In Vivo and In Silico Investigation into Mechanisms of Frequency Dependence of Repolarization Alternans in Human Ventricular Cardiomyocytes

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ABSTRACT

Rationale: Repolarization alternans (RA) are associated with arrhythmogenesis. Animal studies have revealed potential mechanisms, but human-focused studies are needed. RA generation and frequency dependence may be determined by cell-to-cell variability in protein expression, which is regulated by genetic and external factors.

Objective: To characterize in vivo RA in human, and to investigate in silico using human models the ionic mechanisms underlying the frequency-dependent differences in RA behaviour identified in vivo.

Methods and Results: In vivo electrograms were acquired at 240 sites covering the epicardium of 41 patients at 6 cycle lengths (600-350ms). In silico investigations were conducted using a population of biophysically-detailed human models incorporating variability in protein expression and calibrated using in vivo recordings. Both in silico and in vivo, two types of RA were identified, with Fork and Eye-type restitution curves, based on RA persistence or disappearance, respectively, at fast pacing rates.

In silico simulations show RA are strongly correlated with fluctuations in sarcoplasmic reticulum (SR) calcium, due to strong release and weak reuptake. Large L-type calcium current (I_{CaL}) conductance is responsible for RA disappearance at fast frequencies in Eye-type (30% larger in Eye-type vs Fork-type, p<0.01), due to SERCA potentiation caused by frequency-induced increase in intracellular calcium. Large I_{NaCa} is the main driver in translating Ca^{2+} fluctuations into RA.

Conclusions: In human in vivo and in silico, two types of RA are identified, with RA persistence/disappearance as frequency increases. In silico, I_{CaL} and I_{NaCa} determine RA human cell-to-cell differences through intracellular and SR Calcium regulation.

Keywords: Alternans, calcium, electrophysiology, modeling.

Nonstandard Abbreviations and Acronyms:
AP    action potential
APA   action potential amplitude
APD   action potential duration
ARI   activation recovery interval
AVR   aortic valve replacement
CABG  coronary artery bypass grafting
Cajsr  JSR calcium level
CaT   calcium transient
CaTD  calcium transient duration
CaT_{max}  systolic Ca^{2+} level
CaT_{min}  diastolic Ca^{2+} level
CL    cycle length
DI  diastolic interval
GK1  K1 channel conductance
GKr  Kr channel conductance
GKs  Ks channel conductance
GNa  fast Na⁺ channel conductance
GNaCa Na⁺/Ca²⁺ exchanger conductance
GNaK Na⁺/K⁺ pump activity
GNaL  late Na⁺ channel conductance
Gto  transient outward potassium channel conductance
ICaL  L-type calcium current
IKr  rapid delayed rectifier K⁺ current
INa  fast Na⁺ current
INaCa Na⁺/Ca²⁺ exchanger current
Jrel  Ca²⁺ release flux
JSR  junctional sarcoplasmic reticulum
Jup  Ca²⁺ reuptake flux
ORd model  O’Hara-Rudy dynamic model
PCa  Ca²⁺ channel permeability, referred to as GCaL in this study
PJrel  Ca²⁺ release permeability via ryanodine receptors to myoplasm
PJup  Ca²⁺ uptake permeability via SERCA from the myoplasm
RA  Repolarization alternans
RMP  resting membrane potential
RyR  ryanodine receptor
SCB  sarcolemmal calcium balance
SERCA  Ca²⁺ reuptake through sarcoplasmic reticulum Ca²⁺ ATPase pump
SR  sarcoplasmic reticulum
SRCB  sarcoplasmic reticulum calcium balance
UPD  upstroke duration
Vmax  peak upstroke voltage
τd  ICaL activation time constant
τf  ICaL inactivation time constant
τj  ICaL recovery from Ca²⁺ dependent inactivation time constant)
INTRODUCTION

Repolarization alternans are stable beat-to-beat oscillations between subsequent action potentials (APs), and are considered as an important risk factor for arrhythmogenesis.1–3 The mechanisms underlying repolarization alternans have been the focus of extensive investigations to unravel their causes, modulators and implications for arrhythmias such as ventricular and atrial fibrillation.1 However, the majority of previous studies have been conducted on animal species including rat, rabbit, cat and dog, and therefore translation to human is compromised by interspecies differences in electrophysiology and calcium handling.

Repolarization alternans at fast pacing rates are known to be promoted by action potential duration (APD) prolongation and steep restitution, through beat-to-beat fluctuations in ionic current availability.4–6 However, APD alternans have also been observed clinically in the absence of APD prolongation and without steep APD restitution curve.7 The new paradigm based on animal studies is now supporting that APD alternans may be caused by fluctuations in calcium handling processes in the individual myocyte.1,8–10

Important issues on APD alternans mechanisms still remain unresolved particularly in human. Firstly, the disturbances in the Ca\(^{2+}\) regulatory system responsible for calcium and APD alternans are likely to be multifactorial and modulated by a combination of Ca\(^{2+}\) transport processes. The most likely mechanism points towards Ca\(^{2+}\) alternans caused by fluctuations in sarcoplasmic reticulum (SR) Ca\(^{2+}\) content (rat experimental study)11 and/or refractoriness in ryanodine receptors (rabbit isolated cell and whole-heart experimental studies)12,13, with modulating factors also including the strength of Ca\(^{2+}\) reuptake through sarcoplasmic reticulum(SR) Ca\(^{2+}\) ATPase pump (SERCA) (guinea pig experimental study)14,15. The mechanisms in human ventricular myocytes are however unknown.

Secondly, the key mechanisms translating Ca\(^{2+}\) alternans to APD alternans in human ventricular myocytes still need to be identified. A large calcium transient would have opposite effects on L-type calcium current (I\(_{\text{CaL}}\)) (through its calcium-dependent inactivation) and sodium/calcium exchanger (I\(_{\text{NaCa}}\)) (through the potentiation of its forward mode). Therefore the relative effect of intracellular calcium on both currents would determine whether a large calcium transient results in long or short APD. The balance between I\(_{\text{CaL}}\) and I\(_{\text{NaCa}}\) during repolarization may differ in human ventricular cardiomyocytes with respect to other species, and cell-to-cell differences in conductances and permeabilities may modulate their role in APD alternans.

A key challenge in resolving these issues is the interpretation of findings from different animal species and cell types, and also obtained using different experimental conditions and interventions that, by aiming to segregate individual components, perturb the cellular system as a whole, depriving it of the integral phenomenon, as discussed by Valdivia16. Furthermore, even careful studies performed with consistent cell types and experimental conditions exhibit differences both in the manifestation of cardiac alternans and their potential underlying mechanisms17. Species differences and cell-to-cell variability in sarcolemmal conductances and permeabilities determine repolarization differences in a dynamic process modulated by internal and external factors (such as neuronal stimulation and circadian rhythms)17,18,19, which is likely to also determine cell-to-cell differences in the propensity in APD alternans generation.
The aim of this study is two-fold: firstly to characterize potential frequency-dependent differences in APD alternans in vivo in human, and secondly to investigate the role of variability in ionic conductances and permeabilities in determining the different types of APD alternans identified in vivo using in silico human ventricular models. We hypothesize that in human ventricular cardiomyocytes, cell-to-cell variability in $I_{\text{CaL}}$ and $I_{\text{NaCa}}$ can explain the different types of APD alternans generation identified in in vivo recordings. We first characterize in vivo human APD alternans properties using electrophysiological recordings acquired for 6 stimulation frequencies at 240 sites of the epicardium of 41 human ventricles. In order to investigate the ionic mechanisms underlying human cell-to-cell differences in the occurrence and type of APD alternans, the in vivo recordings are used to construct an in silico population of biophysically-detailed models of human ventricular APs, sharing the same equations but with differences in ionic protein densities to mimic cell-to-cell variability (as previously). Both our in silico and in vivo studies show the same two types of APD alternans occurring in human ventricular cardiomyocytes, characterized by an Eye-type and a Fork-type APD restitution curve, with alternans disappearing and remaining at increasingly fast frequencies, respectively. For all in silico human alternans models, SR Ca$^{2+}$ alternans are the primary cause of both types of APD alternans, which are strongly correlated by the balance of sarcolemmal calcium currents at all frequencies. Strong $I_{\text{CaL}}$ is responsible for the disappearance of Eye-type alternans at fast frequencies, due to the potentiation of SERCA caused by frequency dependent Ca$^{2+}$ overload. $I_{\text{NaCa}}$ is the main driver of the calcium to membrane voltage translation of alternans in the human models, and therefore blocking $I_{\text{NaCa}}$ regulates sarcolemmal calcium balance and suppresses alternans generation.

