ABSOLUTE BEAT-TO-BEAT VARIABILITY AND INSTABILITY PARAMETERS OF ECG INTERVALS PREDICT ISCHEMIA-INDUCED VENTRICULAR FIBRILLATION

Ph.D. thesis

Annamária Sarusi, MD

Szeged, Hungary

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Annamária Sarusi, MD

Supervisor: András Farkas, MD, Ph.D.

Doctoral School of Multidisciplinary Medical Sciences Department of Medicine & Department of Pharmacology and Pharmacotherapy

Albert Szent-Györgyi Medical School,
University of Szeged

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LIST OF PUBLICATIONS

The Ph.D. thesis is based on the following 'in extenso' publication:

I.) **Sarusi A**, Rárosi F, Szűcs M, Csík N, Farkas AS, Papp JG, Varró A, Forster T, Curtis MJ, Farkas A. Absolute beat-to-beat variability and instability parameters of ECG intervals: biomarkers for predicting ischaemia-induced ventricular fibrillation.
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III.) Takács H, Kui P, Farkas AS, **Sarusi A**, Forster T, Papp JG, Varró A, Curtis MJ, Shattock MJ, Farkas A.
Ventricular cycle length irregularity affects the correlation between ventricular rate and coronary flow in isolated, Langendorff perfused guinea pig hearts.
J Pharmacol Toxicol Methods. 2016 Jan-Feb;77:45-52.
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IV.) Kui P, Orosz S, Takács H, **Sarusi A**, Csík N, Rárosi F, Csekő C, Varró A, Papp JG, Forster T, Farkas AS, Farkas A.
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V.) Papp H, **Sarusi A**, Farkas AS, Takacs H, Kui P, Vincze D, Ivany E, Varro A, Papp JG, Forster T, Farkas A.
Hyperventilation assists proarrhythmia development during delayed repolarization in clofilium-treated, anaesthetized, mechanically ventilated rabbits.
J Physiol Pharmacol. 2016 Oct;67(5):731-737.
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VI.) Papp H, **Sarusi A**, Farkas AS, Polyák, Papp JGy, Varró A, Farkas A.
New proarrhythmia model based on reduced repolarization reserve in isolated guinea pig hearts.
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VII.) Polyák A, Topal L, Zombori-Tóth N, Tóth N, Prorok J, Kohajda Z, Déri S, Demeter-Haludka V, Hegyi P, Venglovecz V, Ágoston G, Husti Z, Gazdag P, Szlovák J, Árpádffy-Lovas T, Naveed M, **Sarusi A**, Jost N, Virág L, Nagy N, Baczkó I, Farkas AS, Varró A.
Cardiac electrophysiological remodeling associated with enhanced arrhythmia susceptibility in a canine model of elite exercise.
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Quotable abstracts

1. **Sarusi, A**; Farkas, A; Orosz, Sz; Forster, T; Varró, A; Farkas, A
   New in vitro proarrhythmia model that is based on reduced repolarization reserve
   Interventional Medicine and Applied Science 2011; 3: 3 pp. 157-157., 1 p.

2. **Sarusi, A**; Forster, T; Curtis, MJ; Farkas, A
   The absolute beat-to-beat variability and instability parameters of repolarization predict phase-1 ischaemic VF.
   Acta Physiologica 2011; 202: Suppl. 684 Paper: P73, 1 p.

3. **Sarusi, A**; Forster, T; Curtis, MJ; Farkas, A
   The absolute variability predicts phase I ischaemic ventricular fibrillation
   Cardiologia Hungarica 2011; 41: Suppl. F pp. F42-F43, 1 p.

4. **Sarusi, A**; Farkas, A; Orosz, Sz; Forster, T; Varró, A; Farkas, A
   Screening proarrhythmia - validation of new biomarkers and a new in vitro model based on reduced repolarization reserve
   Cardiologia Hungarica 2012; 42: Suppl. A pp. A34-A35, 1 p.

5. **Sarusi, A**; Farkas, A S; Orosz, Sz; Forster, T; Varró, A; Farkas, A
   Validation of a new in vitro proarrhythmia model based on reduced repolarization reserve
   Cardiovascular Research 2012; 93: Suppl. 1. pp. S104-S104. Paper: P532, 1 p.

6. Papp, H; **Sarusi, A**; Farkas, A; Forster, T; Varró, A; Farkas, A
   A new in vitro guinea-pig proarrhythmia model based on reduced repolarization reserve. Examination of the sensitivity of the model to drug-induced arrhythmias
   Cardiologia Hungarica 2013; 43: Suppl. B pp. B26-B27, 1 p.

7. Kui, P; Orosz, Sz; **Sarusi, A**; Csekő, Cs; Forster, T; Varró, A; Farkas, A; Farkas, A
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8. Iványi, E; **Sarusi, A**; Csík, N; Farkas, A; Forster, T; Varró, A; Farkas, A
   The proarrhythmic effects of sodium channel blockers can be represented by our newly developed biomarkers, the 'absolute' beat-to-beat variability and instability parameters
   Cardiologia Hungarica 2013; 43: Suppl. B pp. B19-B19, 1 p.

