

Altered atrial cytosolic calcium handling contributes to the development of postoperative atrial fibrillation

Funsho E. Fakuade^{1,2†}, Vanessa Steckmeister^{1,2†}, Fitzwilliam Seibert^{1,2}, Judith Gronwald^{1,2}, Stefanie Kestel^{1,2}, Julia Menzel^{2,3}, Julius Ryan D. Pronto^{1,2}, Karim Taha^{4,5}, Fereshteh Haghighi^{2,6}, George Kensah^{2,6}, Charles M. Pearman⁷, Felix Wiedmann^{8,9}, Arco J. Teske⁴, Constanze Schmidt^{8,9}, Katharine M. Dibb⁷, Ashraf El-Essawi^{2,6,10}, Bernhard C. Danner^{2,6}, Hassina Baraki^{2,6}, Blanche Schwappach^{2,3}, Ingo Kutschka^{2,6}, Fleur E. Mason^{1,2}, and Niels Voigt^{1,2*}

¹Institute of Pharmacology and Toxicology, University Medical Centre Göttingen, Robert-Koch-Straße 40, 37075 Göttingen, Germany; ²DZHK (German Centre for Cardiovascular Research), Partner Site Göttingen, Germany; ³Department of Molecular Biology, University Medical Centre, Humboldtallee 23, 37075 Göttingen, Germany; ⁴Department of Cardiology, University Medical Centre, Heidelberglaan 100, 3508 GA Utrecht, The Netherlands; ⁵Netherlands Heart Institute, Holland Heart House, Moreelsepark 1, 3511 EP Utrecht, The Netherlands; ⁶Department of Thoracic and Cardiovascular Surgery, University Medical Centre Göttingen, Robert-Koch-Straße 40, 37075 Göttingen, Germany; ⁷Unit of Cardiac Physiology, Division of Cardiovascular Sciences, Manchester Academic Health Science Centre, University of Manchester, Manchester, UK; ⁸Department of Cardiology, University Medical Center Heidelberg, Heidelberg, Germany; ⁹DZHK (German Center for Cardiovascular Research), Partner Site Heidelberg/Mannheim, Germany; and ¹⁰Department of Thoracic and Cardiovascular Surgery, Klinikum Braunschweig, Braunschweig, Germany

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Aims

Atrial fibrillation (AF) is a commonly occurring arrhythmia after cardiac surgery (postoperative AF, poAF) and is associated with poorer outcomes. Considering that reduced atrial contractile function is a predictor of poAF and that Ca^{2+} plays an important role in both excitation–contraction coupling and atrial arrhythmogenesis, this study aims to test whether alterations of intracellular Ca^{2+} handling contribute to impaired atrial contractility and to the arrhythmogenic substrate predisposing patients to poAF.

Methods and results

Right atrial appendages were obtained from patients in sinus rhythm undergoing open-heart surgery. Cardiomyocytes were investigated by simultaneous measurement of $[\text{Ca}^{2+}]_i$ and action potentials (APs, patch-clamp). Patients were followed-up for 6 days to identify those with and without poAF. Speckle-tracking analysis of preoperative echocardiography revealed reduced left atrial contraction strain in poAF patients. At the time of surgery, cellular Ca^{2+} transients (CaTs) and the sarcoplasmic reticulum (SR) Ca^{2+} content were smaller in the poAF group. CaT decay was slower in poAF, but the decay of caffeine-induced Ca^{2+} transients was unaltered, suggesting preserved sodium-calcium exchanger function. In agreement, western blots revealed reduced SERCA2a expression in poAF patients but unaltered phospholamban expression/phosphorylation. Computational modelling indicated that reduced SERCA activity promotes occurrence of CaT and AP alternans. Indeed, alternans of CaT and AP occurred more often and at lower stimulation frequencies in atrial myocytes from poAF patients. Resting membrane potential and AP duration were comparable between both groups at various pacing frequencies (0.25–8 Hz).

Conclusions

Biochemical, functional, and modelling data implicate reduced SERCA-mediated Ca^{2+} reuptake into the SR as a major contributor to impaired preoperative atrial contractile function and to the pre-existing arrhythmogenic substrate in patients developing poAF.

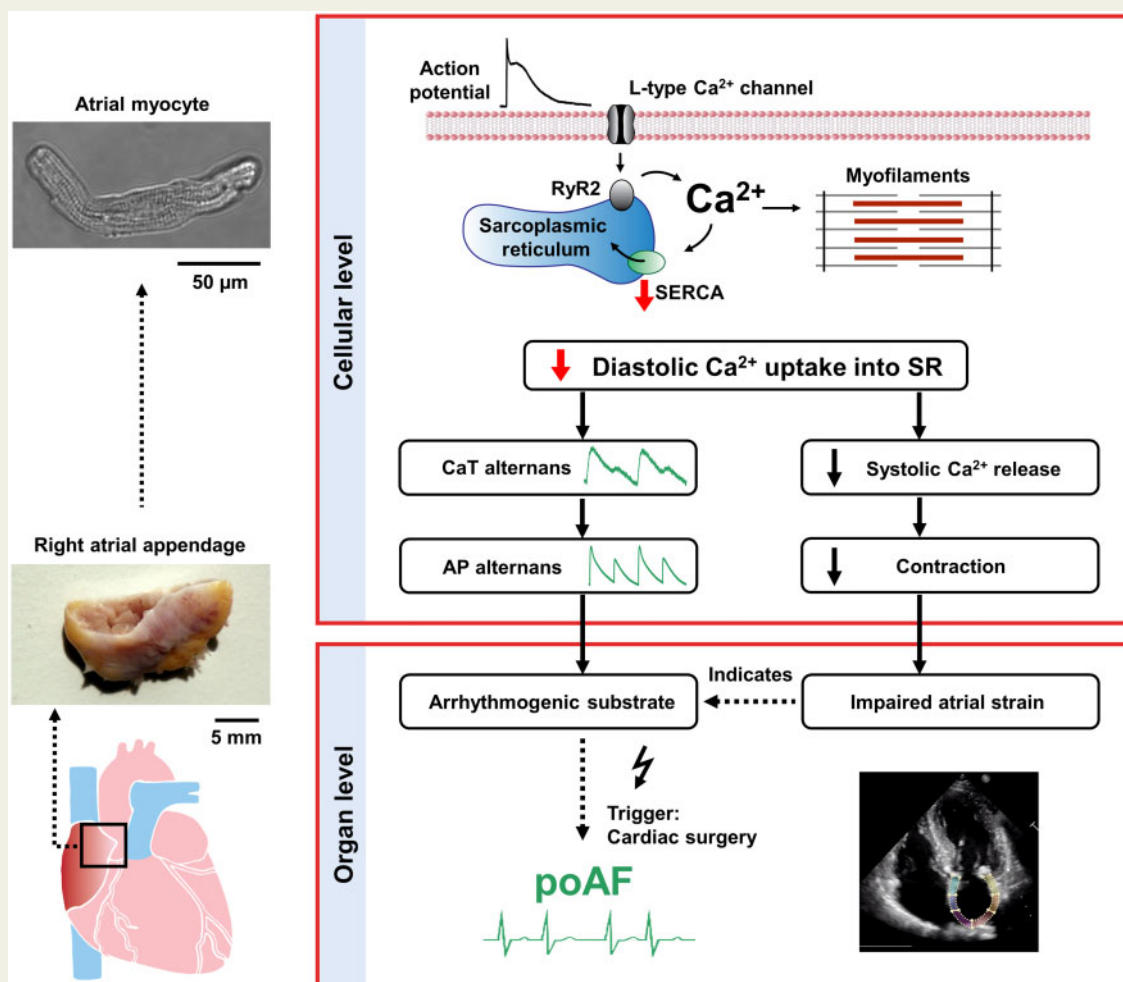
*Corresponding author. Tel: +49 551 3965174; fax: +49 551 3965169, E-mail: niels.voigt@med.uni-goettingen.de

† The first two authors contributed equally to the study.

