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RESEARCH PAPER

Absolute beat-to-beat variability and instability parameters of ECG intervals: biomarkers for predicting ischaemia-induced ventricular fibrillation

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BACKGROUND AND PURPOSE

Predicting lethal arrhythmia liability from beat-to-beat variability and instability (BVI) of the ECG intervals is a useful technique in drug assessment. Most investigators use only arrhythmia-free ECGs for this. Recently, it was shown that drug-induced torsades de pointes (TdP) liability can be predicted more accurately from BVI measured irrespective of rhythm, even during arrhythmias (*absolute* BVI). The present study tested the broader applicability of this assessment by examining whether *absolute* BVI parameters predict another potential lethal arrhythmia, ischaemia-induced ventricular fibrillation (VF).

EXPERIMENTAL APPROACH

Langendorff-perfused rat hearts were subjected to regional ischaemia for 15 min. Absolute BVI parameters were derived from ECG intervals measured in 40 consecutive ventricular complexes (irrespective of rhythm) immediately preceding VF onset and compared with time-matched values in hearts not expressing VF.

KEY RESULTS

Increased frequency of non-sinus beats and 'R on T' arrhythmic beats, shortened mean RR and electrical diastolic intervals, and increased BVI of cycle length and repolarization predicted VF occurrence. *Absolute* BVI parameters that quantify variability of repolarization (e.g. 'short-term variability' of QT interval) had the best predictive power with high sensitivity and specificity. In contrast, VF was not predicted by any BVI parameter derived from the last arrhythmia-free interlude before VF.

CONCLUSIONS AND IMPLICATIONS

The novel *absolute* BVI parameters that predicted TdP in rabbit also predict ischaemia-induced VF in rat, indicating a diagnostic and mechanistic congruence. Repolarization inhomogeneity represents a pivotal biomarker of ischaemia-induced VF. The newly validated biomarkers could serve as surrogates for VF in pre-clinical drug investigations.

Abbreviations

AUC, area under ROC curve; BVI, beat-to-beat variability and instability; DI, diastolic interval; HRV, heart rate variability; LTI, long-term instability; LTV, long-term variability; RMSSD, root mean square of successive differences; ROC curve, receiver operating characteristic curve; RRI, RR instability; SDSD, standard deviation of successive differences; STI, short-term instability; STV, short-term variability; TI, total instability; VPB, ventricular premature beats

Introduction

Predicting the risk of lethal arrhythmias is an important part of selection of drug therapy and is also important for the assessment of the pro-arrhythmic and anti-arrhythmic activity of newly developed drugs (Farkas and Nattel, 2010; Curtis *et al.*, 2013). Unfortunately, there are still no reliable methods or biomarkers that predict the occurrence of the most common cause of sudden cardiac death, ischaemia-induced ventricular fibrillation (VF) (Zipes and Wellens, 1998). Therefore, the present study was designed to determine whether it is possible to predict the likelihood of ischaemia-induced VF by a method developed for predicting the risk of another potential lethal arrhythmia, drug-induced torsades de pointes (TdP).

TdP risk may be predicted from ventricular repolarization variability, but until recently, analysis has been performed only on arrhythmia-free ECGs (Thomsen *et al.*, 2004). Recently, it was shown in a blinded test that beat-to-beat variability of the QT interval measured during stable sinus rhythm did *not* predict TdP in a commonly used *in vivo* model (Vincze *et al.*, 2008; Farkas *et al.*, 2010). However, when beat-to-beat variability and instability (BVI) of the ECG intervals were measured immediately before TdP onset, regardless of whether the rhythm was sinus or irregular, and compared with exact time-matched intervals in animals without TdP, the TdP liability was accurately predicted (Farkas *et al.*, 2010). To achieve this outcome, a method was developed that allows ECG intervals to be measured during an irregular non-sinus rhythm (Farkas *et al.*, 2009; 2010). This allowed established BVI parameters (Farkas *et al.*, 2010) to be derived *irrespective of rhythm*. To avoid confusion with BVI parameters published by others, all of which were derived from intervals obtained only during stable sinus rhythm (*sinus* BVI parameters), we coined the term *absolute* BVI to describe the parameters derived irrespective of rhythm (Farkas *et al.*, 2010).

The mechanism of the maintenance of TdP involves functional re-entrant circuits facilitated by an increase in spatial dispersion of ventricular repolarization (Farkas and Nattel, 2010). *Absolute* BVI parameters quantify electrical instability and define the substrate (electrical inhomogeneity) for re-entrant arrhythmias, for example, TdP (Farkas *et al.*, 2010). Although phase I ischaemia-induced VF (which occurs during the first 30 min of ischaemia) and TdP differ in many respects, functional re-entry is common to both (Jalife and Berenfeld, 2004; Farkas and Nattel, 2010). In view of this, we hypothesized that the new *absolute* BVI biomarkers of TdP liability, validated in rabbits (Farkas *et al.*, 2010), may be adapted to predict VF liability during early ischaemia.

Isolated Langendorff-perfused rat hearts were used in the present study because they are frequently used to explore the mechanisms responsible for the initiation and maintenance of phase I ischaemia-induced VF and to assay non–class III anti-arrhythmic and pro-arrhythmic drugs (Curtis, 1998; Farkas *et al.*, 1999; Farkas and Curtis, 2002; 2003). This model was validated (Curtis, 1998). Although the rat has very different repolarization characteristics compared with the rabbit (Curtis, 1998; Farkas and Curtis, 2003), there is a strong similarity in the underlying mechanisms of VF in all mammalian species and irrespective of the underlying source, wavebreak and re-entry account for the complex pattern of ventricular fibrillation (Tsuchihashi and Curtis, 1991; Noujaim *et al.*, 2007; Vaquero *et al.*, 2008).