METHODS

In vivo data acquisition.
The patient cohort consisted of 41 patients, 32 male 9 female, aged 63 ±13.8 (mean ± SD). 31 patients were having coronary artery bypass grafts (24M, 7F); 6 patients were having aortic valve replacement (4M, 2F); 4 patients were having coronary artery grafts + aortic valve replacement (4M). The subjects were selected at random from the waiting list without specific selection criteria. The study, according to the principles expressed in the Declaration of Helsinki, was approved by the local Hospital Ethics Committee, and written informed consent was obtained from all patients before the study. During cardiac surgery, a multielectrode sock was fitted over the epicardium of both ventricles, and unipolar electrograms were recorded from 240 electrodes. Ventricular pacing was established over a range of 6 cycle lengths (CLs), from 600ms to 350ms, in steps of 50ms.

ARI signal analysis.
Activation recovery intervals (ARIs), an in vivo surrogate of APD, were calculated from the epicardial electrograms as the interval from the minimum derivative during depolarization and the maximum derivative during repolarization (Figure 1A), using custom-written routines in MATLAB (MathWorks, Natick, MA). In vivo ARIs at the different CLs presented rate dependence and variability (Figure 1B), and
both normal and alternans sites were observed in these patients (Figure 1 C-D). Further details of the in vivo ARI analysis are included in Supplemental materials.

**In silico population of human ventricular models.**

In vivo investigations of the ionic mechanisms of cardiac alternans direct in human hearts are currently not possible. An in silico study was therefore performed, that firstly captured the variability in APD rate dependence from the in vivo recordings, and secondly allowed identification of key likely human ionic properties and mechanisms in repolarization alternans generation. The biophysically-detailed O’Hara-Rudy (ORd) model of human ventricular cell electrophysiology was adopted as the basis for the in silico investigations. The ORd model is currently considered the gold standard for human studies of pro-arrhythmia as it is the only one including a description of the main human ionic currents and Ca\(^{2+}\) subsystem constructed and extensively validated based on recordings over 140 human hearts. Importantly, as shown in Supplemental Table I, the ORd is the only model to include a detailed description based on human data for 1) voltage and Ca\(^{2+}\)-dependent inactivation of the L-type Ca\(^{2+}\) current; 2) troponin and Ca\(^{2+}\)/calmodulin-dependent protein kinase II buffering; 3) SR compartmentation; and 4) human Na\(^+\), Ca\(^{2+}\) and voltage dependence of Na\(^+\)/Ca\(^{2+}\) exchanger.

To investigate the implications of variability in conductances and permeabilities in human APD rate dependence, we constructed an in silico population of human ventricular cardiomyocytes models calibrated with the in vivo recordings. Firstly, an initial population of 10000 human AP models was generated with models sharing the same equations, but with cell-to-cell differences in the most important conductances and permeabilities, using Latin Hypercube Sampling. Variability was considered in G\(_{Na}\) (fast Na\(^+\) channel conductance), P\(_{Ca}\) (Ca\(^{2+}\) channel permeability, referred to as G\(_{CaL}\) in this study), G\(_{Ks}\) (Ks channel conductance), G\(_{K1}\) (K1 channel conductance), G\(_{Kr}\) (Kr channel conductance), G\(_{to}\) (transient outward potassium channel conductance), G\(_{NaL}\) (late Na\(^+\) channel conductance), G\(_{NaCa}\) (Na\(^+\)/Ca\(^{2+}\) exchanger conductance), G\(_{NaK}\) (Na\(^+\)/K\(^+\) pump activity), P\(_{rel}\) (Ca\(^{2+}\) release permeability via RyR to cytoplasm) and P\(_{up}\) (Ca\(^{2+}\) uptake permeability via SERCA from the cytoplasm). The initial assumption to be tested by using this population is that cell-to-cell variability in protein density (rather than kinetics) is sufficient to explain differences in APD alternans generation from the in vivo recordings.

As in 22, a range of variation of ±100% from their original value was considered to ensure both overexpression and reduction of conductances and permeabilities. The ±100% range is necessarily an assumption as it cannot be measured in vivo, and voltage clamp data are conducted in isolated cells affected by an aggressive isolation procedure. 29

**Calibration of the human in silico models population.**

The calibration of the in silico human population aimed to select the models yielding APDs with properties in range with the in vivo human recordings for 6 CLs as explained in the Supplemental Material for details. Although in vivo recordings include the effects of gap junctional coupling, computer simulations using the ORd model comparing homogenous tissue and single cell simulations have revealed both negligible differences in APD and consistency in alternans generation in single cell and tissue. 27 We therefore used
single cell computer simulation studies to maximize the computational efficiency of the study and to focus on subcellular to cellular mechanisms of alternans.

**Numerical simulations and statistical analysis.**

All numerical simulations were performed using the open source simulation software Chaste\(^{30}\). Statistical analysis was performed in MATLAB. The Mann-Witney U test was used to determine statistical differences in parameters and biomarkers. Partial correlation was used to determine the relationship between biomarkers and parameters. Pearson correlation was used to calculate the correlations in the study.

**RESULTS**

The population of in silico human ventricular models mimics APD variability in the in vivo recordings and identifies key properties underlying alternans generation.

Figure 2A shows the APs generated using the population of human ventricular models, with models excluded (in blue) and accepted (in red) following calibration with in vivo recordings. Of the initial 10000 models, 2326 human ventricular models were accepted following calibration (including the original ORd), covering a broad range of potential ionic properties values (Supplemental Figures III and IV).

Figure 2B shows the correlation analysis between individual ionic properties and specific AP biomarkers, and it demonstrates that AP properties were often the result of the interplay of several currents. The results are in agreement with established knowledge on the role of specific ionic currents on human electrophysiology: (i) large AP upstroke ($V_{\text{max}}$ and UPD) was related to large $G_{\text{Na}}$, whereas AP amplitude (APA) was also affected by $G_{\text{Cal}}$, $G_{\text{NaK}}$ and smaller $G_{\text{Kr}}$; (ii) the resting membrane potential (RMP) was mainly determined by $G_{\text{K Ki}}$ and $G_{\text{NaK}}$; (iii) higher cytosolic Ca\(^{2+}\) transient levels ($Ca_{\text{T max}}$, $Ca_{\text{T min}}$) were related to larger $G_{\text{Cal}}$ and smaller $G_{\text{NaCa}}$ and $G_{\text{NaK}}$; (iv) shorter Ca\(^{2+}\) transient duration ($Ca_{\text{T D}}$) was related to large $G_{\text{Cal}}$ and $P_{\text{Jup}}$; (v) AP triangulation ($APD_{\text{tri}}$) was mainly determined by $G_{\text{Cal}}$; (vi) APD was positively correlated with $G_{\text{Cal}}$ and negatively correlated with $G_{\text{Kr}}$, $G_{\text{Na}}$ and $G_{\text{K Ki}}$ (Figure 2B).

The human models in the calibrated population were classified into the Normal (2239 out of 2326 models) and Alternans groups (87 out of 2326 models). Figure 2C displays box plots of the 9 biomarkers for Normal and Alternans models. No significant differences in APDs were found between the Normal and Alternans groups, indicating that these APD alternans were not related to prolonged APDs. In contrast, $APD_{\text{tri}}$ tended to be larger in the Alternans group, which suggested that AP morphology may be an indicator of alternans propensity. Although $Ca_{\text{T D}}$ was longer, $Ca_{\text{T max}}$ was found to be significantly smaller in the Alternans than in the Normal models, which further suggested the importance of Ca\(^{2+}\) dynamics in the generation of alternans. In agreement with this, Figure 2D shows that Alternans models exhibited larger $P_{\text{Jrel}}$ and smaller $P_{\text{Jup}}$, as well as larger $G_{\text{Cal}}$, $G_{\text{Kr}}$ and $G_{\text{NaCa}}$, and smaller $G_{\text{Na}}$ than the Normal models.
Two types of alternans are observed in both in vivo and in silico data.