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Graphical Abstract



Keywords

Postoperative atrial fibrillation • Calcium • Action potential • Alternans • Arrhythmia

1. Introduction

Development of atrial fibrillation (AF) within the immediate postoperative period (poAF) is one of the most frequent complications after cardiac surgery, occurring in up to 50% of cases.¹ In more than 90% of these patients, poAF occurs within the first 6 postoperative days and its occurrence is associated with poorer outcomes, particularly a two- to four-fold increased risk of stroke, bleeding complications, and a two-fold increase in all-cause 30-day mortality. In the USA, the annual healthcare expenditure related to the burden of poAF is estimated at over \$1 billion, highlighting poAF as an important socioeconomic problem.¹

Over the last decades, a great deal has been achieved in understanding pathophysiological mechanisms underlying the initiation, maintenance, and progression of AF in general.¹ Yet the high incidence of AF following cardiac surgery persists and remains poorly understood.¹ Clinical AF is initiated when triggers act on an arrhythmogenic substrate. Cardiac surgery can serve as such a trigger, initiating poAF mediated by adrenergic activation or local inflammation, thereby unmasking the pre-existing pro-

arrhythmic substrate.¹ Several studies indicate that poAF is associated with preoperative structural alterations including interstitial fibrosis,^{2,3} cellular hypertrophy, and signs of cellular degeneration,⁴ which are thought to be important determinants of the pre-existing atrial arrhythmogenic substrate.¹ However, in contrast to patients with paroxysmal and long-standing persistent AF, in whom alterations of cellular electrophysiology, such as shortening of action potential duration (APD), contribute to the maintenance of the arrhythmia,⁵ electrical remodelling does not appear to play a major role in the arrhythmogenic substrate predisposing patients to poAF.^{6–9}

During recent years, cytosolic Ca^{2+} handling abnormalities in atrial myocytes have been suggested to play an important role in initiation and maintenance of AF.^{10,11} However, it is currently unknown whether such abnormalities also contribute to the arrhythmogenic substrate predisposing patients to the development of poAF. Recent studies demonstrate that impaired preoperative left and right atrial contractile function represents an independent predictor of poAF in patients undergoing open-heart surgery.^{12,13} Considering that cytosolic Ca^{2+} handling is a

major determinant of contractile function, we hypothesize that alterations in cellular Ca^{2+} dynamics contribute to impaired atrial contractility in patients who proceed to develop poAF.

Here we use right atrial myocytes isolated from patients undergoing open-heart surgery to test the hypotheses that Ca^{2+} handling abnormalities contribute to contractile dysfunction and to the atrial arrhythmogenic substrate in patients who develop poAF.

2. Methods

A detailed description of all methods is provided in the [Supplementary material online](#).

2.1 Speckle-tracking echocardiography

Two-dimensional greyscale images acquired at 50 Hz over three cardiac cycles from a standard apical four-chamber view were obtained from patients in the period prior to cardiac surgery. Using speckle-tracking software (TOMTEC ARENA™ REF TTA2 LOT 31.00), longitudinal deformation of the left atrium (LA) was monitored by point-tracking of six automatically allocated segments of the LA endocardial borders to determine global atrial strain¹⁴ (Figure 1A and [Supplementary material online](#), Video S1).

2.2 Human tissue samples and myocyte isolation

Right atrial appendages were obtained from 202 patients in normal sinus rhythm undergoing cardiac surgery ([Supplementary material online](#), Tables S1–S6 and Figure S1). During the postoperative period, rhythm was monitored with continuous three-lead electrocardiogram recording for 6 days and stored on a central monitoring system (BeneVision Central Monitoring System, Mindray Medical Germany). The recordings were analysed manually by an experienced clinician, who was blinded to the experimental results. PoAF was detected in 90 patients (45%). Patients were assigned to the poAF group if any episode of AF lasting longer than 30 s was documented. Patients were assigned to the control group (Ctrl) if no episode of AF was observed. Patients with a documented episode of AF at any time before or during cardiac surgery or with AF episodes < 30 s were excluded. Experimental protocols were approved by the ethics committee of the University Medical Center Göttingen (No. 4/11/18) and were performed in accordance with the Declaration of Helsinki. The STROBE checklist used for this study is provided as a [Supplementary material online](#). Each patient gave written informed consent. Excised right atrial appendages were either snap-frozen in liquid nitrogen for biochemical studies or were subjected to a standard protocol^{10,15} for myocyte isolation.

2.3 Intracellular Ca^{2+} measurement and cellular electrophysiology

Only rod-shaped myocytes with clear striations and defined margins were selected for measurements of $[\text{Ca}^{2+}]_i$ and cellular electrophysiology ([Supplementary material online](#), Figures SII and SIII). $[\text{Ca}^{2+}]_i$ of right atrial myocytes was measured using the fluorescent Ca^{2+} indicator fluo-3 according to our previously published protocol.¹⁰ Whole-cell ruptured patch-clamp techniques (voltage- and current-clamp) were used to record membrane currents and action potentials (APs) at 37 °C with simultaneous $[\text{Ca}^{2+}]_i$ measurement.¹⁰ Currents were related to membrane capacitance and expressed in current density (pA/pF).

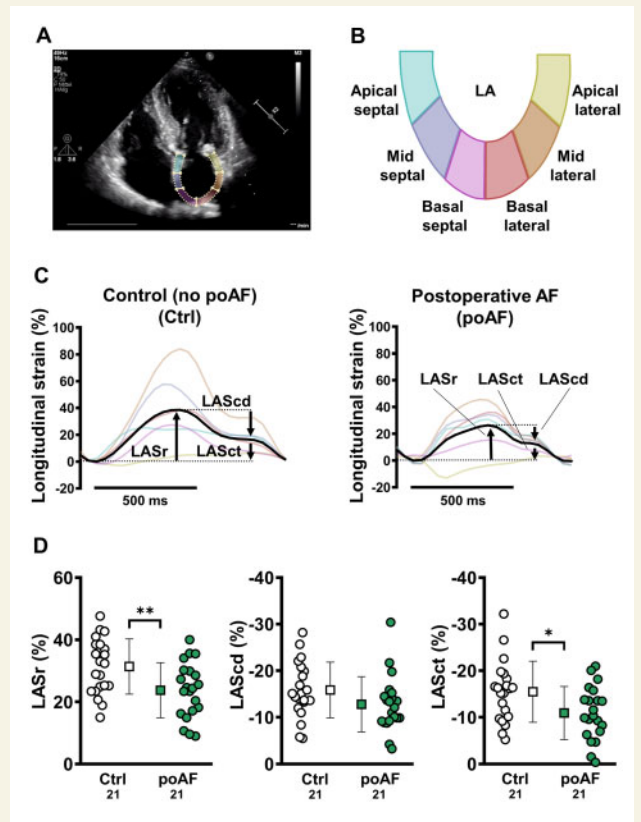


Figure 1 Preoperative speckle-tracking echocardiography to quantify LA strain in patients who do not (Ctrl) and who do develop poAF. (A) Representative apical four-chamber echocardiogram of a poAF patient. The multi-coloured area defines the region of interest along the inner contour of the LA wall. (B) Segment nomenclature of the LA wall. (C) Representative strain curves of Ctrl (left) and poAF (right) patients. Coloured lines show strain of the different segments shown in (B). Global mean strain, denoted by the black line, is used for the measurement of total LASr, LAScd, and LASct as indicated by arrows. (D) Mean \pm standard deviation LASr (left), LAScd (middle), and LASct (right). * $P < 0.05$, ** $P < 0.01$, vs. Ctrl. n = patients. Comparison using the unpaired Student's t -test. Ctrl, control; LA, left atrial; LAScd, left atrial conduit strain; LASct, left atrial contraction strain; LASr, left atrial reservoir strain; poAF, postoperative atrial fibrillation.

2.4 AP and Ca^{2+} transient alternans

Alternans of APs and Ca^{2+} transients (CaTs) occurring at different stimulation frequencies were quantified with a discrete Fourier transform spectral method using modified custom-written software, as previously described.¹⁶

2.5 Biochemical studies

Membranes were isolated from frozen tissue by differential centrifugation and solubilized at 1 mg/mL of total protein in solubilization buffer. Expression of key Ca^{2+} handling proteins was determined using immunoblotting techniques and normalized to total protein load as indicated by REVERT™ total protein staining (LI-COR Biotechnology, USA).

The messenger RNA (mRNA) levels of key Ca^{2+} handling proteins were measured by real-time reverse transcriptase-polymerase chain reaction.