The novel ECG analysis method used in the present investigation does not require a regular rhythm or a stable sinus rhythm for implementation. The reason this would have value is that VF is likely to occur in hearts that already have an irregular rhythm, and it is well known that predicting lethal arrhythmias (i.e. TdP) is achieved more accurately if analysis of risk is undertaken without excluding ECG signal during non-sinus rhythm (Farkas *et al.*, 2010).

The present data were derived from an earlier investigation (Farkas and Curtis, 2002) with ECG records analysed by the new method. Thus, the present investigation is a test of proof of concept that meets the 3Rs objective of animal research. The analysis found that *absolute* BVI parameters predicted ischaemic VF, whereas *sinus* BVI parameters did not.

Methods

Animals and general experimental methods

For full methodical details, see the published study from which the raw ECGs were obtained for processing (Farkas and Curtis, 2002). The animal-handling protocol was in accordance with the Guidance of the Operation on the Animals (Scientific Procedures) Act 1986, London, UK. Male rats were anaesthetized with 60 $mg \cdot kg^{-1}$ (i.p.) pentobarbitone and hearts were excised and Langendorff perfused with Krebs solution modified to contain (in mM) NaCl 118.5; $NaHCO_3$ 25.0; $MgSO_4$ 1.2; NaH_2PO_4 1.2; $CaCl_2$ 1.4; KCl 3 and glucose 11.1, delivered at 37°C and pH 7.4. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). A unipolar ECG was recorded by implanting an electrode into the region to become ischaemic. Regional ischaemia was induced by tightening a coronary occluder that had been placed on the left main coronary artery.

Experimental protocol

Hearts (n = 24) were perfused for an initial 10 min with modified Krebs solution, and then the left main coronary artery was occluded for 15 min.

'VF+' and 'VF–' groups

Ventricular premature beats (VPBs) and VF were defined according to the Lambeth conventions (Walker *et al.*, 1988). The onset time of the first VF was determined. Hearts with (n = 14) or without VF (n = 10) during ischaemia were allocated into the 'VF+' and 'VF–' groups respectively. This was a sufficient number of samples for statistical analysis between groups.

Measurement of the ECG intervals

ECG intervals were measured with investigators blinded to VF occurrence. The RR, QR, RT, QT and electrical diastolic interval (DI) were measured by manual positioning of on-screen marker lines. For definition of the ECG intervals measured, see Table 1. Three vertical marker lines were located in each ventricular cycle, the first at the beginning of the QRT complex, the second at the peak of the R wave and the third at the end of the T wave (Figure 1). LabChart (ADInstruments Ltd, Oxford, UK) was set to provide the distance between consecutive markers.

In rats, prominent transient outward K^+ current (I_{to}) causes a rapid, phase 1 ventricular repolarization that merges into the depolarization (Gussak *et al.*, 2000). Therefore, the QRS and the T wave are not separable, and incorporate into a QRT wave. QT interval is commonly used to describe the time to repolarization, but it is also altered by variations in depolarization. Therefore, we also determined the RT interval, which better estimates the time to repolarization than the QT, as the RT interval contains less overlap of the depolarization (the QR interval).

During ventricular arrhythmias, the T wave frequently overlaps the QRT wave of the next ventricular complex. The QRT complex that interrupts the T wave of the preceding beat is the 'R on T' arrhythmic beat. When these events occurred, the end of the QT interval was the point where the QRS complex of the subsequent R on T beat started. Also, when an R on T arrhythmic beat chopped off the end of the T wave, a validated extrapolation method (Farkas *et al.*, 2004) was used to determine the extrapolated QT interval; the end of the T wave was extrapolated from the asymptote of repolarization. The extrapolated QT interval was labelled QTx (Figure 2).

Table 1

Definitions of the ECG intervals measured

ECG interval	Definition
RR	Cycle length; interval between the peaks of two consecutive R waves
QR	Interval between the first deviation of the ventricular (QRT) complex from the isoelectric line and the peak of the R wave
RT	Interval between the peak of the R wave and the end of the T wave
QT	Interval between the first deviation of the QRT complex from the isoelectric line and the end of the T wave. The end of the T wave is the point, where the curve of the T wave returns to the isoelectric line. When an R on T arrhythmic beat chops off the end of the T wave, then the end of the T wave is the point where the QRS complex of the subsequent R on T beat starts.
QTx	Interval between the first deviation of the QRT complex from the isoelectric line and the end of the extrapolated T wave. The end of the T wave is the point where the extrapolated curve of the T wave returns to the isoelectric line.
DI	Electrical diastolic interval; interval between the end of the T wave and the beginning of the next ventricular (QRT) complex. When the end of the T wave was obliterated by the subsequent QRT complex, the electrical diastolic interval was arbitrarily assigned 10^{-6} ms to allow analysis.

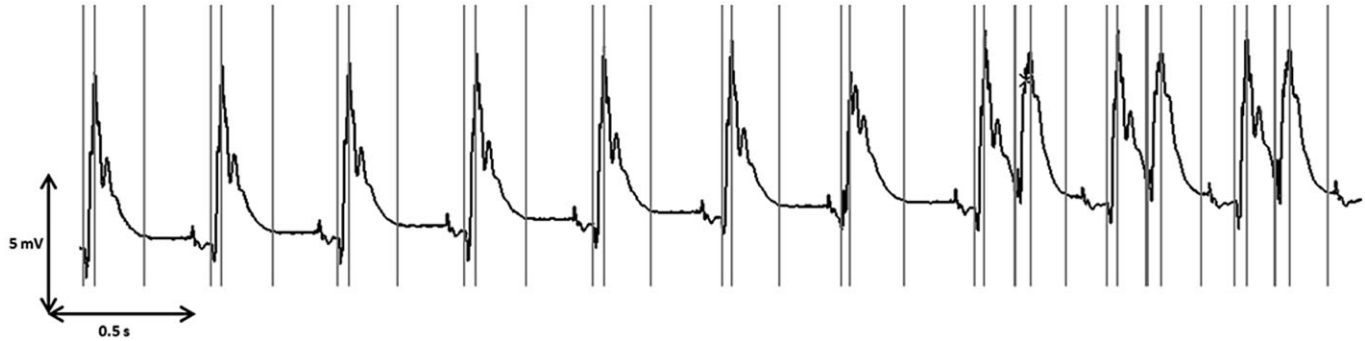

Figure 1

The vertical marker lines applied to measure ECG intervals in an ECG section in rat isolated hearts.