The analysis of APD alternans in silico and in vivo revealed similar patterns, and in both cases, two types were identified as illustrated in Figure 3. Eye-type APD alternans were characterized by the disappearance of APD alternans at increasingly fast pacing rates (closed restitution bifurcation), whereas Fork-type APD alternans models displayed stable alternans at increasingly fast frequencies. In the in silico population of models, 14 human models displayed Eye-type restitution, and 47 models were Fork-type for the frequencies tested both in silico and in vivo. In silico, we were able to increase frequency to confirm that most Fork-type restitution curves (44 out of 47) remained open when the pacing CLs were further decreased to 200ms. In addition, 26 models displayed calcium alternans with APD alternans smaller than 5ms in amplitude, named as CaT alternans models hereafter.

Both in silico and in vivo, APD alternans initiation started at longer CL in Eye-type alternans than in Fork-type alternans (median CL for APD alternans initiation: 550ms and 500ms in vivo, 475ms and 350ms in silico, respectively; with statistical differences p<0.001). Furthermore, both types of alternans occurred at similar diastolic interval (DI) and APD values than those exhibited by Normal models (Figure 3B), which further supports the independence of APD alternans from APD or DI values. The fact that similar patterns of alternans are observed both in silico and in vivo recordings confers credibility to mechanistic investigations using the population of in silico human cardiomyocyte models.

APD alternans in the human ventricular models initiate following the loss of SR calcium content balance.

As shown in Figure 4A-B, both Eye-type and Fork-type alternans models displayed larger SR Ca\(^{2+}\) release (P_{rel}) and smaller Ca\(^{2+}\) uptake (P_{up}) permeabilities than Normal models. Based on these data, we hypothesize that APD alternans initiation in the in silico human cardiomyocytes is caused by fluctuations in junctional SR (JSR) calcium content due to the inability of SERCA (J_{up}) to balance RyR Ca\(^{2+}\) release (J_{rel}) at fast frequencies. If found in the human models, the mechanisms would be consistent with some previous measurements in rat and rabbit isolated cardiomyocytes.\(^ {11,12}\)

For all Alternans models, the SR calcium balance (SRCB) was calculated as the integral of calcium ions uptaken by SERCA (J_{up}) minus those released by RyR (J_{rel}) over one beat at each CL (Supplemental Table II). For both Eye-type and Fork-type models, SRCB magnitude displays beat-to-beat fluctuations during APD alternans, with two consecutive beats leading to similar SRCB magnitudes but of different sign (Supplemental Figure V). Figure 4C shows the magnitude of SRCB for one short APD beat for each CL for Eye-type and Fork-type alternans models. For the CLs leading to APD alternans, SRCB magnitude increases above zero for both Eye and Fork-type alternans models (Figure 4C), and its magnitude strongly correlates with the APD alternans magnitude (correlation coefficient ranging 0.86-0.96 for all CLs).

The primary role of oscillations in calcium dynamics in generating APD alternans was confirmed by conducting simulated AP clamp experiments. We imposed the AP clamp of two identical long beats (L+L) and two identical short beats (S+S) to the Eye-type and Fork-type alternans models displaying the biggest
alternans amplitudes (Supplemental Figure VI). In the absence of APD alternans (imposed by the AP clamp), the Ca$^{2+}$ alternans still persisted, which supported that the oscillations of SR Ca$^{2+}$ content existed independently of APD alternans. Therefore, our results support that APD alternans in the human models initiate due to the fluctuations in SR calcium content, which was then transferred to the membrane potential as APD alternans.

*Strong* $I_{\text{NaCa}}$ *and fluctuations in SCB result in APD alternans in both Eye-type and Fork-type human models, and strong $I_{\text{Cal}}$ restores SCB suppressing APD alternans at fast pacing rates for Eye-type models.*

We then investigated the mechanisms underlying the translation from calcium alternans to APD alternans by further examining ionic differences between Normal and Alternans models. As shown in Figure 5A-B, the analysis of the in silico population reveals that the conductance of $I_{\text{NaCa}}$ is significantly larger in both Eye-type and Fork-type models than in Normal models, whereas the $I_{\text{Cal}}$ conductance is larger in Eye-type models compared to its similar magnitude in Fork-type and Normal models. A stronger $I_{\text{NaCa}}$ in the human models would be expected to maximize the gain from calcium fluctuations to APD alternans, and this would be similar to findings in guinea pig myocytes$^{31}$. We therefore hypothesized that SRCB fluctuations destabilize the intracellular calcium balance, which then propagates to the membrane potential in the form of APD alternans through a strong $I_{\text{NaCa}}$ in Eye-type and Fork-type models.

Figure 5C shows, for Eye-type and Fork-type alternans models, the sarcolemmal Ca$^{2+}$ balance (SCB) quantified as the integration of all the sarcolemmal calcium currents over one beat for each CL (Supplemental Table II). As for SRCB, SCB magnitudes of the two alternating beats were practically equal but with different signs, which indicated that the overall calcium amount during the two beats was balanced (Supplemental Figure VII). As for SRCB, a strong correlation was found between the magnitudes of SCB and APD alternans in Fork and Eye-type alternans (correlation coefficient from 0.80 to 0.95).

The larger $G_{\text{CaL}}$ in the Eye-type models resulted in stronger $I_{\text{Cal}}$ and also larger CaT values than in Fork-type models, particularly for short CL <400ms (Supplemental Figure VIII). This leads to the enhancement of SERCA at fast frequencies, which allowed for restoring SRCB and suppressing SR content fluctuations at fast pacing rates for Eye-type models.

*A fine balance in sarcolemmal currents, SR and intracellular calcium mechanisms determines APD alternans in human ventricular myocytes.*

Figure 6 illustrates the network of events explaining alternans generation in the human ventricular myocytes. Figure 6A shows the time course of the transmembrane potential, $C_{\text{asr}}$, $J_{\text{rel}}$, $J_{\text{up}}$, intracellular CaT, and sarcolemmal calcium currents for two consecutive beats for a representative Eye-type model for a long CL with no alternans (CL=600ms), for a fast CL resulting in alternans (CL=500ms, middle column), and for a faster CL with no alternans (CL=350ms, right column). Figure 6B provides a schematic representation of the ionic mechanisms involved, summarizing the ionic mechanisms for long versus short APD beats.
Beat to beat fluctuations in the magnitude of all properties are only observed in the central panels of Figure 6A, as fast and slow pacing rates lead to alternans disappearance in the Eye-type model (left and right columns, respectively). During the long APD beat (blue dashed lines, first row), CaJSR (second row) reach a low level following SR release (third row), and then it progressively recovers due to SR reuptake (fourth row). However, the next beat starts before the CaJSR levels has reached its initial value and this results in a lower CaJSR level at the start of the next beat (second row, compare red solid and blue dashed lines). The consequence for the next beat is a lower Jrel (third row, red solid lines), leading to a higher minimum CaJSR value (second row). The reuptake gradually recovers CaJSR content, which in this beat reaches a higher level at the end than at the start of the beat (red solid line, second row). The next beat would therefore start with higher CaJSR as in the blue dashed line, continuing the oscillations in CaJSR and SRCB as identified in Figure 4.

The beat-to-beat fluctuation in Jrel leads to intracellular Ca2+ level oscillations (fifth row, middle column), which further results in the alternation of calcium related sarcolemmal currents such as Ical (sixth row, middle column) and INaCa (seventh row, middle column). For the beat with a higher initial CaJSR level and stronger Jrel (dashed blue lines), the amplitude of Ca2+ is also higher. The fluctuation in intracellular Ca2+ content does not affect the Ical amplitude, in agreement with a number of studies showing peak Ical is unchanged during alternans. However, it leads to a faster calcium induced inactivation of Ical (sixth row), therefore reducing the overall inward current. However, this is overridden by the calcium induced potentiation of the forward-mode activity of INaCa (seventh row, middle column), which implies an increased inward current, and results in longer APD (first row). INaCa is therefore the main electrogenic mechanism driving APD alternans in the human ventricular models.

The third column in Figure 6A illustrates the mechanisms underlying the disappearance of CaJSR fluctuations at faster CL for Eye-type models. As the CL is further decreased (third column), Ca2+ concentration increases due to the well-known Ca2+ accumulation at fast pacing rates, as reproduced by the models (fifth row). Increased Ca2+ levels enhance SR reuptake (fourth row) and speed up the recovery of CaJSR levels (second row) enabling for CaJSR levels to reach their initial values at the end of each beat. Therefore, fluctuations in CaJSR levels disappear at fast pacing rates as a result of rate dependent calcium accumulation. Eye-type models display stronger Ical conductances than Normal and Fork-type models, and this also results in larger intracellular calcium levels at fast pacing rates (Supplemental Figure VIII). This is the reason why APD alternans are suppressed at fast pacing rates in Eye-type models.