2.6 Computational modelling

APs and corresponding CaTs were simulated based on the mathematical model described by Courtemanche *et al.*¹⁷. The sarcoplasmic reticulum (SR) Ca^{2+} uptake compartment parameter was adjusted by +20%, -20%, and -40% to simulate altered SR Ca^{2+} -ATPase (SERCA) activity.

2.7 Statistical analysis

Summarized data are reported as mean \pm standard error of the mean, unless otherwise specified. Continuous data with sample sizes $n \geq 30$ were assumed to be normally distributed, while the distribution of read-outs between $n = 10$ –30 was analysed using the Shapiro–Wilk normality test. Normally distributed data were compared using the paired and unpaired two-tailed Student's *t*-test. Differences between unpaired data with unequal variances were assessed using the Welch's *t*-test, which is indicated in the legends of all affected figures. Non-normally distributed data and all data sets with $n < 10$ were compared with the Mann–Whitney *U* test. The Fisher's exact test was applied for differences in categorical data. Confounding effects of patient age were determined by the analysis of covariance. Kaplan–Meier curves were compared using the Gehan–Breslow–Wilcoxon test. *P*-value < 0.05 was considered statistically significant.

3. Results

3.1 Speckle-tracking echocardiography reveals reduced atrial function in poAF patients

Left atrial (LA) function was measured in preoperative patients through cardiac cycle-spanning segmental strain quantification and subsequent global averaging via a speckle-tracking echocardiography technique by an operator blinded to clinical data (Figure 1). Forty-two patients had an eligible preoperative echocardiogram for speckle-tracking analysis, of which 21 developed poAF and 21 did not. Patients who went on to develop poAF exhibited significantly reduced left atrial global contraction strain (LASct) before cardiac surgery, compared with Ctrl ($-10.94 \pm 5.69\%$ vs. $-15.50 \pm 6.56\%$, $P < 0.05$, $n = 21$ vs. 21, Figure 1D). Measured during atrial systole until mitral valve closure, impaired LASct indicates overall reduced atrial systolic function in these patients. Reservoir strain (LASr) relating to ventricular systole and atrial filling was also reduced in poAF patients (poAF: $23.75 \pm 8.89\%$, $n = 21$; Ctrl: $31.37 \pm 8.91\%$, $n = 21$), whereas conduit strain (LAScd), which occurs during the early ventricular filling phase, was unaltered. Left atrial diameter was similar between patient groups (poAF: 42.2 ± 6.3 mm, $n = 21$; Ctrl: 39.9 ± 6.5 mm, $n = 21$).

3.2 Reduced $I_{\text{Ca,L}}$ -triggered Ca^{2+} transient in atrial myocytes from poAF patients

To investigate whether impaired excitation–contraction coupling in atrial myocytes contributes to impaired left atrial contractility, we measured L-type Ca^{2+} current ($I_{\text{Ca,L}}$)-triggered CaT in right atrial myocytes. No significant difference between membrane capacitance of poAF (90.38 ± 5.92 pF, $n/N = 35/22$) (myocytes/patients) and Ctrl (95.97 ± 5.79 pF, $n/N = 78/38$; $P = 0.558$) myocytes was observed, indicating comparable cell size. $I_{\text{Ca,L}}$ was induced by a voltage-step protocol (0.5 Hz stimulation frequency) and was measured simultaneously with CaT in fluo-3-loaded right atrial myocytes, as shown by the representative traces in Figure 2A. Neither the peak amplitude nor the integral of

$I_{\text{Ca,L}}$ was different in poAF vs. Ctrl (Figure 2B). Diastolic $[\text{Ca}^{2+}]_i$ was comparable in both groups, however, systolic $[\text{Ca}^{2+}]_i$ tended to be lower in poAF and CaT amplitude was significantly smaller in poAF vs. Ctrl (149.30 ± 14.20 vs. 224.01 ± 16.69 nmol/L, $n/N = 35/22$ poAF vs. 78/38 Ctrl, $P < 0.001$, Figure 2C). The time constant of CaT decay (τ), measured by fitting a single exponential curve to the CaT decay, was found to be significantly higher in poAF vs. Ctrl, equating to a slower rate of decay in poAF (527.38 ± 45.31 vs. 405.44 ± 18.77 ms, $n/N = 35/22$ poAF vs. 78/38 Ctrl, $P < 0.05$, Figure 2D).

β -Adrenergic stimulation has been implicated in the pathogenesis of poAF¹⁸ and is known to influence excitation–contraction coupling via protein kinase A (PKA)-mediated phosphorylation of related proteins.¹⁹ We investigated the response of atrial myocytes to β -adrenergic stimulation using $1 \mu\text{mol/L}$ isoprenaline. In the presence of isoprenaline, $I_{\text{Ca,L}}$ and the triggered CaT amplitude were significantly increased in both Ctrl and poAF, while a significant decrease in the time constant (τ) of CaT decay was observed (Supplementary material online, Figure SIV). $I_{\text{Ca,L}}$ peak amplitude was similar between poAF and Ctrl in both the presence and absence of isoprenaline. However, before the application of isoprenaline, a tendency towards a reduced CaT amplitude was observed in poAF compared to Ctrl and in the presence of isoprenaline, CaT was significantly smaller in poAF. The time constant (τ) of CaT decay was increased in poAF compared to Ctrl, regardless of whether isoprenaline was present.

3.3 Slower SR Ca^{2+} uptake contributes to smaller SR Ca^{2+} content in atrial myocytes from poAF patients

CaT amplitude is governed by various factors such as $I_{\text{Ca,L}}$ and SR Ca^{2+} content. Considering that $I_{\text{Ca,L}}$ was similar between groups, the SR Ca^{2+} content was subsequently measured. An $I_{\text{Ca,L}}$ -activating protocol (see above) was applied to atrial myocytes for 3–5 min, after which stimulation was terminated and caffeine (10 mmol/L) was applied to induce complete Ca^{2+} release from the SR (Figure 3A). Both the amplitude of the resulting Ca^{2+} transient and the resulting charge accumulation [via sodium-calcium exchanger (NCX)-mediated inward current] were significantly smaller in poAF compared to Ctrl, indicating smaller SR Ca^{2+} content in poAF (amplitude: 0.63 ± 0.06 vs. $0.97 \pm 0.08 \mu\text{mol/L}$, $P < 0.001$; charge: 1.53 ± 0.08 vs. 1.80 ± 0.06 pC/pF, $P < 0.01$, $n/N = 35/22$ poAF vs. 78/38 Ctrl, Figure 3B).

The time constant of decay (τ) of the caffeine-induced CaT (a measure of NCX function), however, was comparable between the two groups (Figure 3C) and we did not detect altered contribution of the plasmalemmal Ca^{2+} -ATPase to cytosolic Ca^{2+} removal (Supplementary material online, Figure SV). In accordance, the slope of the line relating I_{NCX} to $[\text{Ca}^{2+}]_i$ during decay of the caffeine-induced CaT was comparable between both groups, suggesting similar Ca^{2+} -dependence of NCX activity (Figure 3D and E). Protein levels of NCX1 and mRNA (SLC8A1) were not found to be different in poAF vs. Ctrl (Figure 3F and Supplementary material online, Figure SVI).