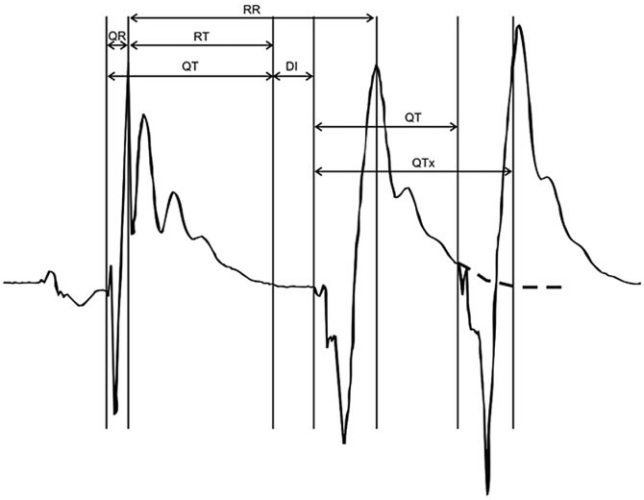

Figure 2

Rat ECG intervals. The first QRT is a normal sinus complex with complete repolarization. The second is a VPB that does not interrupt the repolarization process of the previous QRT. The third QRT is an 'R on T' arrhythmic beat that interrupts repolarization. RR, QR, RT, QT and DI intervals are labelled. The extrapolation method is shown in the second QRT. The T wave was extrapolated from the repolarization profile and the extrapolated QT interval is labelled 'QTx'.

Table 2

Derived BVI parameters

Abbreviation	Derived parameter
SD	Standard deviation
RMSSD	Root mean square of the successive differences
SDSD	Standard deviation of the successive differences
STV	Short-term variability
LTV	Long-term variability
TI	Total instability
LTI	Long-term instability
STI	Short-term instability
Inst.	Instability

Obtaining absolute and sinus BVI parameters

To determine BVI values, all analyses were based on samples of 40 consecutive QRTs. From each QRT, the RR, QR, RT, QT (or QTx) and DI intervals were measured, then the mean and the standard BVI parameters were derived as described previously (Brennan *et al.*, 2001; Hondeghem *et al.*, 2001; Thomsen *et al.*, 2004; van der Linde *et al.*, 2005) (Table 2). The following BVI parameters were calculated.

Root mean square (RMSSD) and standard deviation (SDSD) of successive differences. One approach to characterize the beat-to-beat variability of an ECG interval is to take its successive differences ($\Delta d_j = d_{j+1} - d_j$; $0 \le j \le N-2$, where d_j represents the duration of the interval and N is the total number of intervals) and calculate the root mean square (RMSSD) and the standard deviation (SDSD) of these differences (Brennan *et al.*, 2001): $\mathrm{RMSSD} = \sqrt{E([\Delta d]^2)}$, and $\mathrm{SDSD} = \sqrt{E([\Delta d]^2) - E^2(\Delta d)}$, where E denotes the mean value.

Short-term variability (STV). In terms of a Poincaré plot, which is a plot of the value of an ECG interval (d_{i+1}) against the preceding value (d_i), one can visualize the short-term variability as the mean perpendicular distance between the points of the plot and the $d_{i+1} = d_i$ line. This corresponds to the following formula as described by Thomsen *et al.* (2004): $\mathrm{STV} = \frac{1}{N\sqrt{2}} \sum_{i=0}^{N-2} (d_{i+1} - d_i)$, where d_i represents the sequence of the ECG interval durations and N is the total number of intervals.

Long-term variability (LTV). In the framework outlined earlier, LTV is the mean distance (measured parallel to the $d_{i+1} = d_i$ line in the Poincaré plot) between the individual interval durations (d_i) and their mean value [$E(d)$] as described by Thomsen *et al.* (2004): $\mathrm{LTV} = \frac{1}{N\sqrt{2}} \sum_{i=0}^{N-2} [d_{i+1} + d_i - 2E(d)]$.

Total instability (TI), long-term instability (LTI) and short-term instability (STI). TI, LTI and STI values were derived from the Poincaré plot by applying a complex mathematical analysis. For the exact mathematical descriptions of TI, LTI and STI parameters, see van der Linde *et al.* (2005).

Instability. The instability of an ECG interval was calculated as the difference between the upper quartile (the upper boundary of the lowest 75% of interval values) and the lower quartile (the upper boundary of the lowest 25% of interval values) (Hondeghem *et al.*, 2001).

The BVI parameters were defined as *sinus* when the 40 consecutive QRTs for analysis were selected to be all of sinus rhythm, and *absolute* when they were selected irrespective of the rhythm (arrhythmic traces were included) at predetermined time points (Farkas *et al.*, 2010). *Sinus* BVI parameters were determined for the last arrhythmia-free period in sinus rhythm either immediately before VF in the 'VF+' group or before the end of 15th minute of ischaemia in the 'VF–' group. *Absolute* BVI parameters were determined during the last minute before coronary occlusion, the seventh minute of the ischaemia, and immediately before VF occurrence in the 'VF+' group or at an equivalent time point in the 'VF–' group.