9. Kui, P; Orosz, Sz ; **Sarusi, A**; Csekő, Cs; Forster, T; Varró, A; Farkas, A; Farkas, AS
   Validation of an isolated guinea pig heart proarrhythmia model with reduced-repolarisation reserve
   Cardiologia Hungarica 2014; 44: Suppl. E pp. E30-E31, 1 p.

10. Kui, P; Orosz, Sz; **Sarusi, A**; Csekő, Cs; Forster, T; Varró, A; Farkas, A; Farkas, AS
    New in vitro model for proarrhythmia screening: I_{Ks} inhibition potentiates the QTc prolonging effect of I_{Kr} inhibitors in isolated guinea pig hearts
    Cardiovascular Research 2014; 103: Suppl.1 pp. S116-S116. Paper: P640, 1 p.

ACRONYMS AND ABBREVIATIONS

BVI: beat-to-beat variability and instability

VPB: ventricular premature beats

DI: diastolic interval

RMSSD: root mean square of successive differences

SDSD: standard deviation of successive differences

STV: short-term variability

LTV: long-term variability

TI: total instability

LTI: long-term instability

STI: short-term instability

ROC curve: receiver operating characteristic curve

AUC: area under ROC curve

RRI: RR instability

HRV: heart rate variability

APD: action potential duration

ABSTRACT

Background: ECG interval measurement is possible during arrhythmias. Beat-to-beat variability and instability (BVI) of ECG intervals measured irrespective of rhythm (*absolute* BVI) predict drug-induced torsades de pointes (TdP) more accurately than the same variables derived exclusively during sinus rhythm (*sinus* BVI) in rabbits. We have tested whether this approach predicts another stochastic arrhythmia event, ventricular fibrillation (VF), in a different pathophysiological setting.

Methods and Results: Langendorff perfused rat hearts were subjected to regional ischemia for 15 min. *Absolute* BVI parameters were derived from ECG intervals measured in 40 consecutive ventricular complexes (irrespective of the rhythm) immediately preceding VF onset and compared with values in time-matched ECGs in hearts that did not express VF. 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 were associated with VF occurrence. *Absolute* BVI parameters that quantify variability of repolarization (e.g. 'short-term variability' of QT interval) had the best predictive power with very high sensitivity and specificity. In contrast, VF was not predicted by any BVI parameter derived exclusively from sinus rhythm.

Conclusions: The novel *absolute* BVI parameters that predicted TdP liability in rabbits also predict VF liability during regional ischemia in rat hearts, indicating a diagnostic and mechanistic congruence. Repolarization inhomogeneity appears to play a pivotal role in ischemic VF induction since *absolute* BVI parameters that quantify repolarization variability had outstanding predictive power.

INTRODUCTION

In most cases, the cause of sudden cardiac death is acute ischaemia-induced ventricular tachyarrhythmia, primarily VF [1]. Predicting whether VF will occur during acute ischaemia has proven to be a difficult challenge. In the present investigation, it has been examined whether a method of predicting TdP liability, validated in rabbits [2], may be adapted to predict VF liability during early ischaemia.

The ability of ventricular repolarization variability to predict drug-induced TdP liability has been tested in different models, but until recently, analysis has been performed only on arrhythmia-free ECGs [3]. 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* proarrhythmia model [2], [4]. However, when beat-to-beat variability and instability of the ECG intervals were measured immediately before TdP onset, regardless of whether the rhythm was sinus or disorganized, and compared with exact time-matched intervals in animals without TdP, the TdP liability was accurately predicted [2]. To achieve this outcome, a method was developed to allow ECG intervals to be measured during disorganized non-sinus rhythm [2], [5]. This allowed established BVI parameters [2] to be derived *irrespective of rhythm*. To avoid confusion with published BVI parameters described by others, all of which are derived during stable rhythm (*sinus* BVI parameters), we coined the term "*absolute*" to describe the BVI parameters derived irrespective of rhythm [2].

The mechanism of the maintenance of TdP involves functional re-entrant circuits facilitated by an increase in the spatial dispersion of ventricular repolarization [6]. *Absolute* BVI parameters quantify electrical instability and define the substrate (electrical inhomogeneity) for re-entrant arrhythmias, e.g. TdP [2]. Although ischaemic VF and TdP differ in many respects, functional re-entry is common to both [6], [7]. In view of this, and in view of the absence of a reliable method for predicting ischaemia-induced VF liability, it has been tested whether the new arrhythmia biomarkers, the *absolute* BVI parameters, predict the occurrence of phase I ischaemia-induced VF (that which occurs during the first 30 min of ischaemia [8]) in isolated rat hearts. Thus, the present study provides a robust test for the hypothesis that *absolute* BVI parameters may be used to predict arrhythmia liability in a context wider than just a TdP-liable scenario.