To exclude a role for increased SR Ca^{2+} leak in lowering SR Ca^{2+} content in poAF, total SR Ca^{2+} leak was quantified using the tetracaine method of Shannon *et al.*²⁰ (Figure 4A). SR Ca^{2+} leak was not different in the poAF group (Figure 4B). In accordance, occurrence of spontaneous Ca^{2+} release events (Supplementary material online, Figure SVII), as well as protein and mRNA expression and phosphorylation of ryanodine receptor channels (RyR2, Figure 4C and D and Supplementary material

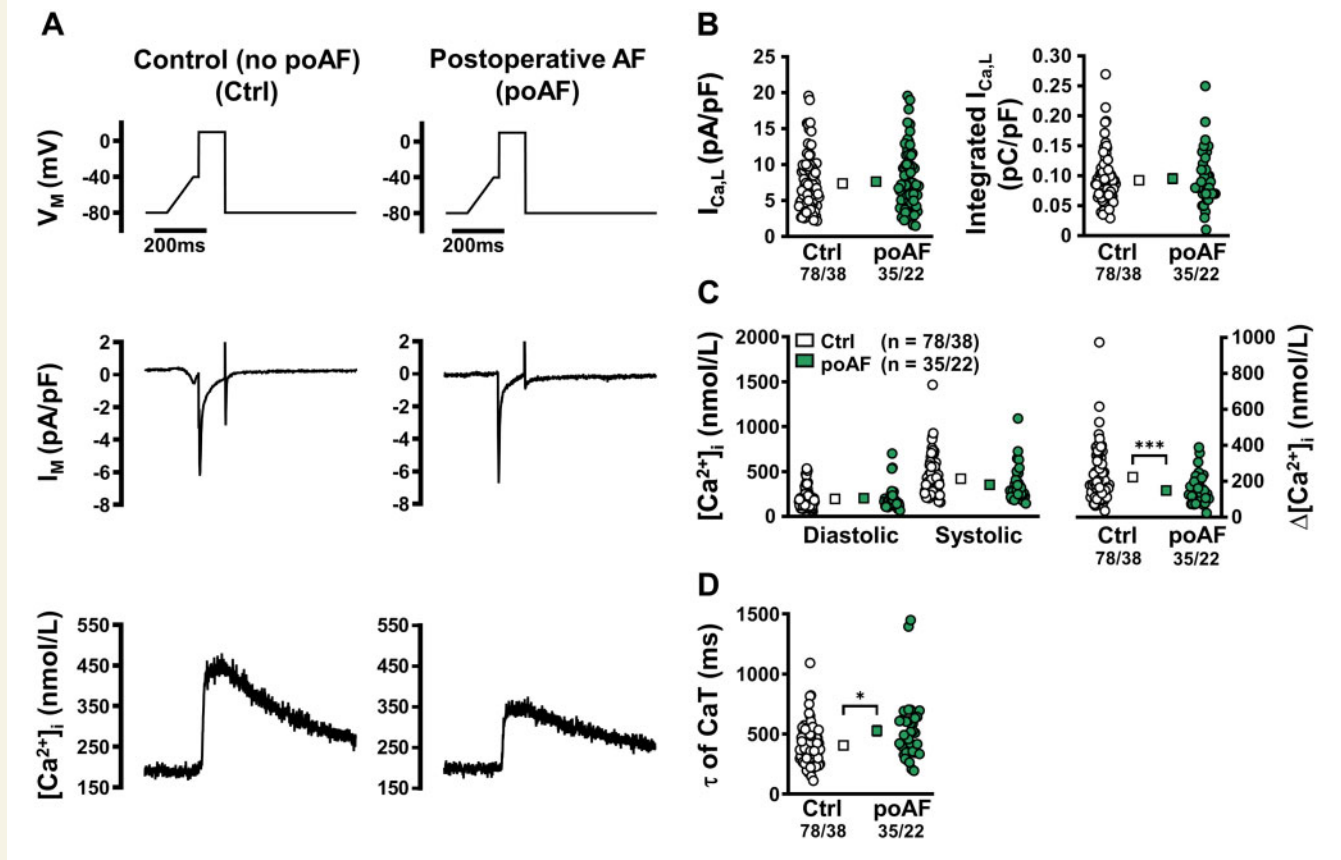


Figure 2 I_{CaL} -triggered CaT in atrial myocytes from patients who do not (Ctrl) and who do develop poAF. (A) Voltage-clamp protocol (0.5 Hz, upper), representative simultaneous recordings of I_{CaL} (middle), and triggered CaT (lower) in Ctrl (left) and poAF (right) myocytes. (B) Mean \pm standard error of the mean (SEM) peak I_{CaL} (left) and integrated I_{CaL} (right). (C) Mean \pm SEM diastolic and systolic $[Ca^{2+}]_i$ (left) and resulting CaT amplitude (right). (D) Mean \pm SEM time constant of decay (τ) of I_{CaL} -triggered CaT. * P < 0.05, *** P < 0.001 vs. Ctrl. n/N = number of myocytes/patients. Comparison using the unpaired t -test with Welch's correction (C right and D). CaT, Ca^{2+} transients; Ctrl, control; poAF, postoperative atrial fibrillation.

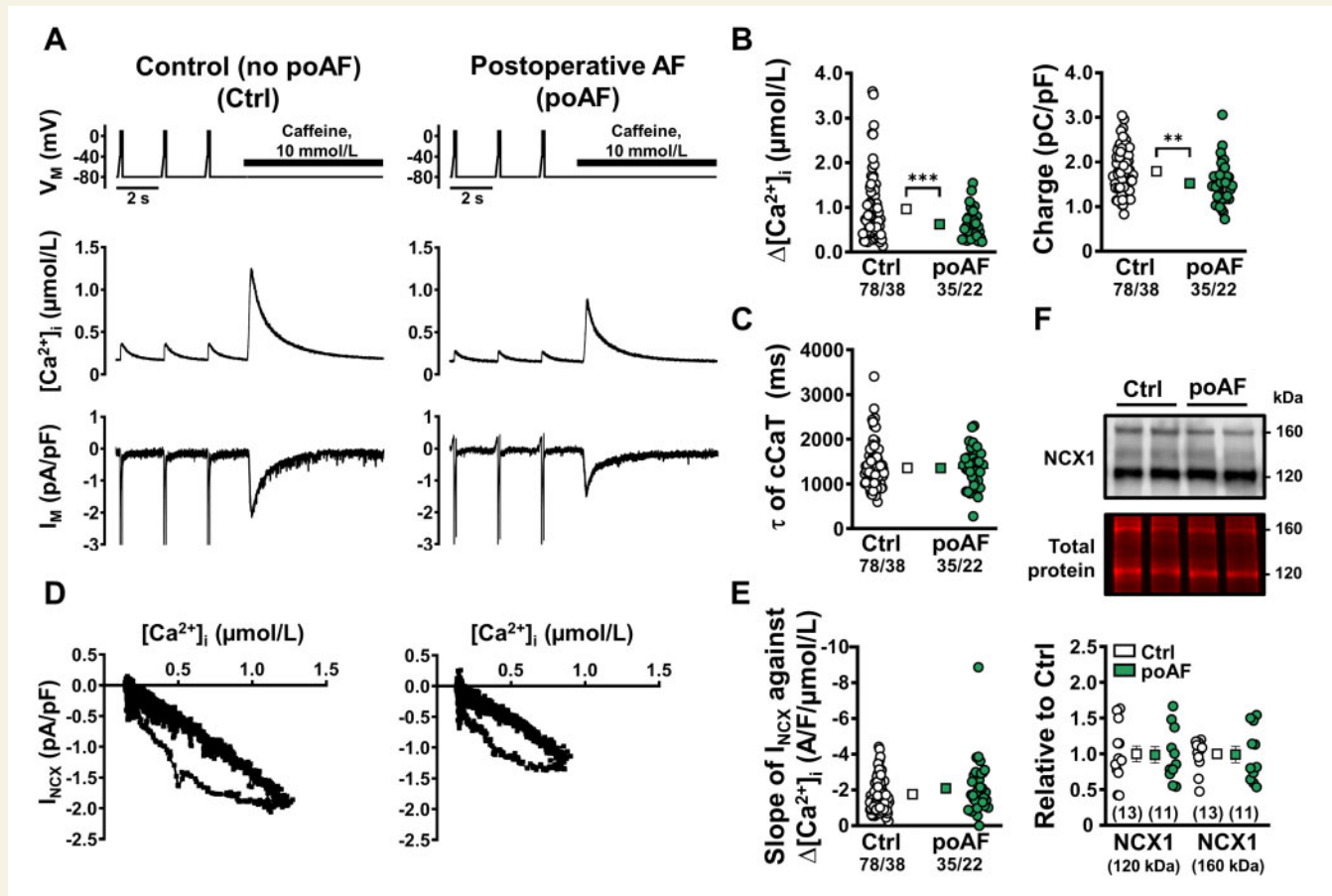
online, Figure SIV), were not found to be different in poAF compared to Ctrl.