The percentage frequency of arrhythmic beats (defined as VPBs or individual QRT complexes in a run of a salvo or VT) and 'R on T' arrhythmic beats were calculated as number per 40 beats times 100.

Morphological characterization of the arrhythmic beats before VF. The arrhythmic beats were analysed for R voltage and QR, QT, QTx, RT and coupling intervals (RR) from data on the last 40 arrhythmic beats immediately before VF occurrence in the 'VF+' group of hearts or at equivalent time points in the 'VF–' group of hearts. Groups were compared in terms of mean and SD of 40 beats.

Statistics

Continuous data were expressed as mean ± SEM. Between-group comparisons were made by Mann–Whitney test. VF frequencies were compared by Fisher's exact test. Differences were considered statistically significant when $P < 0.05$. Receiver operating characteristic (ROC) curve analysis (Akobeng, 2007) was performed to determine the predictive power of the ECG variables, derived BVI parameters and the % frequencies of arrhythmic beats. Area under ROC curve (AUC) and confidence interval for AUC were calculated by using IBM SPSS Statistics 20 software (IBM Corporation, Armonk, NY, USA). Parameters having AUC above 0.8 were considered to have a validated predictive value for VF occurrence. The optimal cut-off values were determined using Youden indexes (Akobeng, 2007). Sensitivity of a parameter was estimated as the fraction of 'VF+' hearts that had greater value (or in case of the *absolute* mean ECG interval parameters, smaller value) than the cut-off value. Specificity was estimated as the fraction of 'VF–' hearts that had smaller value (or in case of the *absolute* mean ECG interval parameters, greater value) than the cut-off value (Akobeng, 2007).

Results

Supplementary data are presented in the Supporting Information.

Absolute mean ECG intervals

Before coronary occlusion, there were no significant differences in mean RR, QR, RT, QT, QTx, DI values between the 'VF+' and 'VF–' groups. The mean RR interval was significantly shorter in the 'VF+' group immediately before VF compared with the equivalent time point in the 'VF–' group. The RT, QT and DI intervals were also shorter in the 'VF+' group at this time (Figure 3). ROC analysis showed that among the derived *absolute* mean ECG interval parameters, only the mean RR and the mean DI had higher AUC values than 0.8 (indicative of high predictive power for VF occurrence) and only these two intervals predicted VF with relatively high sensitivity and specificity (Table 3 and Supporting Information Table S1).

Absolute BVI parameters of the ECG intervals

Before coronary occlusion and during the seventh minute of the ischaemia, for each ECG interval (RR, QT, etc.) the derived *absolute* BVI values were small and did not differentiate between 'VF+' and 'VF–' groups. For each ECG interval, all derived BVI values increased during ischaemia in the 'VF+' and 'VF–' groups, but the increases were significantly greater in the 'VF+' group (Figures 4 and 5; Supporting Information Table S2). This was especially the case for the *absolute* BVI parameters for repolarization (e.g. STV for QT, STI for QT), indicative of an immensely increased temporal variability and instability in repolarization before VF occurrence. ROC analysis showed that repolarization-related *absolute* BVI parameters had high predictive power for VF occurrence, with AUC values of every BVI parameter for QT and RT greater than 0.8; the STV, RMSSD, SDSD and STI for the RT and QT intervals had outstanding sensitivity (93%) and specificity (80%) (Table 3 and Supporting Information Table S3). The AUC values of the *absolute* BVI parameters of the extrapolated QT interval (QTx) were lower than the values for the non-extrapolated QT, indicating a relatively lower predictive power (Supporting Information Table S3).

Three RR interval instability values (TI, LTI and STI) were significantly greater in 'VF+' hearts compared with 'VF–' hearts (Figures 4 and 5; Supporting Information Table S2). However, the predictive power of RR instability (AUC) was unequivocally less than the predictive power of QT and RT instability (Table 3 and Supporting Information Table S3).

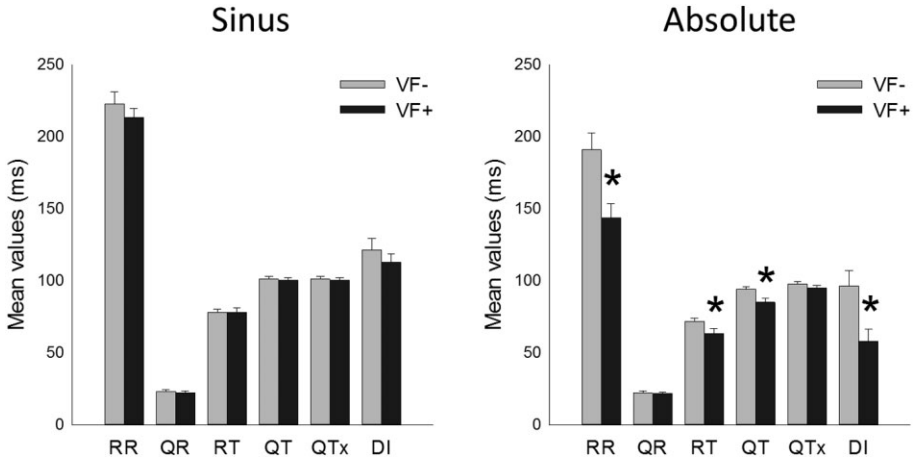

Figure 3

The *sinus* and *absolute* mean ECG intervals. (A) Values were measured in sinus rhythm in the last arrhythmia-free period either before VF in the 'VF+' group or before the end of the 15th minute of ischaemia in the 'VF–' group. (B) Values were measured irrespective of the rhythm immediately before VF in the 'VF+' group or at equivalent time point in the 'VF–' group. All values shown as mean ± SEM. *$P < 0.05$ versus the 'VF–'.