Our simulations also explain the mechanisms underlying the occurrence of CaT alternans without significant APD alternans (< 5ms) in the 26 models. Calcium fluctuations were due to the same mechanisms as in the Eye-type and the Fork-type APD alternans models, but the magnitude of the oscillations in SRCB was smaller and did not result in significant APD alternans due to a modest GNaCa similar to the one in Normal models.
ICaL kinetics variation can affect alternans by regulating SCB and SRCB.

Given the role of ICaL in modulating SCB and its importance in alternans generation, we investigated the effects of variations in ICaL kinetics in modulating APD alternans and the sarcolemmal and SR calcium balance. Simulations were conducted for varying ICaL activation, inactivation and recovery from Ca2+ dependent inactivation time constants in representative models, including the Eye-type and Fork-type models displaying the largest APD alternans. In this new set of simulations we also considered the original ORd model and the two models in the Normal population exhibiting the longest and shortest APD values, respectively. Variations in kinetics time constants of ±50% were considered to investigate theoretical mechanisms rather than representing specific pathological situations.

Alternations of ICaL kinetics did not produce alternans in any of the Normal models considered. However, as shown in Figure 7, in both the Eye-type and Fork-type alternans models, variations particularly in ICaL inactivation kinetics modulate the propensity of alternans generation. In all cases, APD alternans magnitude was still very strongly correlated with SCB and SRCB (R<sup>2</sup> > 0.96), further supporting the mechanisms unraveled in the previous sections. For the Eye-type model, slower ICaL inactivation kinetics (increase in τf and τj) decreased APD alternans because it increased an already strong ICaL, leading to further Ca2+ accumulation and increased SERCA activity, therefore stabilizing SRCB and SCB. For the Fork-type model however, the biggest effect was seen for fast inactivation (decrease in τf and τj) which decreased APD alternans by decreasing ICaL and consequently Jrel, making it easier for SERCA to stabilize SRCB. The ICaL conductance is therefore key to determining the effect of its inactivation kinetics in alternans generation, as it modulates the balance between the effect of ICaL on both Jrel and the intracellular Ca2+ content, both of which are frequency dependent.

INaCa modulation prevents APD alternans in human ventricular myocytes.

Based on our results, one of the fundamental events in the propagation of intracellular Ca2+ alternans to APD alternans is the extrusion of the over-released JSR calcium through INaCa, which is stronger in Alternans than in Normal models. In addition, INaCa is also a crucial regulator of SCB, which is a fundamental indicator of alternans even after introducing ICaL kinetics variation. Therefore, we explored the effects of suppressing the up-regulated INaCa in all types of Alternans models. Figure 8 shows the resulting percentage of alternans types as well as the change of SCB and SRCB after different INaCa interventions. Reducing the enhanced INaCa in Alternans models by only 20% successfully converted 63% of the APD Alternans models into Normal models, whereas 60% INaCa reduction completely suppressed APD alternans (Figure 8A). INaCa modulation eliminated APD alternans by reducing the fluctuation in both SCB and SRCB (Figure 8B-C). In fact, INaCa inhibition only moderately shortens APD and increases the magnitude of the intracellular Ca2+ transient (Supplemental Figure IXA, B). In addition, the maximum Ca2+ level in JSR also increased (Supplemental Figure IXC).
DISCUSSION

In this in vivo and in silico human study, we unraveled the mechanisms and network of events leading to the occurrence of two types of APD alternans identified in novel human ventricular electrophysiological data. Variability in ionic conductances and permeabilities is shown to determine how human membrane kinetics translates calcium fluctuations into APD alternans. This study presents two main methodological novelties including the focus on human both in vivo and in silico, and the investigation of the mechanisms of frequency dependence of APD alternans without significant APD prolongation and long diastolic intervals. Our main new findings are:

1) Both in vivo and in silico human ventricular cardiomyocytes reveal the existence of two types of APD alternans with Eye-type (closed bifurcation) and Fork-type restitution (open bifurcation) curves. Both types of APD alternans are observed for long diastolic intervals (>270ms) and with normal (rather than prolonged) APD. Similarities between human in vivo and in silico alternans support the critical role of cellular processes of individual myocytes in RA generation, and lend credibility to the computational investigations on the underlying mechanisms.

2) In the absence of APD prolongation, APD alternans in the in silico human cardiomyocyte population are consistently associated with fluctuations in SR Ca\(^{2+}\) content even taking into account ionic variability. The relative balance in flux densities between weak SERCA reuptake, strong RyR release and strong \(I_{NaCa}\) extrusion determines the occurrence of alternans. Therefore, variability in ionic conductances and fluctuations do not explain alternative potential sources of Ca\(^{2+}\) alternans (such as RyR refractoriness), which still remain to be shown in human ventricular myocytes.

3) At increasingly fast frequencies, APD alternans disappear in Eye-type cardiomyocytes due to a strong \(I_{CaL}\), which leads to a frequency-induced increase in intracellular calcium levels that promotes SERCA and restores SR content balance.

4) \(I_{CaL}\) conductance determines the effect of alterations in \(I_{CaL}\) inactivation kinetics in APD alternans, as it determines the balance between \(I_{CaL}\) effects directly on SCB and indirectly in SRCB through intracellular Ca\(^{2+}\) content and \(J_{rel}\).

5) Targeting \(I_{NaCa}\) sarcolemmal Ca\(^{2+}\) extrusion, as an indirect strategy to regulate intracellular Ca\(^{2+}\) cycling, successfully restores SR content balance and suppresses alternans generation in the human ventricular myocytes in agreement with previous rat and guinea pig studies (Supplemental Table III), which supports the potential of \(I_{NaCa}\) as a promising anti-arrhythmic target in human.

Fluctuations in SR Ca\(^{2+}\) content as a primary cause of APD alternans in human ventricular cardiomyocytes in silico.

Recent studies have reported that although there is bi-directional coupling between membrane voltage and CaT, APD alternans tend to be the secondary consequence of Ca\(^{2+}\) cycling disturbances.\(^{14}\) The relationship between SR Ca\(^{2+}\) load and Ca\(^{2+}\) release on calcium alternans was proposed by Eisner et al.\(^{32}\) A steep Ca\(^{2+}\) release-SR Ca\(^{2+}\) load relationship was used to explain the generation of calcium alternans.\(^{32}\) Our in silico analysis also revealed weaker Ca\(^{2+}\) reuptake and stronger Ca\(^{2+}\) release in all types of Alternans
models (Figure 4), even considering variability in ionic conductances and permeabilities in the simulations. The role of SR Ca\(^{2+}\) reuptake in our results in human is consistent with the experimental observation that overexpression of SERCA2a suppresses alternans\(^{15,33,34}\) whereas the suppression of SR Ca\(^{2+}\) release has also been shown to inhibit APD alternans in rabbit myocytes.\(^{14}\)

In our human ventricular Alternans models, fluctuations in SR Ca\(^{2+}\) content lead to Ca\(^{2+}\) and APD alternans, and this is in agreement with recordings in rat and rabbit isolated cell experiments.\(^{11,13}\) Recordings in rabbit myocytes and intact hearts\(^{12,13}\) have shown that in some cardiomyocytes, Ca\(^{2+}\) alternans can occur in the absence of SR Ca\(^{2+}\) content fluctuations due to RyR refractoriness during fast pacing. This was not observed in our human population triggering the following thoughts. Firstly, new experiments are required to evaluate the potential contribution of RyR refractoriness to alternans in human ventricular myocytes. Secondly, the human population considered variability in ionic conductances and permeabilities as well as frequency dependence of calcium dynamics. Indeed the ORd model used to construct the population is able to reproduce key properties of Ca\(^{2+}\) cycling rate dependence as measured in human experiments, including frequency modulation of SR Ca\(^{2+}\) release, uptake and content, modulated by Ca\(^{2+}\)/calmodulin-dependent protein kinase II. However, SR Ca\(^{2+}\) content fluctuations were consistently observed during APD alternans. This suggests that if RyR refractoriness is shown to be a mechanism of RA in human in future studies, the in silico framework would need to be updated to reflect the new mechanisms, as it cannot be explained by differences in ionic protein expression in the current framework. Future experimental and theoretical studies are required to evaluate the need for updates in the complex calcium cycling framework, such as the calcium release units, to address the contributions of the ‘3R’ theory from calcium alternans to APD alternans.\(^{35,36}\)