SERCA function was subsequently calculated to ascertain if any alteration could underlie slower decay of systolic CaT and smaller SR Ca^{2+} content in poAF. The function of SERCA, expressed as the rate constant k_{SERCA} , was calculated by subtracting the rate constant of decay of the caffeine-induced CaT (k_{caff} , inverse of cCaT τ) from that of the systolic CaT (k_{syst} , inverse of systolic CaT τ , Figure 5A and B), as previously described.²¹ k_{SERCA} was found to be significantly lower in poAF vs. Ctrl (1.48 ± 0.17 vs. 2.08 ± 0.15 s^{-1} , n/N = 35/22 poAF vs. 78/38 Ctrl, P < 0.01, Figure 5B). Furthermore, although SERCA2a mRNA levels (ATP2A2) were similar in Ctrl and poAF patients, SERCA2a protein content, analysed by western blot, was revealed to be significantly lower in poAF (Figure 5C). This points to posttranslational modifications of SERCA2a that may contribute to reduced SERCA-mediated Ca^{2+} extrusion in poAF. These results are likely to underlie both the slower decay rate of the systolic CaT and the smaller SR Ca^{2+} load in poAF. mRNA and protein levels and phosphorylation of phospholamban (PLB) and mRNA levels of sarcolipin (SLN) were comparable in both groups (Figure 5D and Supplementary material online, Figure SVI), indicating that any reduction of SERCA activity was independent of these regulatory proteins.

In addition to altered activity of Ca^{2+} removal mechanisms, increased intracellular Ca^{2+} buffering has also been shown to reduce both amplitude and the rate of decay of systolic Ca^{2+} transients.²² The intracellular Ca^{2+} buffering properties of myocytes from Ctrl and poAF patients were estimated from the caffeine-induced Ca^{2+} transient and associated NCX-mediated inward current (Supplementary material online, Figure SVIII). NCX current was integrated cumulatively to provide an index of 'total' Ca^{2+} , which was plotted against the decay phase of the caffeine-induced Ca^{2+} transient ('free' Ca^{2+}), thereby generating buffer curves which were fitted with a hyperbolic function.²³ The extrapolated B_{max} and k_d values were not different between poAF and Ctrl.

3.4 Enhanced susceptibility to CaT and AP alternans in atrial myocytes from poAF patients

Reduced SERCA activity has previously been associated with alternans in ventricular myocytes.²⁴ Alternans describes the observation that, under certain conditions, the shape of the CaT and AP varies on a beat to beat basis between two contrasting states. To investigate whether this is also the case in atrial myocytes, computational modelling was performed



with the atrial-specific Courtemanche model.¹⁷ A reduction in SERCA activity in this model reduced the stimulation frequency threshold for alternans, both of AP and of CaT, as shown in [Supplementary material online, Figure SIX](#).

In order to further investigate the effect of stimulation frequency on electrophysiology and Ca^{2+} handling, fluo-3-loaded right atrial myocytes from Ctrl and poAF groups were paced at frequencies ranging from 0.25 to 8 Hz in current-clamp to elicit APs and accompanying CaTs. Resting membrane potential (RMP), mean AP duration at 90% repolarization (APD_{90}), maximal slope of AP restitution curve, and diastolic $[Ca^{2+}]_i$ were not significantly different in poAF vs. Ctrl ([Figure 6](#)). CaT amplitude, however, was found to be significantly smaller over the frequencies tested in poAF ([Figure 6D](#)), in agreement with the voltage-clamp experiments.

The incidence of alternans was investigated at each stimulation frequency. [Figure 7A](#) shows example AP and CaT alternans at 4 Hz stimulation. 54% of Ctrl myocytes and 63% of poAF myocytes developed AP alternans. CaT alternans was observed in 17% and 42% of myocytes from Ctrl and poAF patients, respectively. Kaplan–Meier analysis over the whole range of frequencies revealed higher susceptibility to AP and

CaT alternans in poAF patients ([Figure 7B](#)). In addition, and in agreement with results of computational modelling, threshold for AP alternans was significantly lower in the poAF group, i.e. in the presence of reduced SERCA function (2.62 ± 0.52 vs. 5.15 ± 0.68 Hz; $n/N = 12/10$ poAF vs. $13/12$ Ctrl, $P < 0.01$, [Figure 7C](#)). Threshold for CaT alternans showed a tendency to be lower in poAF ([Figure 7C](#)).

4. Discussion

In the present study, we describe Ca^{2+} handling abnormalities which contribute to the atrial arrhythmogenic substrate predisposing patients to the development of poAF. In addition, this is also likely to lead to impaired atrial contractile function in these patients. Our data show the absence of electrical remodelling at the time of cardiac surgery in atrial myocytes from patients who proceed to develop poAF. In contrast, pre-operative echocardiography revealed impaired atrial contractile function in poAF patients and reduction of SR Ca^{2+} release in atrial myocytes, attributable to reduced SR Ca^{2+} load and impaired diastolic SR Ca^{2+}

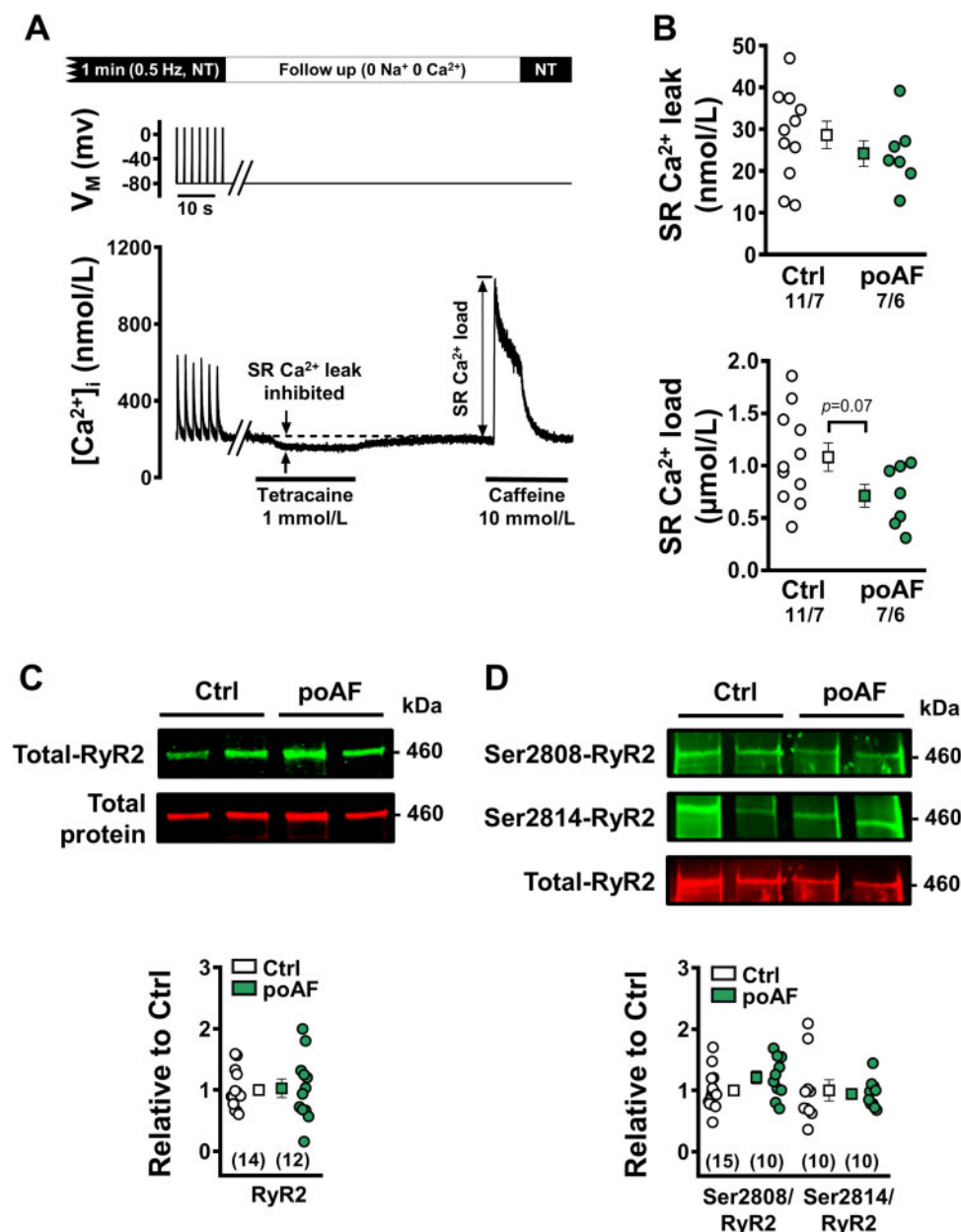
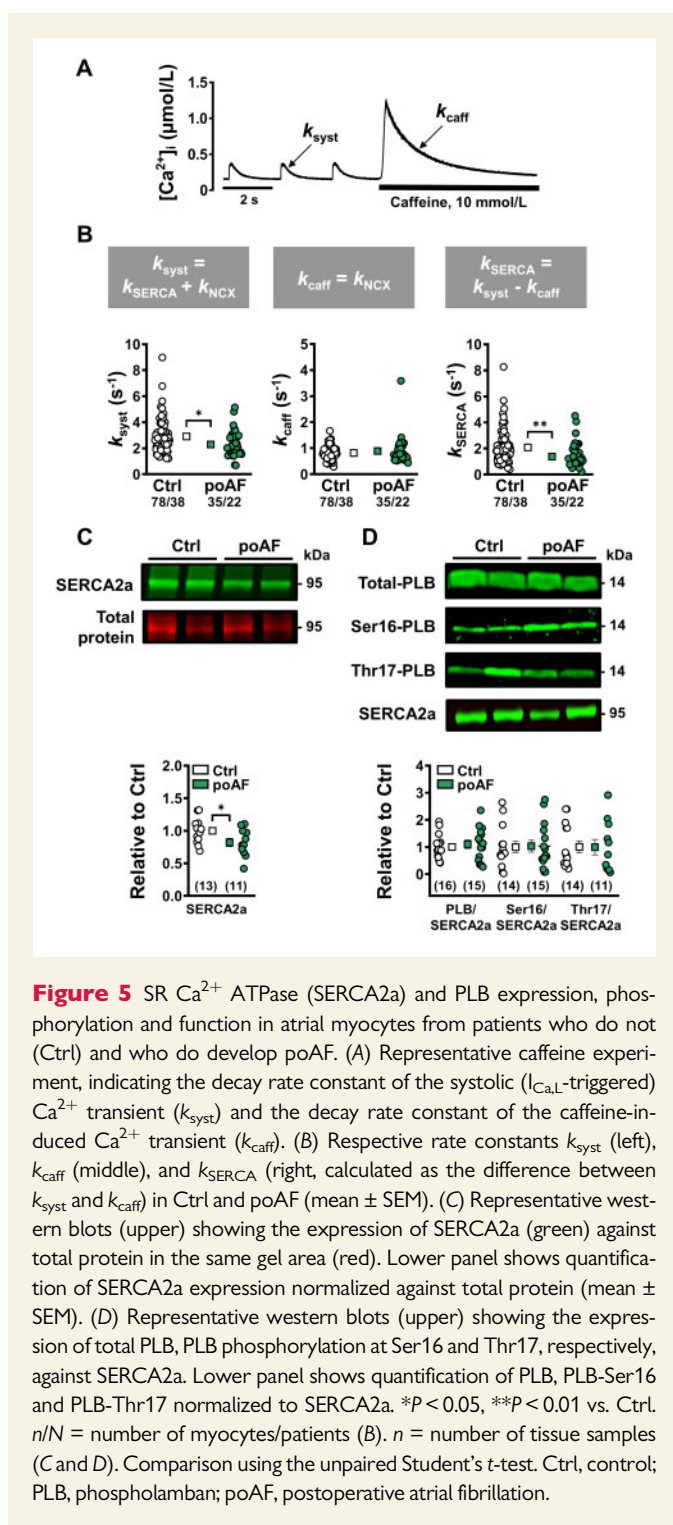