Table 3

Predictive power, sensitivity and specificity of some of the parameters measured irrespective of the rhythm

Parameter	AUC	Sensitivity (%)	Specificity (%)
STV RT	0.886	93	80
STV QT	0.886	93	80
RMSSD QT	0.879	93	80
SDSD QT	0.879	93	80
STI QT	0.879	93	80
STI RT	0.871	93	80
RMSSD RT	0.864	93	80
SDSD RT	0.864	93	80
AB	0.829	86	70
Mean RR	0.814	93	70
Mean DI	0.807	86	70
'R on T'	0.804	71	70
LTI RR	0.800	86	80
STI RR	0.800	79	80

Parameters (derived from 40 consecutive ventricular complexes immediately before VF or at an equivalent time point in the 'VF–' group) were arranged in decreasing order of AUC. AB: frequency of arrhythmic beats, 'R on T': frequency of 'R on T' arrhythmic beats. *P* values for all AUCs are <0.05. For the *P* value, the null hypothesis was: true area = 0.5. For the cut-off values, confidence intervals and the predictive power of all other parameters measured, see Supporting Information Tables S1, S3 and S4.

Equivalent analysis of ECG intervals selected for sinus rhythm

The *sinus* mean ECG intervals and the *sinus* BVI parameters sampled from the last arrhythmia-free period either before VF in the 'VF+' group or before the end of 15th minute of ischaemia in the 'VF–' group notably revealed no significant differences between the 'VF+' and 'VF–' groups (Figures 3 and 5).

Frequency of arrhythmic beats and R on T arrhythmic beats

Before coronary occlusion and in the seventh minute of ischaemia, there were close to zero arrhythmic beats in all hearts, and there was no significant difference between the 'VF+' and 'VF–' groups. Before VF, the frequencies of arrhythmic beats and 'R on T' arrhythmic beats were high in both groups, and significantly greater in the 'VF+' group versus the 'VF–' group (Figure 6). However, the predictive power (AUC of the ROC curve), and the sensitivity and specificity of the frequencies of the arrhythmic beats and 'R on T' arrhythmic beats as predictors of VF (Table 3 and Supporting Information Table S4) were lower than the equivalent values for many of the *absolute* BVI parameters, especially those for ventricular repolarization (Table 3 and Supporting Information Table S3).

Morphological characterization of arrhythmic beats

Morphology of a VPB depends on its origin. The more variation in origin per cluster of VPBs, the larger the morphological variability. In order to quantify morphological variability, the voltage of the R wave and the QR, QT, QTx, RT and coupling interval of the last 40 arrhythmic beats before VF were measured. There was no significant difference in the morphology (any variable) of the arrhythmic beats between the 'VF–' and 'VF+' groups (data not shown).

Discussion

The *absolute* BVI parameters that refer to repolarization had a very high predictive power with high sensitivity and specificity for VF occurrence. In contrast, the variability and instability parameters measured in sinus rhythm did not predict VF. The frequency of the arrhythmic beats and 'R on T' arrhythmic beats increased significantly before VF occurrence. The increase in the *absolute* BVI parameters only partly resulted from the high number of arrhythmic beats as the frequency of arrhythmic beats alone was a weaker predictor of VF than many *absolute* BVI parameters.

Absolute BVI parameters predict drug-induced TdP (Farkas *et al.*, 2010). *Absolute* BVI parameters quantify electrical instability that correlates to substrate inhomogeneity, a key contributing factor to functional re-entry (Farkas *et al.*, 2010). As functional re-entry is common to TdP and phase I ischaemia-induced VF, it was hypothesized that *absolute* BVI parameters may predict phase I ischaemia-induced VF. The present data prove this hypothesis and show that repolarization-related *absolute* BVI parameters predict phase I ischaemia-induced VF with high sensitivity and specificity. The following sections consider the significance of the predictive properties of *absolute* BVI parameters.

BVI parameters measure temporal variability that correlates with spatial electrical inhomogeneity, the common substrate of phase I VF and TdP

It is widely accepted that ischaemic VF and TdP are re-entry arrhythmias (Gray *et al.*, 1995; Jalife and Berenfeld, 2004). *Spatial* electrical inhomogeneity provides the common substrate for TdP and phase I VF, although the mechanism of *spatial* inhomogeneity is different (Farkas and Nattel, 2010; Di Diego and Antzelevitch, 2011). BVI parameters quantify *temporal* variability and instability of the ECG intervals, therefore, one can question how these parameters are related to *spatial* electrical inhomogeneity of the myocardium. Earlier investigations showed that *temporal* (beat-to-beat) variability of repolarization caused by ischaemia or VPBs occurs inhomogeneously between the different regions of the heart, which produces *spatial* inhomogeneity in the myocardium and can lead to unidirectional block and VF (Hashimoto *et al.*, 1984; Rubenstein and Lipsius, 1995). This may explain the power of the absolute BVI parameters to predict the occurrence of phase I ischaemia-induced VF and TdP.