**Sarcolemmal calcium balance translates Ca\(^{2+}\) fluctuations into APD alternans in human ventricular cardiomyocytes.**

Our human ventricular population shows that I\(_{\text{NaCa}}\) is larger in Alternans than in Normal models. Furthermore, we found that the increase of CaT amplitude and long APD beats were in-phase (Figure 6), as was shown by Wang *et al.* in intact rabbit hearts.\(^{13}\) An increase in CaT levels can induce both calcium-induced I\(_{\text{Cal}}\) inactivation (decrease inward current and shortening of APD) and increase of forward-mode I\(_{\text{NaCa}}\) (increase in inward current and prolongation of APD). Therefore, higher CaT corresponding to longer APD in our human models suggests that the forward-mode I\(_{\text{NaCa}}\) plays a more dominant role in prolonging APD for high calcium levels in the human ventricular myocytes, as in \(^{37}\). Our simulations suggest that I\(_{\text{NaCa}}\) modulation may effectively inhibit alternans occurrence in human ventricular cardiomyocytes. This is in agreement with the efficacy of I\(_{\text{NaCa}}\) block against Ca\(^{2+}\) oscillations and APD alternans in rat and guinea pig studies (see Supplemental Table III). Regulation of Ca\(^{2+}\) extrusion through I\(_{\text{NaCa}}\) may substantially differ in animals and human, and our study is the first human based investigation to support the relevance of I\(_{\text{NaCa}}\) block potential against repolarization alternans in human. Our in silico predictions could be further tested in future experimental studies, given in addition the recent availability of novel specific inhibitors of the sodium-calcium exchanger.\(^{38}\)
Furthermore, interventions that promote the forward mode of I_{NaCa} may promote APD alternans. This can also be caused through its sensitivity to Na^+, as for example described in a theoretical study using canine models which shown that suppressing I_{Na} can produce larger APD alternans. In our simulation results, fluctuations in I_{Na} and Na^+ concentration were tightly linked with the alternation of I_{NaCa} during APD alternans, supporting the additional sensitivity of APD alternans to sodium content.

Even though peak I_{CaL} does not fluctuate during APD alternans in the human ventricular models (as in experiments), we show that both conductance and kinetics of I_{CaL} modulate APD alternans in human ventricular myocytes both through the direct electrogenic effect of I_{CaL} on membrane potential, and through indirect effects on intracellular Ca^{2+} content (which determines SR release and uptake). Strong I_{CaL} as in Eye-type models results in larger Ca^{2+} accumulation at fast pacing rates, which promotes SERCA and leads to re-stabilisation of SR content and disappearance of APD alternans. Alterations in I_{CaL} inactivation kinetics can also modulate APD alternans, and their effect is different depending on the overall conductance of I_{CaL}, as shown in Figure 7.

Limitations.

Rather than a single in silico model, the present study is built on a population-based in silico and in vivo approach, allowing the investigation of different alternans types in human ventricular cells and their common underlying mechanisms. Still, there are several limitations in this work: 1) in vivo information from AVR or CABG patients were used in this study, and we did not attempt to specifically model the pathologies of each patient. Instead, we varied the ionic properties of the ORd model in a wide range to investigate the contribution of variability in ionic conductances and permeabilities to explaining different APD alternans regimes. The in silico human models did predict similar alternans patterns at similar CLs to the in vivo data, supporting the validity of the methodology used. While the authors believe that ORd is currently the best model for the purposes of this study, the findings might be model specific. 2) Only epicardial models and recordings were considered in the study due to the difficulties in acquiring simultaneous epicardial and endocardial recordings in vivo in human. 3) Although ARI is widely accepted as a surrogate for APD, as all indirect measurements it may be affected by a bias. 4) In this study, we only considered the variability in ionic conductances and permeabilities. However, variability may also exist in current kinetics as a result of differences in protein structure and conformation (especially in the presence of genetic mutations). It is possible that alternans can also emerge from the kinetics of some currents, and for example RyR refractoriness as was shown in some rabbit ventricular cardiomyocytes. Further experiments would need to confirm the contribution of RyR mechanisms in human. 5) Repolarization alternans in whole-ventricles are caused and modulated by a variety of factors, including gap junctional coupling, tissue heterogeneity and conduction velocity restitution (through for example I_{Na} recovery from inactivation). Further studies could focus on determining the interaction of the calcium-driven mechanisms of Eye-type and Fork-type alternans unraveled in our study with those additional factors in tissue.
ACKNOWLEDGEMENTS
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DISCLOSURES
None.

REFERENCES


DOI: 10.1161/CIRCRESAHA.115.307836
FIGURE LEGENDS

**Figure 1.** In vivo recordings of activation recovery interval (ARI) used for the calibration of the population of human ventricular models. **A:** ARI as an in vivo surrogate of APD. Red dots represent activation and depolarization times, whereas grey dots represent recovery and repolarization times (adapted from 25). **B:** Rate dependence and variability in in vivo ARIs aggregated from the different patients as a function of decreasing pacing cycle length. **C:** Unipolar electrograms and corresponding sequence of ARIs from an alternans-susceptible site. **D:** Unipolar electrograms and corresponding sequence of ARIs from an alternans-resistant site. Dots and crosses on the electrograms represent activation and recovery times, respectively.

**Figure 2.** Population of human ventricular models calibrated with in vivo recordings. **A:** Action potentials of accepted and rejected models for a CL of 600ms. **B:** Partial correlation coefficients (PCC) between action potential biomarkers and current conductance parameters. **C:** Action potential biomarkers for Normal and Alternans models for a CL of 350ms. Biomarkers values have been normalized against maximum values in all accepted models. **D:** Distribution of ionic conductances scaling factors for Normal and Alternans models with respect to their original value in the ±100% range (0 to 2). Symbols indicate statistical significance levels (*: p<0.05; **: p<0.01; ***: p<0.001).

**Figure 3.** Types of APD alternans in vivo and in silico. **A:** Representative restitution curves of APD (ARI) versus CL exhibiting Eye-type and Fork-type alternans in vivo (upper panels) and in silico (lower panels) data. **B:** Representative restitution curves of APD (ARI) versus DI exhibiting normal condition, Eye-type and Fork-type alternans in vivo (upper panels) and in silico (lower panels) data.

**Figure 4.** SR Ca²⁺ cycling properties and fluctuations in Alternans models. **A, B:** Comparison of RyR release (P_{rel}, A) and SERCA uptake (P_{up}, B) in normal, Eye-type and Fork-type alternans models. The vertical axis shows parameters scaling. Symbols indicate statistical significance levels (*: p<0.05; **: p<0.01; ***: p<0.001). **C:** Sarcoplasmic reticulum Ca²⁺ balance (SRCB) magnitudes for Eye-type and Fork-type alternans under all considered CLs.

**Figure 5.** Sarcolemmal Ca²⁺ balance (SCB) in alternans models. **A, B:** Comparison of L-type Ca²⁺ channel conductance (G_{CaL}, A) and Na⁺/Ca²⁺ exchanger conductance (G_{NaCa}, B) in normal, Eye-type and Fork-type alternans models. **C:** Sarcolemmal calcium balance (SCB) magnitudes for Eye-type and Fork-type alternans under all considered CLs.

**Figure 6.** Ionic mechanisms resulting in APD alternans in human in silico ventricular cardiomyocytes. **A:** From top to bottom, transmembrane potential (V_m), Ca²⁺ concentration in JSR (Ca_{JSR}), Ca²⁺ release via RyR (J_{rel}), Ca²⁺ reuptake via SERCA (J_{up}), intracellular Ca²⁺ transient (CaT), L-type Ca²⁺ current (I_{CaL}) and Na⁺/Ca²⁺ exchanger current (I_{NaCa}) in a representative Eye-type model before the generation of alternans (CL=600ms, left column), during alternans (CL = 500ms, middle column) and alternans disappearance (CL=350ms, right column). **B:** Schematic diagram illustrating the network of events explaining APD
alterans for long and short beats: In (1), larger/smaller Ca_{JSR} levels at the start of the beat, results in larger/smaller J_{Na} and intracellular Ca^{2+} levels (2), leading to (3) increase/decrease in inward current through the I_{NaCa} forward mode, and a smaller decrease/increase in inward current through I_{CaL} calcium induced inactivation.