Figure 4 SR Ca^{2+} leak and ryanodine receptor channel (RyR2) function in atrial myocytes from patients who do not (Ctrl) and who do develop poAF. (A) Voltage-clamp protocol (upper) and $[Ca^{2+}]_i$ (lower) in a representative Ctrl experiment, showing the method for SR Ca^{2+} leak and SR Ca^{2+} content quantification in human atrial myocytes using the tetracaine protocol developed by Shannon *et al.*²⁰ (B) Mean \pm standard error of the mean (SEM) total SR Ca^{2+} leak in Ctrl and poAF patients (upper) and SR Ca^{2+} load quantified using caffeine-triggered Ca^{2+} transient amplitude (lower). (C) Representative western blots (upper) showing the expression of total RyR2 (green) against total protein in the same gel area (red). Lower panel shows quantification of total RyR2 expression normalized to total protein (mean \pm SEM). (D) Representative western blots (upper) showing RyR2 phosphorylation at Ser2808 and Ser2814 (green) against total expression of RyR2 (red). Lower panel shows quantification of Ser2808/Ser2814 RyR2 phosphorylation levels normalized against total RyR2 (mean \pm SEM). n/N = number of myocytes/patients (B). n = number of tissue samples (C and D). Comparison using the Mann–Whitney U test. Ctrl, control; poAF, postoperative atrial fibrillation; SR, sarcoplasmic reticulum.

uptake. Computational modelling suggests that the latter also predisposes atrial myocytes from poAF patients to CaT and AP alternans. Accordingly, atrial myocytes from poAF patients were found to have higher susceptibility and lower threshold frequencies for alternans both of CaT and of AP. We thereby provide the first evidence that CaT and

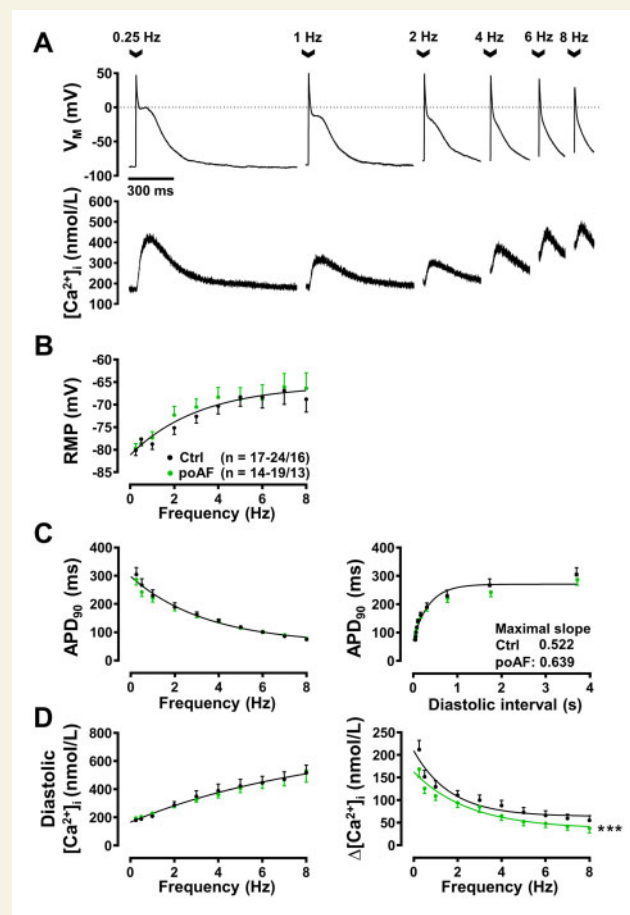
AP alternans can occur in human atrial myocytes. Together these data point to impaired SR Ca^{2+} uptake as a common underlying mechanism which contributes both to the impaired pre-existing atrial contractile function as an independent risk factor of poAF and to the arrhythmogenic substrate, which predisposes patients to the development of poAF.