Absolute BVI parameters are better predictors of VF than frequency of non-sinus beats

Arrhythmic beats may not only trigger arrhythmias but also increase substrate inhomogeneity, facilitating VF (Chen *et al.*,

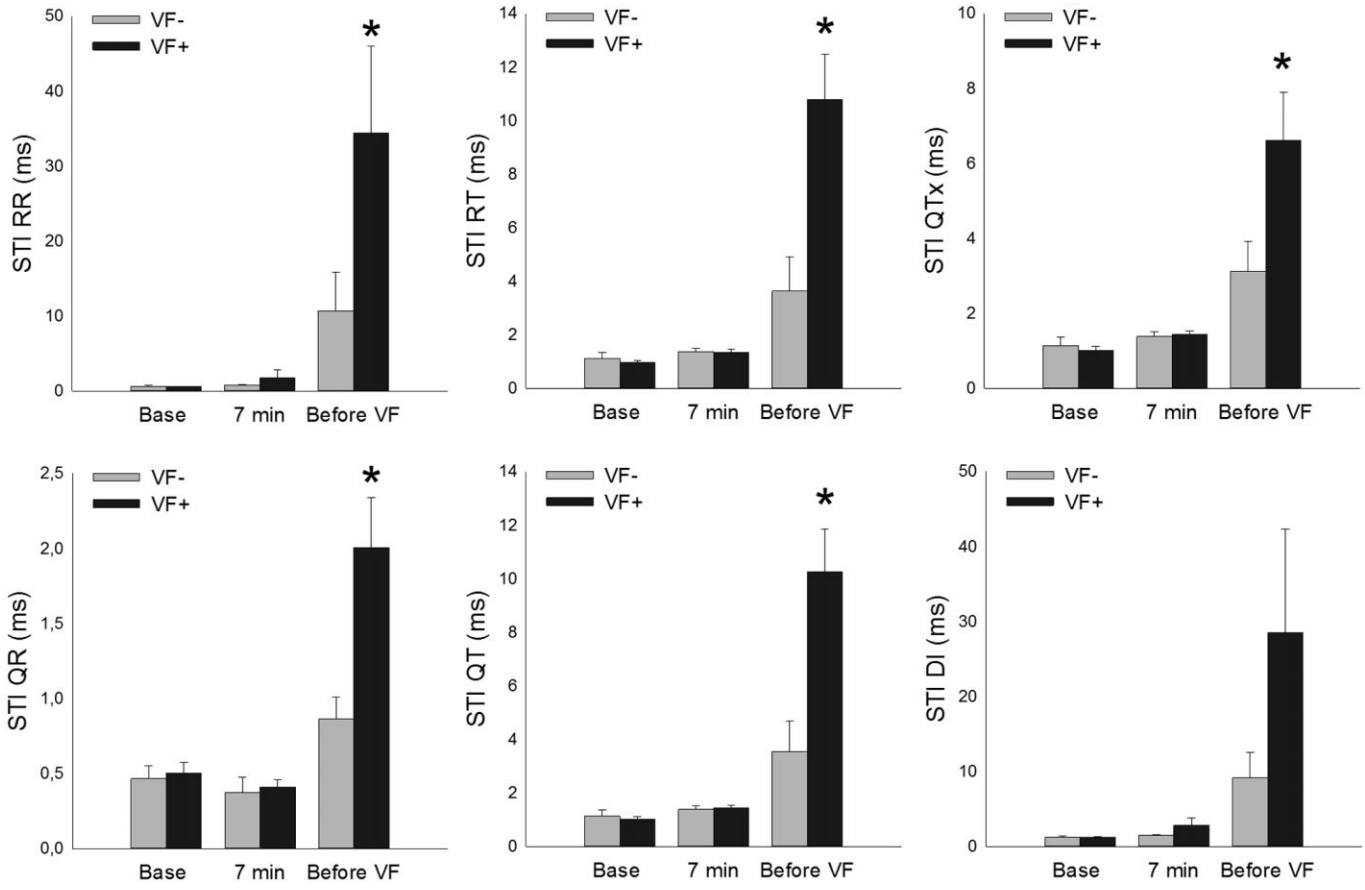

Figure 4

The *absolute* short-term instability (STI) parameters of the ECG intervals in the last minute before coronary occlusion (Baseline), in the seventh minute of the ischaemia (7 min), and immediately before VF occurred in the 'VF+' group or at an equivalent time point in the 'VF–' group (Before VF). All values are mean ± SEM. *$P < 0.05$ versus the 'VF–'.

2011). Therefore, one would anticipate that the frequency of arrhythmic beats would be an accurate predictor of re-entrant arrhythmias, for example, VF and TdP, but the present results and earlier findings have shown that this is not the case. Arrhythmic beat frequency *per se* contributes to TdP development, but does not predict TdP occurrence in anaesthetized rabbits (Farkas *et al.*, 2010). However, the more *variable* the arrhythmic beats in terms of coupling interval and shape, the greater the incidence of TdP. Thus, the more disorganized the ventricular rhythm, the greater the *absolute* BVI parameters and the probability of TdP (Farkas *et al.*, 2010). *Absolute* BVI variables predict TdP more accurately than arrhythmic beat frequency (Farkas *et al.*, 2010).

In the present study, arrhythmic beats were similar in the 'VF+' and 'VF–' groups in terms of coupling interval and morphology, but the frequencies of arrhythmic beats and 'R on T' beats predicted VF occurrence. However, frequencies were not the best predictors of VF; the *absolute* BVI parameters that refer to repolarization had greater sensitivity and specificity. Since inhomogeneity is determined by many factors, any parameter that quantifies only a single contributory factor (e.g. the frequency of arrhythmic beats) has *a priori* only limited power to predict VF and TdP. In contrast *absolute* BVI parameters measure the sum of every effect that increases substrate inhomogeneity, explaining why *absolute* BVI parameters were found to have superior predictive power for the occurrence of VF (and TdP).