**Figure 7. Effects of varying I_{CaL} kinetics on the Eye-type and Fork-type models with biggest alternans magnitudes.** Correlation between APD alternans magnitudes and SCB magnitudes in the Eye-type model with biggest alternans (A) and the Fork-type model with biggest alternans (B). Correlation between SRCB magnitudes and APD alternans magnitudes in the biggest Eye -type model (C) and the biggest Fork-type model (D). Stars, squares and diamonds represent the variation of ICaL activation (τ_{a}), inactivation (τ_{f}) and recovery from Ca^{2+} dependent inactivation (τ_{j}) time constants, respectively. Colors represent changes in time constants magnitude. The red circle with a square inside represents the original kinetics.

**Figure 8. Suppression of APD alternans by I_{NaCa} inhibition.** A: Percentage of different types of alternans models under 20%, 40% and 60% I_{NaCa} block. B, C: Effects of I_{NaCa} suppression on regulating the SCB and SRCB, respectively.
Novelty and Significance

What Is Known?

- Repolarization alternans are stable beat-to-beat fluctuations between subsequent action potentials (APs), and are regarded as an important risk factor for arrhythmogenesis.
- Animal experiments have revealed potential mechanisms of alternans associated with AP prolongation, fluctuations in sarcoplasmic reticulum (SR) calcium content and/or refractoriness in ryanodine receptors; however, characterization and investigation of alternans in human are lacking.
- Understanding sources and modulators of variability in human electrophysiology and alternans occurrence is a key challenge that requires alternative approaches to controlled laboratory techniques which aim to suppress variability experimentally and statistically.

What New Information Does This Article Contribute?

- Repolarization alternans in human are characterized and investigated using a combined in vivo and in silico methodology based on a population of 2326 human ventricular in silico cell models calibrated with in vivo electrophysiological recordings obtained in 41 patients (not exhibiting prolonged AP).
- Two types of AP alternans are identified in vivo and in silico for long diastolic intervals with Eye-type (closed bifurcation) and Fork-type (open bifurcation) restitution curves, determined by differences in rate-dependent regulation of intracellular calcium level by L-type calcium current (I_{CaL}) (stronger in cardiomyocytes displaying Eye-type alternans).
- Repolarization alternans in all in silico human cardiomyocytes are consistently associated with fluctuations in SR calcium content translated to the transmembrane potential through a strong sodium-calcium exchanger current (I_{NaCa}).

Repolarization alternans are closely associated with the development of life-threatening arrhythmias in patients, but mechanistic investigations in human are both key and missing. Cell-to-cell variability in ionic conductances and permeabilities determines repolarization differences in a dynamic process modulated by internal and external factors to the cell, and which are likely to also determine cell-to-cell differences in the propensity in alternans generation. Our study presents two main methodological novelties, including the focus on in vivo and in silico human investigations, and on the mechanisms modulating variability in the frequency dependence of repolarization alternans without significant AP prolongation. Both in vivo and in silico human ventricular data reveal the existence of two types of alternans, differentiated by their persistence/disappearance as frequency increases. The magnitude of I_{CaL} regulates the disappearance of alternans at fast pacing rates in human ventricular cardiomyocytes. In silico analysis reveals that, even considering ionic variability, repolarization alternans are consistently associated with SR calcium fluctuations caused by loss of balance between SR calcium release and SR calcium reuptake, and translated to repolarization alternans by a strong I_{NaCa}. Reducing I_{NaCa} is an effective strategy to restore SR calcium balance and to suppress alternans generation in the in silico human cardiomyocytes.
Figure 2

A) Accepted models, Excluded models, Original ORD model

B) PCC Value

C) Box plots for various parameters

D) Comparison between Normal and Alternans
Figure 4

(A) PJrel

(B) PJup

(C) SRCB Magnitudes (nmol)

- Normal
- Eye type alternans
- Fork type alternans

Eye type alternans
Fork type alternans

CL = 350, 400, 450, 500, 550, 600ms
A) Alternans model

<table>
<thead>
<tr>
<th></th>
<th>Before Alternans</th>
<th>Alternans</th>
<th>Alternans Disappear</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_m$</td>
<td><img src="image" alt="Graph" /></td>
<td><img src="image" alt="Graph" /></td>
<td><img src="image" alt="Graph" /></td>
</tr>
<tr>
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<tr>
<td>$\text{Jrel}$</td>
<td><img src="image" alt="Graph" /></td>
<td><img src="image" alt="Graph" /></td>
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</tr>
<tr>
<td>$\text{Cap}$</td>
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<tr>
<td>$\text{ICaL}$</td>
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<td><img src="image" alt="Graph" /></td>
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<tr>
<td>$\text{INaCa}$</td>
<td><img src="image" alt="Graph" /></td>
<td><img src="image" alt="Graph" /></td>
<td><img src="image" alt="Graph" /></td>
</tr>
</tbody>
</table>

Time (ms): 0 100 200 300 400 500 600

$\Delta \text{APD}=7.45\text{ms}$

B) Short beat vs. Long beat

- **Short beat**
  - ![Diagram](image)

- **Long beat**
  - ![Diagram](image)
Figure 7

A) Biggest Eye type model

\[ R^2 = 0.9651 \]

B) Biggest Fork type model

\[ R^2 = 0.9915 \]

C) \[ R^2 = 0.9670 \]

D) \[ R^2 = 0.9948 \]
Figure 8
In Vivo and In Silico Investigation into Mechanisms of Frequency Dependence of Repolarization Alternans in Human Ventricular Cardiomyocytes
Xin Zhou, Alfonso Bueno-Orovio, Michele Orini, Ben Hanson, Martin P Hayward, Peter Taggart, Pier D Lambiase, Kevin Burrage and Blanca Rodriguez

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Supplemental Material

1. CHOICE OF MODEL OF HUMAN VENTRICULAR ELECTROPHYSIOLOGY

In silico investigations into the network of mechanisms underlying the generation of the two types of repolarization alternans in human ventricular cardiomyocytes were based on the O’Hara et al. model (ORd). Even though several human ventricular models have been published in the past, the ORd model is now the gold standard for human studies of pro-arrhythmia, the only one extensively constructed and validated based on recordings over 140 human hearts, and the only model dimmed suitable for regulatory used by the USA Food and Drug Administration (see CiPA initiative). Importantly, the ORd model is the only one to include detailed formulations for the Ca\(^{2+}\) subsystem and Ca\(^{2+}\) extrusion dynamics based on human electrophysiology data. This is supported by the comparison to other models of the human ventricular action potential published prior to the ORd model, and specifically the well-known Grandi-Bers (GB) and Ten Tusscher-Panfilov (TP) models. Table I summarises the key differences in intracellular Ca\(^{2+}\) regulation between the three models.

<table>
<thead>
<tr>
<th>Human ventricular calcium cycling and extrusion</th>
<th>ORd</th>
<th>GB</th>
<th>TP</th>
</tr>
</thead>
<tbody>
<tr>
<td>I(_{\text{CaL}}) Ca(^{2+})-dependent inactivation</td>
<td>Yes</td>
<td>Partly</td>
<td>No</td>
</tr>
<tr>
<td>Ca(^{2+})-TRPN buffering</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Ca(^{2+}) bufferung (I(_{\text{CaL}}, \text{RyR}, \text{SERCA}, \text{ICaK}, \text{ICaNa}))</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Sarcoplasmic reticulum compartmentalisation</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Human Na(^{+}), Ca(^{2+}) and voltage dependence of I(_{\text{NaCa}}) Ca(^{2+}) extrusion</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
</tbody>
</table>

Table I: Calcium regulation in latest models of human ventricular electrophysiology. Yes/Partly/No refers to the presence of a given attribute in each of the considered models based on human data. TRPN: troponin; CaMK: Ca\(^{2+}\)/calmodulin-dependent protein kinase II; ORd: O’Hara et al. model (2011); GB: Grandi et al. model (2010); TP: ten Tusscher-Panfilov model (2006).