4.1 Comparison with previous studies

It is well accepted that re-entry is the major mechanism of AF maintenance.^{1,11} Re-entry induction requires an appropriate vulnerable substrate and a trigger which initiates re-entry within this substrate.⁵

A re-entry-favouring substrate is determined by slow conduction and short refractoriness.^{1,11} Long-standing persistent AF, but not paroxysmal AF (pAF), is associated with electrical remodelling of various ion currents,⁵ e.g. reduced I_{CaL} ^{6,10} and increased I_{K1} ,^{25,26} leading to re-entry-promoting shortening of the atrial APD.¹⁰ Slow impulse



propagation due to fibrosis or impaired electrical coupling between myocytes facilitates re-entry by allowing more time for tissue to regain excitability, contributing to the arrhythmogenic substrate.^{1,11} Spatially discordant electrical alterations in excitability (alternans) cause electrical heterogeneity which favours re-entry, thus promoting AF maintenance.^{27,28}

Although the presence of a pre-existing AF-maintaining substrate in patients developing poAF has been shown,²⁹ its molecular basis is largely unknown. Several studies indicate that development of poAF is associated with preoperative alterations in extracellular matrix, such as increased interstitial fibrosis,² impaired connexin expression,³⁰ cellular

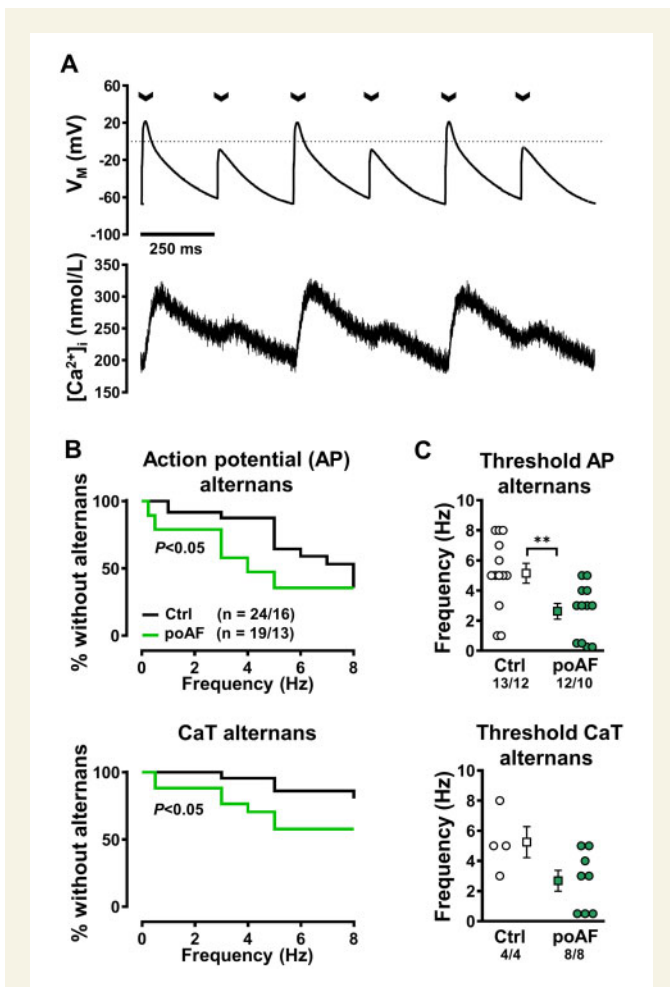


Figure 7 Occurrence of alternans in atrial myocytes from patients who do not (Ctrl) and who do develop poAF. (A) Representative traces of concordant alternans in AP (amplitude and repolarization alternans; upper) and CaT (amplitude and diastolic alternans; lower) at 4 Hz measured from a patient proceeding to develop poAF. (B) The first occurrence of frequency-dependent alternans as a Kaplan–Meier plot in AP (upper panel) and CaT (lower panel). (C) Alternans threshold frequency. Data are mean \pm standard error of the mean. Kaplan–Meier curves compared with the Gehan–Breslow–Wilcoxon test. $^{**}P < 0.01$ vs. Ctrl. n/N = number of myocytes/patients. Alternans threshold frequency compared using the Mann–Whitney U test. AP, action potentials; CaT, Ca^{2+} transients; Ctrl, control; poAF, postoperative atrial fibrillation.

Long-standing persistent AF ('chronic': cAF) and pAF are associated with pronounced Ca^{2+} handling abnormalities which promote ectopic firing.^{10,11} In both cAF and pAF, there is evidence for increased incidence of Ca^{2+} -driven delayed afterdepolarizations (DADs), leading to cellular triggered activity. These are thought to be caused by increased SR Ca^{2+} leak with enhanced incidence of spontaneous SR Ca^{2+} release events (SCaEs).^{10,11} In the present study, we did not find evidence for increased SR Ca^{2+} leak or increased incidence of SCaEs in myocytes from poAF patients at the time of surgery. This is in accordance with our data showing unaltered RyR2 expression and phosphorylation in poAF patients (Figure 4 and Supplementary material online, Figure SVI) and with a previous report documenting unaltered RyR2 mRNA levels.³¹ Taken together, a preoperative increase in the incidence of cellular Ca^{2+} -dependent DADs and triggered activity does not appear to contribute to the arrhythmogenic substrate in patients developing poAF.

4.2 Novel findings and potential clinical implications

To the best of our knowledge, we provide the first comprehensive study investigating pre-existing arrhythmogenic alterations in atrial Ca^{2+} homeostasis in patients developing poAF.

Here we propose that the impaired SR Ca^{2+} uptake associated with increased cellular susceptibility to CaT and AP alternans contributes to atrial arrhythmogenesis in patients developing poAF by creating an arrhythmogenic substrate. In addition, impaired diastolic Ca^{2+} reuptake into the SR leads to reduced SR Ca^{2+} load and a subsequent reduction in systolic Ca^{2+} release, thereby providing a cellular correlate for the clinical observation that atrial contractile dysfunction is an independent risk factor for the development of poAF.^{12,13} Based on our findings we propose that reduced SERCA activity is a common cellular mechanism underlying both impaired contractile function and atrial arrhythmogenesis.

In the current study, we provide the first demonstration of CaT and AP alternans in human atrial myocytes. Within atrial tissue, spatially discordant electrical alterations in excitability and electrical heterogeneity can be caused by cellular AP alternans. This favours re-entry and promotes AF maintenance.^{27,28} Accordingly, patients with a history of cAF and pAF exhibit alternans of monophasic APs of greater magnitude and at lower stimulation frequencies.³² We demonstrate here that right atrial myocytes from patients undergoing open-heart surgery and developing poAF are more susceptible to AP alternans at the time of surgery. The canonical mechanism of AP alternans suggests that it arises because of the extent to which APs shorten in response to changes in the preceding diastolic interval.²⁴ Accordingly, we plotted APD₉₀ vs. the preceding diastolic interval, but did not observe differences in the resulting AP restitution curve (Figure 6C). In addition, maximum slope of the AP restitution curves did not exceed 1, which has been suggested as a prerequisite for alternans based on AP restitution (Figure 6C). More recent work suggests that CaT alternans drives AP alternans, i.e. beat to beat alterations in the cytosolic CaT are translated into alterations of AP shape due to the interaction of $[\text{Ca}^{2+}]_i$ with Ca^{2+} -dependent ion channels and transporters, such as $\text{I}_{\text{Ca,L}}$ -channels and NCX. $[\text{Ca}^{2+}]_i$ -driven alternans is enhanced by factors increasing SR Ca^{2+} release and by factors reducing Ca^{2+} sequestration from the cytosol, for example, increased SR Ca^{2+} leak and reduced SERCA activity, respectively.²⁴ At the time of surgery, we observed no difference either in RyR2 expression and phosphorylation or in diastolic SR Ca^{2+} leak in atrial myocytes from poAF patients compared to Ctrl (Figure 4 and Supplementary material online, Figures SVI and

hypertrophy,⁴ and signs of cellular degeneration.^{1,4} In contrast, altered cellular electrophysiology does not appear to contribute to the preoperative substrate of poAF.¹ Here, we found no differences in APD and $\text{I}_{\text{Ca,L}}$ between poAF and Ctrl groups. Furthermore, frequency-dependence of APD and RMP was unaltered in poAF patients. These findings agree with previous work showing unchanged expression and activity of depolarizing sodium and Ca^{2+} currents (I_{Na} and $\text{I}_{\text{Ca,L}}$)⁹ as well as unchanged repolarizing potassium currents^{7–9} resulting in comparable APD and RMP. Only one previous report has demonstrated increased $\text{I}_{\text{Ca,L}}$ in poAF patients.⁶ The reasons for this discrepancy are unclear but may include differences in clinical characteristics and experimental approaches.⁹

SVII). These findings point against altered SR Ca^{2+} release as a contributor to increased alternans susceptibility in poAF. In contrast, our *in vitro* and *in silico* data suggest that slower Ca^{2+} reuptake into the SR, which was consistent even during β -adrenergic stimulation, underlies the increased alternans susceptibility of atrial myocytes from poAF patients.