Instability of the cycle length in irregular rhythms predicts VF

Three *absolute* instability parameters of the cycle length (TI, LTI, STI) predicted phase I VF. Previously, Lemmert *et al.* (2010) measured RR instability (RRI) irrespective of the rhythm among patients with acute ST elevation myocardial infarction. Although they found that VF patients had an increased RRI and also more VPBs, multivariate analysis revealed that only the RRI was independently associated with VF occurrence, which is in agreement with the present results and shows that irregularity in cycle length caused by ventricular arrhythmic activity may contribute to the initiation of VF (Farkas *et al.*, 2009; 2010).

Heart rate variability (HRV) can be confused with *absolute* BVI of RR intervals. However, HRV refers to the beat-to-beat variability of the cycle length (RR interval) in *sinus rhythm in vivo* and is a biomarker of parasympathetic tone (Kleiger *et al.*, 1987). *Absolute* BVI parameters are measured *irrespective of*

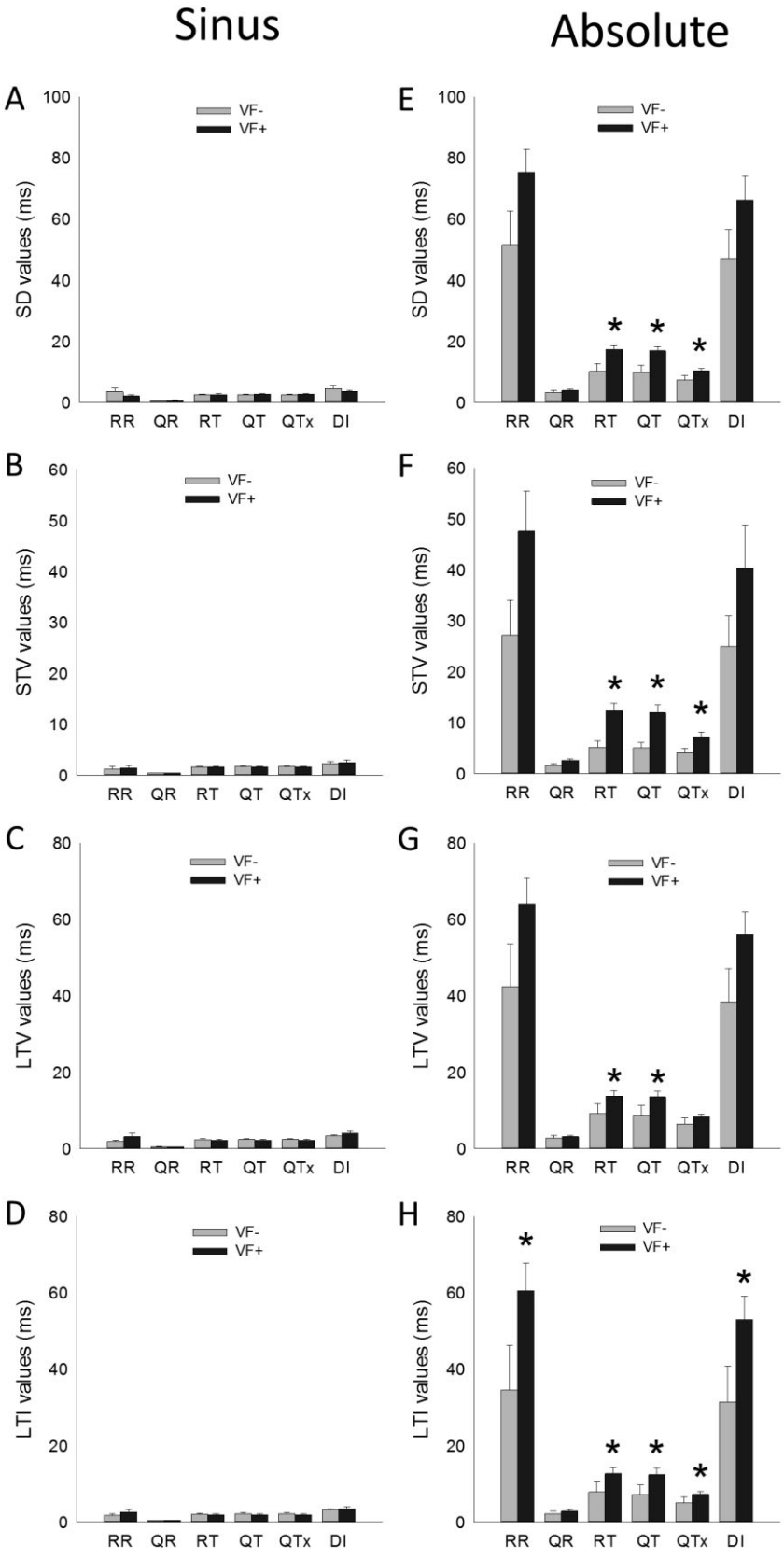

Figure 5

The *sinus* and *absolute* SD, STV, LTV and LTI parameters. (A–D) Values were determined in the last arrhythmia-free period either before VF in the 'VF+' group or before the 15th minute of ischaemia in the 'VF–' group. (E–H) Values were determined irrespective of the rhythm immediately before VF in the 'VF+' group or at an equivalent time point in the 'VF–' group. All values shown as mean ± SEM. *$P < 0.05$ versus the 'VF–'.