As shown in Table I:

- Ca\(^{2+}\)-dependent inactivation (CDI) of I\(_{\text{CaL}}\) is included in both the GB and the ORd models. However, the ORd model is the only one to include the formulation based on new recordings in undiseased human ventricular myocytes, in the presence of Ca\(^{2+}\) or Ba\(^{2+}\) as charge carriers to separate CDI from voltage dependent inactivation (VDI). The TP model only incorporates a VDI of I\(_{\text{CaL}}\).
- Both the ORd and GB models account for troponin cytosolic Ca\(^{2+}\) buffers. However, only the ORd contemplates the Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaMK) regulation of the human ventricular Ca\(^{2+}\) cycling, in particular through CaMK modulation of the I\(_{\text{CaL}}\) current, sarcoplasmic reticulum (SR) Ca\(^{2+}\)-release through RyRs, and SR Ca\(^{2+}\)-reuptake by SERCA. CaMK buffering was found to play an important role for Ca\(^{2+}\) cycling, through the modulation of the Ca\(^{2+}\) transient amplitude, diastolic Ca\(^{2+}\) levels, rate dependence of junctional SR Ca\(^{2+}\) content and its evacuation, and magnitudes of RyR and SERCA Ca\(^{2+}\) fluxes.
- Only the ORd model contemplates a functional subdivision of the SR into a junctional (JSR) and network (NSR) compartments. In particular, the existence of a JSR subspace is of critical importance in calcium dynamics, as it serves as the effective Ca\(^{2+}\) pool sensed for the release of RyRs.
- Only the Na\(^{+}\)/Ca\(^{2+}\) exchanger of the ORd model, key for intracellular Ca\(^{2+}\) extrusion, has been formulated using measurements from undiseased human ventricular myocytes. Specifically, it allows for replicating the charge and Ca\(^{2+}\) flux reversal potentials of the exchanger, Na\(^{+}\) leak in the absence of Ca\(^{2+}\) exchange, and the Na\(^{+}\), Ca\(^{2+}\) and voltage dependent properties of the I\(_{\text{NaCa}}\) current as observed in the non-failing human ventricle.

2. BIOMARKER CALCULATION OF SIMULATED ACTION POTENTIALS

The stimulation protocol in the simulations mimicked the one applied in vivo. In silico models were stimulated for 1000 beats at each of the CLs in a step protocol from 600ms to 350ms to reach their steady states. For each of the human models, the following biomarkers were calculated at steady state for each of the 6 considered CLs: APD (at three repolarization levels: APD\(_{30}\), APD\(_{80}\), APD\(_{90}\), APD\(_{\text{tri}}\) (triangulation), CaTD (calcium transient duration), UPD (upstroke duration), V\(_{\text{max}}\) (peak upstroke
voltage), RMP (resting membrane potential), APA (action potential amplitude), CaT_{max} (systolic Ca^{2+} level) and CaT_{min} (diastolic Ca^{2+} level). See Supplemental Table II for a detailed description of biomarkers calculation. Peak current and flux magnitudes were calculated as the maximum absolute value of their current densities during the AP. We also calculated two additional property referred to as sarcoplasmic reticulum calcium balance (SRCB) and sarcolemmal calcium balance (SCB), defined as the overall Ca^{2+} flow through the SR and cell membrane, respectively (Supplemental Table II). The occurrence of APD (or CaTD) alternans was defined as a difference greater than 5ms between APD_{80} (or CaTD_{80}) in the last two APs of the pacing train.

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Calculation method</th>
</tr>
</thead>
<tbody>
<tr>
<td>RMP (resting membrane potential)</td>
<td>Minimum membrane voltage</td>
</tr>
<tr>
<td>V_{max} (peak upstroke voltage)</td>
<td>Maximum membrane voltage</td>
</tr>
<tr>
<td>APA (action potential amplitude)</td>
<td>V_{max}-RMP</td>
</tr>
<tr>
<td>Upstroke potential</td>
<td>RMP+APA*(100-repolarization level)%</td>
</tr>
<tr>
<td>Transmembrane threshold (at 30, 80 or 90% repolarization level)</td>
<td>Time at which membrane voltage rises to Upstroke potential</td>
</tr>
<tr>
<td>UT (upstroke time)</td>
<td>Time at which membrane voltage rises to Transmembrane threshold</td>
</tr>
<tr>
<td>DT (depolarization time)</td>
<td>Time at which membrane voltage falls back to Transmembrane threshold</td>
</tr>
<tr>
<td>RT (repolarization time)</td>
<td></td>
</tr>
<tr>
<td>UPD (upstroke duration biomarker)</td>
<td>UT-DT</td>
</tr>
<tr>
<td>APD (at 30, 80 or 90% repolarization level)</td>
<td>RT-DT</td>
</tr>
<tr>
<td>DI (diastolic interval)</td>
<td>Cycle length – APD_{90} of the previous beat</td>
</tr>
<tr>
<td>APD_{90} (triangulation biomarker)</td>
<td>APD_{90}/APD_{80}</td>
</tr>
<tr>
<td>CaT_{min} (diastolic calcium level)</td>
<td>Minimum value of intracellular Ca^{2+} transient concentration</td>
</tr>
<tr>
<td>CaT_{max} (systolic calcium level)</td>
<td>Maximum intracellular Ca^{2+} transient concentration</td>
</tr>
<tr>
<td>CaTD (at repolarization level 30 or 80)</td>
<td>Similar to APD, but based on intracellular Ca^{2+} transient</td>
</tr>
<tr>
<td>Initial alternans cycle length (CL)</td>
<td>The longest CL for alternans occurrence</td>
</tr>
<tr>
<td>Sarcolemmal calcium balance (SCB)</td>
<td>SCB = \int \left[-(I_{CaL} + I_{pCa} + I_{cab}) + 2 \times I_{NaCa}\right] dt</td>
</tr>
<tr>
<td>SCB magnitude</td>
<td>Absolute value of SCB</td>
</tr>
<tr>
<td>Sarcoplasmic reticulum calcium balance (SRCB)</td>
<td>SRCB=\int(I_{up} * V_{cell,NSR} - I_{rel} * V_{cell,JSR}) dt</td>
</tr>
<tr>
<td>SRCB magnitude</td>
<td>Absolute value of SRCB</td>
</tr>
</tbody>
</table>

Table II: Calculation of action potential biomarkers.

3. CALIBRATION OF THE HUMAN IN SILICO MODELS POPULATION
With our methodology, we specifically propose the investigation of variability in in vivo human ventricular rate dependence using in silico investigations with the population of human ventricular models. The in vivo recordings were analysed and the histograms shown in Supplemental Figure I show the distribution of ARI values for each of the CLs from 600 to 350ms. A rigorous analysis of the data was conducted, as described in Supplemental Figure II, to obtain physiological ranges of ARI variability in vivo for each CL while avoiding including possible outliers. This was done by fitting the aggregated ARIs at each CLs to a skewed normal distribution, and the cumulative distribution function was used to obtain 95% physiological ARI ranges to exclude the effects of extreme values but to still consider the variability in the data, as one of the key goals of our study.
The initial 10000 models in the human population were then filtered to only retain the models yielding APD values within the 95% in vivo ARI ranges for each of the CLs (Filter 1). In addition, we also ensured that each of the APD restitution curves was monotonically decreasing until the eventual occurrence of alternans (Filter 2). This calibration for rate dependence based on the in vivo data was crucial as it allowed retaining critical information on in vivo human rate dependence in the in silico study, an aspect that is key for the study of repolarization alternans.

In addition to calibrating the in silico population with the human in vivo rate dependence data (Filters 1 and 2), we also considered the following filters based on well-known properties of undiseased human ventricular cardiomyocytes reported in the literature:

- Filter 3 (resting potential in undiseased human ventricular cardiomyocytes): RMP between -100 and -64mV (thresholds computed as mean ± 2SD of experimental data by Li et al.).
- Filter 4 (upstroke amplitudes in undiseased human ventricular cardiomyocytes): V_max greater than 0mV.
- Filter 5 (upstroke duration in undiseased human ventricular cardiomyocytes): UPD smaller than 10ms.
- Filter 6 (resting Ca²⁺ levels in undiseased human ventricular cardiomyocytes): CaT_min between 21 and 285nM (thresholds computed as mean ± 2SD of experimental data by Piacentino et al.).

