waveforms. Representative recordings are shown in Figure 5C. For currents elicited by atrial waveforms, A545P + KChIP2 had significantly higher peak-current density compared with WT + KChIP2 (Figure 5D). This is in agreement with the findings using the standard step protocol (Figure 4).

Computer simulation of the effect of the A545P mutation (in the presence of KChIP2) demonstrated that, in the model, the mutation results in a shortening of the action potential duration through an increased $I_{to}$ (Figure 6).

4. Discussion

We identified a novel mutation in KCND3 in a young patient with lone AF. The mutation resulted in an amino-acid substitution A545P in KV4.3. The mutation was not found in 216 Danish controls or in 6500 exomes from the EVS. No non-synonymous mutations were found in KCNIP2. Electrophysiological investigations revealed a gain-of-function of A545P compared with WT. In addition, computer simulations suggested that the mutation results in a shortening of the atrial action potential. These findings provide the first evidence associating KV4.3 gain-of-function mutations to the pathogenesis of AF.
AF was not observed in other family members. Yet, common SNPs have been shown to modify the risk of AF, which may explain the absence of AF in family members or in the development of AF in our index patient. In line with this, in a previous study from our group, the patient carrying mutation K\(_{\text{v}}\)4.3 A545P has been genotyped in the three most important AF loci identified in GWAS (Olesen et al.). The patient was homozygous for the risk allele at both the ZFHX3/rs2106261 and KCNN3/rs13376333 loci, indicating that the patient is also predisposed by common variants with relative low effect sizes.

\(I_{\text{to}}\) is expressed in both atrial and ventricular tissue of larger mammals. In both tissues, \(I_{\text{to}}\) determines the phase 1 repolarization and affects calcium handling. During chronic AF, \(I_{\text{to}}\) has repeatedly been reported to be reduced (reviewed in Workman et al.). Besides a reduction in \(I_{\text{to}}\), chronic AF is associated with a marked shortening of the action potential duration, a decrease in effective refractory period, and a hyperpolarization of the resting membrane potential due to an increase in inward rectifying K\(^+\) currents. Blocking of K\(^+\) currents and thereby prolongation of atrial repolarization and refractoriness can terminate AF. In line with this, a modelling study has shown that reduction of \(I_{\text{to}}\) terminates rotor activity, suggesting that reduced \(I_{\text{to}}\) during AF may be a potential protective mechanism. The patients harbouring mutations in K\(_{\text{v}}\)4.3 reported earlier did not have documented AF. However, a mutation in KCNE3 resulting in increased K\(_{\text{v}}\)4.3 currents in a heterologous expression system has been linked to early-onset lone AF, further supporting the hypothesis that increased \(I_{\text{to}}\) can be involved in the pathogenesis of AF. Our computer simulations of the A545P gain-of-function mutation suggest a possible mechanism as we observed a marked reduction in the duration of the action potential. A shorter action potential may in turn decrease the wavelength needed for a re-entrant circuit to form and thereby create a substrate for AF.

In the ventricles, the presence of a prominent \(I_{\text{to}}\) in the mid- and epicardial layers results in a marked phase 1 repolarization and a spike-and-dome morphology of the action potentials, whereas \(I_{\text{to}}\) is small/absent in the endocardium. Pharmacological activation of \(I_{\text{to}}\) using the compound NS5806 results in a marked augmentation of the phase 1 repolarization as well as an increase in APD in the mid- and epicardium. These changes are consistent with BrS and mutations in K\(_{\text{v}}\)4.3 or in the accessory subunits KCNE3 and KCNE5 resulting in increased \(I_{\text{to}}\) have previously been linked to BrS, however, flecainide testing of the patient in this study was negative.

Guidicessi et al. described a patient harbouring a K\(_{\text{v}}\)4.3 G600R (referring to the long K\(_{\text{v}}\)4.3 variant) mutation. The patient had a spontaneous Brugada ECG pattern as well as a prolonged QTc interval. Similar to the G600R mutation associated with BrS and LQT, the A545P mutation results in larger peak-current density and a slowing of current decay. However, the patient in this study had a QTc interval of 382 ms and thus belongs to the 4% of the population with the shortest QTc. The finding of short QTc in our patient is surprising. Based on pharmacological studies using NS5806 in canine ventricular cardiomyocytes, we found that increasing \(I_{\text{to}}\) peak-current density and slowing of \(I_{\text{to}}\) decay prolonged APD in the mid- and epicardial cells which led to a QT prolongation. For the patients carrying KCNE3 or KCNE5 mutations described earlier, no abnormalities of QTc intervals were reported.

Similar to the previously reported K\(_{\text{v}}\)4.3 gain-of-function mutations V392I, L450F, and G600R, A545P is found in the C-terminus of K\(_{\text{v}}\)4.3. Though the main interaction between KChIP2 and K\(_{\text{v}}\)4.3 takes place in the proximal N-terminus of K\(_{\text{v}}\)4.3 parts of the K\(_{\text{v}}\)4.3 C-terminus have been suggested to stabilize the interaction. The increase in peak-current density and slowing of inactivation could suggest an improved interaction with KChIP2. However, the effect of the mutation on peak-current density was independent of the presence of KChIP2. The C-terminus also harbours several sites involved in post-translational modifications by PKA, PKC, PKG, ERK, and Ca\(^{2+}/\) calmodulin-dependent protein kinase II (CaMII) (reviewed in Niwa and Nerbonne). Serine at position 550, in close proximity of A545P mutation on peak-current density and slowing of \(I_{\text{to}}\) decay prolonged APD in the mid- and epicardial cells which led to a QT prolongation. The finding of short QTc in our patient is surprising. Based on pharmacological studies using NS5806 in canine ventricular cardiomyocytes, we found that increasing \(I_{\text{to}}\) peak-current density and slowing of \(I_{\text{to}}\) decay prolonged APD in the mid- and epicardial cells which led to a QT prolongation.
with our previous findings where gain-of-function mutations in genes encoding potassium channel subunits (KCNC1, KCNA5, KCNJ2, KCNE1, and KCNE3) were associated with AF. Our finding thereby supports the hypothesis that shortening of the atrial action potential could be a general mechanism that increases the susceptibility for AF.

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