In agreement with previous studies¹ we did not detect differences in expression and phosphorylation of the inhibitory SERCA-regulator PLB, suggesting that reduced expression of SERCA2a itself, which we observed in right atrial membrane preparations, may be a major contributor to reduced SR Ca^{2+} uptake in poAF patients (*Figure 5* and *Supplementary material online, Figure SVI*). This is supported by the unchanged overall intracellular Ca^{2+} buffering in poAF patients observed in our study, which if increased, could delay the uptake of Ca^{2+} into the SR,²² due to possible alterations in both the Ca^{2+} sensitivity of the myofilaments and mitochondrial uptake, amongst other factors.³³ Furthermore, the activity of SERCA2a, which is enhanced via PKA phosphorylation of PLB upon β -adrenergic stimulation, was lower in poAF patients compared to Ctrl regardless of isoprenaline presence, further corroborating the role of reduced expression of SERCA2a in the decreased SR Ca^{2+} uptake observed in poAF. Although we did not detect significant alterations in mRNA level of the atrial-specific SERCA2a inhibitor SLN, similar to previous studies our data show a strong tendency towards reduced SLN mRNA levels, which may reflect a compensatory mechanism to minimize the effects of reduced SERCA2a expression.³⁴ The mechanisms underlying reduced SERCA2a expression in poAF patients remain unknown and may involve environmental and genetic factors or predisposing diseases. Unaltered SERCA2a mRNA levels in patients developing poAF (*Supplementary material online, Figure SVI*) suggest the involvement of posttranslational mechanisms. SERCA2a expression is regulated in part by binding of small ubiquitin-like modifiers (SUMO), which stabilize SERCA2a protein structure.³⁵ SUMOylation of SERCA2a has been shown to be reduced in patients with heart failure and contributes to reduced SERCA2a expression. Whether a similar mechanism may contribute to impaired atrial SERCA2a expression in patients developing poAF needs to be investigated in future studies.

Finally, reduced SERCA2a expression may contribute to preoperative atrial contractile dysfunction observed in patients developing poAF,^{12,13} thereby linking atrial contractile dysfunction and atrial arrhythmogenesis. Atrial mechanical dysfunction has recently been suggested as a key determinant of atrial cardiomyopathy, a term describing an atrial phenotype which may promote the occurrence of adverse consequences such as thromboembolic stroke independent of AF.³⁶ Occurrence of poAF is not only associated with an increased risk of early stroke and mortality after surgery but also with an increased long-term risk of stroke.¹ Whether atrial contractile dysfunction due to reduced SERCA activity (atrial cardiomyopathy) predisposes to stroke, independent of AF development, remains to be investigated.

Targeting impaired SERCA2a function may represent an important therapeutic approach to improve atrial contractile function and prevent development of poAF and related negative outcomes. Several concepts to increase ventricular SERCA2a expression and function have been investigated in patients with heart failure. In particular, the effects of adeno-associated viral SERCA2a gene transfer³⁷ and pharmacological activation of SERCA2a with istaroxime were investigated in phase-2 trials, revealing improved systolic function.^{38,39} These approaches could therefore guide future studies to prevent poAF in patients undergoing open-heart surgery or improve atrial contractile function in patients with atrial cardiomyopathy.

4.3 Potential limitations

In the present study, only the right atrial appendages from patients undergoing open-heart surgery were used because of limited availability of human atrial tissue. Our findings may therefore not apply fully to other atrial regions. Furthermore, in our cohort we analysed atrial strain only in the LA due to unfavourable sonographic conditions in the right atrium. However, a recent study demonstrated altered right atrial strain parameters in patients developing poAF, suggesting that impaired atrial contractile function is a general preoperative phenomenon in both atria of poAF patients and that the molecular alterations we observed in right atrial appendages are likely to be similar in left atrial regions.¹³ Nevertheless, future studies investigating both left and right atrial strain in patients developing poAF are necessary.

Here, we identified potential arrhythmogenic mechanisms in isolated atrial cardiomyocytes which may contribute to poAF development. There are additional factors (genetic, type of surgery, autonomic nervous system, inflammation) predisposing patients to the development of poAF and we do not claim that the properties studied here account fully for the arrhythmogenic phenotype in poAF patients.¹ Pre-existing fibrosis, for example, has been identified as an important contributor to the re-entry-promoting substrate in patients developing poAF.² Interestingly, neither left atrial diameter (*Supplementary material online, Tables SI–SVII*) nor left atrial conduit strain (*Figure 1*) was significantly different between poAF and Ctrl, suggesting the absence of any important structural remodelling in our poAF population. Furthermore, age has been identified as an important risk factor for poAF, and here, patients used for voltage-clamp experiments and strain analysis were significantly older (*Supplementary material online, Tables SIII and SIII*). However, further analysis revealed that CaT amplitude and decay, as well as strain parameters, did not correlate with age in these experiments (*Supplementary material online, Figures SX and SXI*), suggesting that impaired SR Ca^{2+} release and subsequent reduction in atrial contractile function are independent risk factors for poAF development.

The methods applied in the present study are mainly designed to detect alterations in global cytosolic Ca^{2+} handling. Based on our experiments we conclude that reduced SERCA2a expression is a major contributor to slower Ca^{2+} reuptake into the SR. However, we cannot exclude that reduced cytosolic $[\text{Ca}^{2+}]$ within the specific SERCA2a microdomain may also contribute to this observation. Furthermore, while slowing of the decay of the Ca^{2+} transient can occur due to factors other than SERCA, our data do not support roles for SR Ca^{2+} leak (*Figure 4*), cytosolic Ca^{2+} buffering (*Supplementary material online, Figure SVIII*), or altered t-tubule density (*Supplementary material online, Figure SIII*). Again, we cannot exclude local alterations on a subcellular level within the specific RyR2 microdomain.⁴⁰ In addition, since RyR2 expression and phosphorylation were evaluated only in a subset of patients, we cannot exclude that statistical power was not sufficient and alterations in these values may be detected in a larger cohort.

In the present work, we largely focus on Ca^{2+} handling abnormalities contributing to the pre-existing cellular substrate predisposing patients to the development of poAF. However, activation of the autonomic nervous system and inflammation are the most accepted acute factors associated with cardiac surgery in triggering poAF.¹ β -Adrenoceptor stimulation and pro-inflammatory cytokines such as tumour necrosis factor alpha and interleukin-1 β have been shown to increase the incidence of SCAEs in ventricular and atrial myocytes⁴¹ and may thereby contribute to the initiation of poAF.¹ The impact of inflammatory mediators on cytosolic Ca^{2+} handling in patients with and without the development of

poAF is beyond the scope of the current project and should be addressed in future detailed studies.

5. Conclusions

In this study, we evaluated pre-existing Ca^{2+} handling abnormalities underlying impaired atrial contractility and atrial arrhythmogenesis in poAF patients. Reduced SERCA activity appears to be an important cause of impaired excitation–contraction coupling and contributes to the arrhythmogenic substrate in these patients. Our data will help to develop new patient-tailored preventive and therapeutic strategies, which could target not only the immediate postoperative period but also provide long-term protection of patients exhibiting specific risk factors such as impaired atrial contractility.

Data availability

All available data are incorporated into this article and its online [supplementary material](#).

Supplementary material

[Supplementary material](#) is available at *Cardiovascular Research* online.

Authors' contributions

F.E.F., F.E.M., and N.V. designed the studies. F.E.F., V.S., F.S., J.G., S.K., J.M., J.R.D.P., K.T., F.H., G.K., C.M.P., A.J.T., K.M.D., B.S., F.E.M., and N.V. performed the research and analysed the data. F.W., C.S., A.E.-E., B.C.D., H.B., and I.K. provided expertise about human heart samples and clinical data analysis. F.E.F., F.S., F.E.M., and N.V. wrote the manuscript, and all authors contributed to the final version.

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Translational perspective

Development of atrial fibrillation (AF) within the immediate postoperative period (poAF) represents one of the most frequent complications after cardiac surgery and is associated with poorer outcomes. Our results suggest that reduced Ca^{2+} uptake into the sarcoplasmic reticulum (SR), associated with increased cellular susceptibility to Ca^{2+} -transient and action potential alternans, contributes to the arrhythmogenic substrate predisposing patients to the development of poAF. Therefore, modulation of SERCA activity may represent a novel mechanistic target to prevent the development of poAF. Furthermore, we show that the impaired SR Ca^{2+} uptake contributes to reduced systolic Ca^{2+} release and impaired atrial contractility in poAF patients. Atrial contractility may therefore represent an important factor for identification of patients at risk for poAF development.