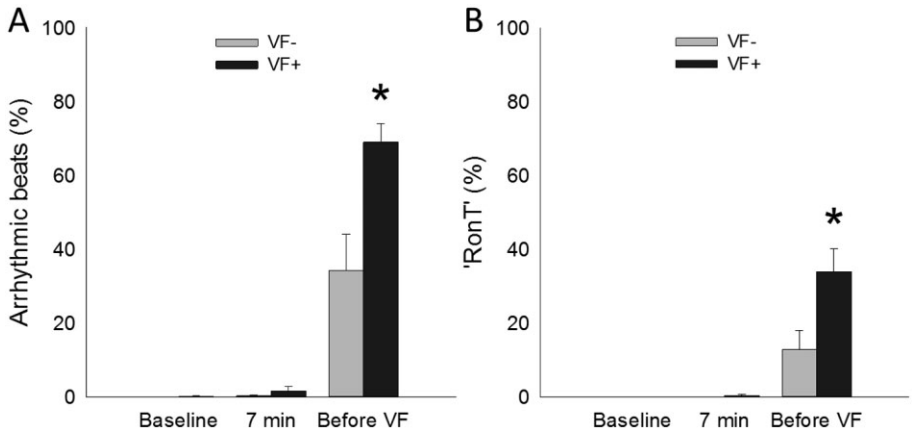

Figure 6

The frequency of arrhythmic beats (A) and 'R on T' arrhythmic beats (B) in 40 consecutive ventricular beats (shown as % of number of beats). All values are shown as mean ± SEM. *$P < 0.05$ versus the 'VF–'.

rhythm. Thus, *absolute* BVI parameters are affected by various factors, including the frequency, coupling interval and shape of the arrhythmic beats. Moreover, *absolute* BVI data (present study) come from isolated, denervated hearts, thus, the present data are free of autonomic influence. Decreased HRV is a long-term predictor of sudden cardiac death among patients with chronic heart disease (Kleiger *et al.*, 1987). In contrast, *absolute* BVI parameters and RRI (see above) are short-term predictors of VF in acute myocardial ischaemia.

Variability of repolarization in irregular rhythms predicts VF

Recently, there has been increased interest in validating changes in repolarization dynamics and variability to predict sudden cardiac death with many different algorithms examined (Hondeghem *et al.*, 2001; Thomsen *et al.*, 2004; van der Linde *et al.*, 2005). Almost all the methodologies consider only sinus rhythm or use a regular pacing protocol to determine repolarization instability (Thomsen *et al.*, 2004).

Absolute BVI parameters are measured without excluding ECG signals during non-sinus rhythm. The present results show that *absolute* BVI parameters that refer to repolarization had outstanding predictive power with great sensitivity and specificity for VF occurrence. VPBs cause instability in repolarization and action potential duration, and lead to heterogeneous distribution of DIs (Laurita *et al.*, 1998). Consequentially, conduction blocks occur, facilitating re-entry (Gilmour *et al.*, 2007). Chen *et al.* (2011) incorporated VPBs in the evaluation of the QT instability before ventricular tachycardia (VT) onset. They showed that an increased frequency of VPBs and an increased instability in QT interval dynamics measured irrespective of the rhythm preceded sustained VT onset in patients with acute myocardial infarction (Chen *et al.*, 2011). The results of Chen *et al.* (2011) and the present data with *sinus* and *absolute* BVI parameters emphasize that methods used for prediction of severe ischaemia-induced arrhythmias should not focus only on sinus or regular rhythm. In contrast, repolarization instability has high predictive power for occurrence of ischaemic VT or VF when determined irrespective of rhythm.

ECG analysis of intervals during irregular rhythm elaborates the mechanism of phase I ischaemia-induced VF

Analysis of ECG intervals during irregular rhythm revealed that a significant increase in frequency of the arrhythmic beats and 'R on T' VBPs, a significant shortening of the mean cycle length and electrical DIs, an increased BVI of cycle length and repolarization predicted phase I ischaemia-induced VF. These findings elaborate the mechanism of ischaemia-induced VF and accord with the results of earlier investigations. Laurita *et al.* (1998) showed that single and multiple VPBs increase spatial inhomogeneity of repolarization. Ischaemia *per se*, even without arrhythmic beats, produces great beat-to-beat variability of repolarization in the ischaemic area, while repolarization remains stable in non-ischaemic myocardium, resulting in a great spatial inhomogeneity between ischaemic and non-ischaemic myocardium (Dilly and Lab, 1988). Spatial inhomogeneity of repolarization caused by arrhythmic beats and ischaemia can provide the substrate for re-entry. Arrhythmic beats can also serve as triggers for re-entry (Chen *et al.*, 2011). High frequency of arrhythmic beats shortens average cycle length, whereas short cycle length aids the generation of delayed afterdepolarization-induced VPBs (Antzelevitch and Burashnikov, 2011). Also, short cycle length results in short DI. A greater variability of the duration of the repolarization together with a short DI facilitates re-entry.

Limitations and clinical value

Since repolarization in the rat ventricle differs substantially from that of man (Iost *et al.*, 1998; Gussak *et al.*, 2000), the present results may not be extrapolated directly to man.

Predicting VF more accurately during the minute before it occurs may not have obvious clinical value. However, the *absolute* BVI variables that we have validated could serve as surrogates for VF in pre-clinical drug investigations discovery, meeting a 3Rs objective (refinement). Furthermore, the validated biomarkers would be useful later in translational research to identify likely benefits of a new drug against VF in the larger low risk human population.

Conclusion

The novel *absolute* BVI parameters that predicted TdP liability in rabbits also predict VF liability during regional ischaemia in rat hearts, indicating a diagnostic and mechanistic congruence. Repolarization inhomogeneity appears to play a pivotal role in ischaemic VF induction since *absolute* BVI parameters that quantify repolarization variability had outstanding predictive power with high sensitivity and specificity. The newly validated biomarkers could serve as surrogates for VF in preclinical drug investigations.

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Conflict of interest

None.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

http://dx.doi.org/10.1111/bph.12579

Table S1 Power of *absolute* mean ECG intervals to predict VF.
Table S2 The *absolute* BVI parameters of the ECG intervals.
Table S3 Predictive power of the *absolute* BVI parameters of the ECG intervals.
Table S4 Predictive power of the frequencies of arrhythmic beats and 'R on T' arrhythmic beats.