It is also important to stress that our approach does not aim to find a 1:1 match between the in silico and in vivo data, but rather to provide a tool to explore variability in human electrophysiology. This means that for example, a same model could indeed be representative of the rate dependent behavior of several sites in vivo. With the calibration with ensure that the human models in the population are representative of physiology variability in the in vivo data and cover a wide range of possible underlying combinations of ionic properties as illustrated Supplemental Figure III and analysed in the main body of the Manuscript. Supplemental Figure IV shows the effect of each of the Filters in constraining the distribution of each of the ionic properties. The consideration of a wide range of variability adds two advantages to the study. Firstly, we evaluate the consistency of the mechanisms of different alternans types when variability in ionic currents is considered within the population. Secondly, the calibrated population supports the model independency of the findings with respect to the parameter values, which is often compromised in studies using a single action potential model. Indeed in our study, we use over 2000 models to investigate the consistency in the mechanisms of alternans in human, all of them displaying physiological human electrophysiology consistent with the in vivo recordings too.

4. CALCULATION OF IN VIVO ALTERNANS AND IN VIVO RESTITUTION CURVES

The ventricles were stimulated from the apex of the left ventricle. The pattern of activation was consistent for different cycle lengths, being the correlation between the activation sequences for different S1 very high. In unipolar electrograms recorded in vivo, restitution curves illustrating Eye or Fork-type alternans are constructed as follows:

**Definition of alternans:** APD alternans was identified as being present whenever the beat-to-beat variation of ARI, ΔARI = ARI_i - ARI_{i-1}, exhibited an alternating pattern (long, short, long, short, etc) for at least 7 consecutive beats. Alternans magnitude was then calculated as median (|ΔARI_k|) where k represents the heart beats exhibiting an alternating pattern. The first 7 beats of each train of steady state S1 paced beats are discarded in order not to include alternans due to fast rate adaptation.

**Classification of Eye/Fork types of alternans**
1. Recordings are divided in recordings showing alternans at least at one cycle length (alternans susceptible site) and recording not showing alternans (alternans resistant site).
2. Recordings corresponding to alternans-susceptible sites are divided into Fork/Eye type:
   a. A site is said to show Fork-type alternans if alternans are present at CL=350ms.
   b. A site is said to show Eye-type alternans if alternans are not present at CL=350ms.

Eye/Fork alternans restitution curve plots
For each site (i.e. channel, electrode etc) ARIs are shown for each cycle length.
  • If at a given cycle length there are no alternans, only one point is plotted: this point corresponds to the median ARI.
  • If at a given cycle length alternans occur then two points are plotted. These 2 points correspond to $\text{ARI}_m \pm \text{ALT}/2$, where $\text{ARI}_m$ is the median ARI calculated over the ARI that are actually alternating, and ALT is the alternans magnitude. The span between the 2 points is equal to the alternans magnitude.

Initial alternans CL for in vivo Eye/Fork alternans: the longest pacing cycle length when alternans occur at a site.

5. ACTION POTENTIAL CLAMP SIMULATION
For each single model, two protocols were generated from its steady state: two consecutive long beats (L+L) or two consecutive short beats (S+S). The simulation started from the end of the 1000th beat, and then either the L+L protocol or the S+S protocol were applied.

6. ICaL KINETICS VARIATION ANALYSIS
$I_{\text{CaL}}$ activation time constant $\tau_d$, inactivation time constant $\tau_f$ and recovery from $\text{Ca}^{2+}$ dependent inactivation time constant $\tau_j$ were decreased ($\times 50\%$ or 75%) or increased ($\times 125\%$ or 150%), and models were still paced for 1000 beats to reach steady states.

7. PREVIOUS STUDIES ON Na+/Ca2+ EXCHANGER BLOCK AND ALTERNANS

<table>
<thead>
<tr>
<th>Authors</th>
<th>Species</th>
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<th>$I_{\text{NaCa}}$ blocker</th>
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<tr>
<td>Schäfer et al.</td>
<td>Rat myocytes</td>
<td>Wet lab</td>
<td>KB-R7943 (reverse-mode blocker)</td>
<td>Inhibition of the reverse mode $I_{\text{NaCa}}$ reduced spontaneous $\text{Ca}^{2+}$ oscillations upon reperfusion.</td>
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<td>(2001)</td>
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<tr>
<td>Satoh et al.</td>
<td>Rat and guinea pig myocytes</td>
<td>Wet lab</td>
<td>KB-R7943 (reverse-mode blocker)</td>
<td>$I_{\text{NaCa}}$ is species-dependent, and blocking reverse-mode $I_{\text{NaCa}}$ abolished spontaneous $\text{Ca}^{2+}$ oscillations upon ischemia/reperfusion.</td>
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<td>(2003)</td>
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<tr>
<td>Wan et al.</td>
<td>Guinea pig myocytes</td>
<td>Wet lab / Modelling</td>
<td>SEA-0400</td>
<td>Coupling from CaT alternans to APD alternans is determined by the relative balance between $I_{\text{NaCa}}$ and $I_{\text{CaL}}$. Under control condition, $I_{\text{NaCa}}$ is the major coupling link.</td>
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<td>(2012)</td>
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Table III: Previous studies on $I_{\text{NaCa}}$ block related to $\text{Ca}^{2+}$ and APD alternans.

8. SUPPLEMENTAL FIGURES
Supplemental Figure I: Distribution of in vivo ARI data from patients. Aggregated ARIs at the different CLs presented a skewed distribution.

Supplemental Figure II: Probabilistic analysis of in vivo ARI data. Aggregated ARIs at the different CLs were fitted by a skewed normal distribution. The cumulative distribution function was used to obtain 95% physiological ARI ranges to exclude the effects of extreme values. Left: histogram of ARI data from all patients at a CL of 400ms. Middle: probability distribution function (PDF) of the original data based on a skewed normal distribution. Right: Selection of 95% data coverage thresholds (black circles) in a reconstructed cumulative distribution function (CDF).
Supplemental Figure III: Histograms of accepted conductance parameters. The distribution of several ionic parameters tended to be asymmetric, such as those for $G_{CaL}$, $G_{NaCa}$, $G_{NaK}$ and $P_{Jup}$, whereas the distribution of $G_{Kr}$ was bell-shaped. Small values in $G_{Na}$ were completely rejected after the calibration, which indicated the irreplaceable role of this Na$^+$ current in the action potential upstroke.
Supplemental Figure IV: Distribution of ionic properties in the population of human models following the different calibration filters. Each column shows from consideration of first Filter 1 to addition of each of the consecutive filters:

- Filter 1 (in vivo ARI value ranges): APD$_{90}$ within the 95% physiological ARI ranges calculated from all patients under each CL.
- Filter 2 (in vivo ARI rate dependence): APD$_{90}$ restitution within the 95% physiological envelope of ARI restitution as calculated from all patients, ensuring a monotonically decreasing restitution curve in all models as CL decreases until alternans occurrence.
- Filter 3 (resting potential in undiseased human ventricular cardiomyocytes): RMP between -100 and -64mV (thresholds computed as mean ± 2SD of experimental data by Li et al.$^4$).
- Filter 4 (upstroke amplitudes in undiseased human ventricular cardiomyocytes): $V_{\text{max}}$ greater than 0mV.
- Filter 5 (upstroke duration in undiseased human ventricular cardiomyocytes): UPD smaller than 10ms.
- Filter 6 (resting Ca$^{2+}$ levels in undiseased human ventricular cardiomyocytes): CaT$_{\text{min}}$ between 21 and 285nM (thresholds computed as mean ± 2SD of experimental data by Piacentino et al.$^5$).
Supplemental Figure V: SRCB analysis between long/short alternating steady-state beats. Sarcoplasmic reticulum Ca$^{2+}$ balance (SRCB) of the final two alternating beats in all APD alternans models (CL=350ms). The SRCB in long and short beats compensates for each other (same magnitudes with opposite signs).
Supplemental Figure VI: Action potential clamp simulations. Two identical long beats (L+L) or two identical short beats (S+S) were applied to Eye-type (A) and Fork-type (B) alternans representative models with biggest alternans amplitude.
Supplemental Figure VII: SCB analysis between long/short alternating steady-state beats. Sarcolemmal Ca$^{2+}$ balance (SCB) of the final two alternating beats in all APD alternans models (CL=350ms). The SCB in long and short beats compensates for each other (same magnitudes with opposite signs).
Supplemental Figure VIII: Rate dependency of ICaL magnitude, CaT magnitude, Jup magnitude and Jrel magnitude.
Supplemental Figure IX: Effects of I_{NaCa} down-regulation on AP, CaT and JSR Ca^{2+} in alternans models. A, B: Effects of I_{NaCa} inhibition by 60% on AP and CaT. C: Maximum JSR Ca^{2+} level in alternans models before and after I_{NaCa} inhibition at a CL=350ms.
SUPPLEMENTAL REFERENCES