Isolated, Langendorff perfused rat hearts have been frequently used to explore the mechanisms responsible for the initiation and maintenance of phase I ischaemic VF and to assay

non-class-III antiarrhythmic and proarrhythmic drugs [8], [9], [10], [11], and the model has been validated [8]. Although the rat has very different repolarization characteristics compared with the rabbit [8], [9], there is a strong similarity in the underlying mechanisms of VF in all mammalian species [12]; irrespective of the underlying source, wavebreak and re-entry account for the complex pattern of ventricular fibrillation [13]. Since the substrate of VF is electrical inhomogeneity in the rat, as it is in bigger mammalian species [12], [14], the rat heart is suitable for testing the *absolute* BVI parameters for VF prediction.

The present data were derived from an earlier investigation [10] with ECG records analyzed by the new method. Thus, the present investigation is a proof of concept, which meets the 3Rs objective of animal research. The analysis found that *absolute* BVI parameters predicted ischaemic VF, whereas sinus BVI parameters did not.

AIMS OF THE STUDY

The present study was designed primarily to test whether the new arrhythmia biomarkers, the *absolute* BVI parameters of the ECG intervals predict the occurrence of phase I ischaemia-induced VF in isolated rat hearts.

We also aimed to examine whether the mean ECG intervals measured irrespective of the rhythm, even during arrhythmias (*absolute* mean ECG intervals), are able to predict phase I ischaemia-induced VF in isolated rat hearts.

This investigation also sought to assess whether mean ECG intervals and derived BVI parameters measured in arrhythmia-free sinus rhythm are capable of predicting phase I ischaemia-induced VF in isolated rat hearts.

Furthermore, the study tested whether the frequency of ventricular premature beats, including ‘R on T’ arrhythmic beats, can predict phase I ischaemic VF in isolated rat hearts.

We also evaluated whether the multiplicity of ventricular premature beats contributes to the development of phase I ischaemic VF in isolated rat hearts.

Finally, we aimed to identify the ECG parameters that had the best predictive power, sensitivity and specificity for ischaemic VF occurrence in isolated rat hearts.

METHODS

3.1. Animals and General Experimental Methods

In the original study performed in isolated, Langendorff perfused rat hearts (n=24) there were two independent drug-free control groups that experienced the same protocol, that is local ischemia for 30 minutes. For methodical details, see the paper of the original study [10]. As there was no significant difference in basic data between the two drug-free control groups, we merged them into one group (explained later).

The animal-handling protocol was in accordance with the Guidance on the Operation of Animals (Scientific Procedures) Act 1986, London, UK. Male Wistar rats were anesthetized, their hearts excised, and perfused according to the Langendorff method, with a modified Krebs solution containing (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. A unipolar ECG was recorded by implanting one stainless-steel wire electrode into the centre of the region to become ischemic, with a second electrode connected to the aorta. Regional ischemia was induced by tightening a traction-type coronary occluder positioned on the left main coronary artery.

3.2. Experimental protocol

All hearts were initially perfused for 5 minutes with modified Krebs solution, then switched to the control solution, which contained the vehicle (ethanol and water, 0.18 mM and 0.06 mM, respectively) of the drugs tested in the earlier investigation. After an additional 5 minutes of perfusion, the left main coronary artery was occluded for 30 minutes.

3.3. Groups

In our earlier study, each control group (following the same protocol) consisted of 12 hearts. During the first 15 minutes, VF occurrence was similar in the two groups: 50% and 66.6% of the hearts. We compared basic data of the hearts from the two groups: coronary flow, RR, PR, and QT_{90} intervals at three determined time points, occluded zone size (in terms of percent of total ventricular weight), and onset time of the first ischemic VF (data not shown). As there were no significant differences in basic data between the two groups, we merged them into one group, resulting in a single group containing a total of 24 hearts.

ECG recordings were replayed, and the onset time of the first ventricular fibrillation (VF) was determined. VF was defined according to the Lambeth Conventions [15]: signal from which individual QRS deflections cannot be distinguished from one another (implying morphological instability) and from which rate can no longer be measured.

VF developed in 58% of the hearts during the first 15 minutes of ischemia. Hearts with and without VF in the first 15 minutes of ischemia were retrospectively allocated into a 'VF+' group containing 14 hearts and a 'VF-' group containing 10 hearts, respectively. Thus, in the first part of the study, group sizes became appropriate to compare data from hearts with and without VF.

In the second part of the study, we made a self-controlled comparison. The two hearts that did not experience VF during the 30-minute ischemic period were excluded from the analysis, and the ECG tracings of the remaining 22 hearts were examined at two predetermined time points. *Absolute* beat-to-beat variability parameters were compared between the last minute before coronary occlusion ('base') and immediately before VF occurred in the 30-minute ischemia after coronary occlusion ('30 min VF').

3.4. Measurement of the ECG intervals

After completing the arrhythmia analysis, ECG intervals were measured by the same trained expert in a blinded manner, without knowledge of whether the heart had experienced VF. The RR, QR, RT, QT, and electrical diastolic interval (DI) were measured by manually positioning on-screen marker lines. For definitions of the measured ECG intervals, see *Table 1*. Three vertical marker lines were placed 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.

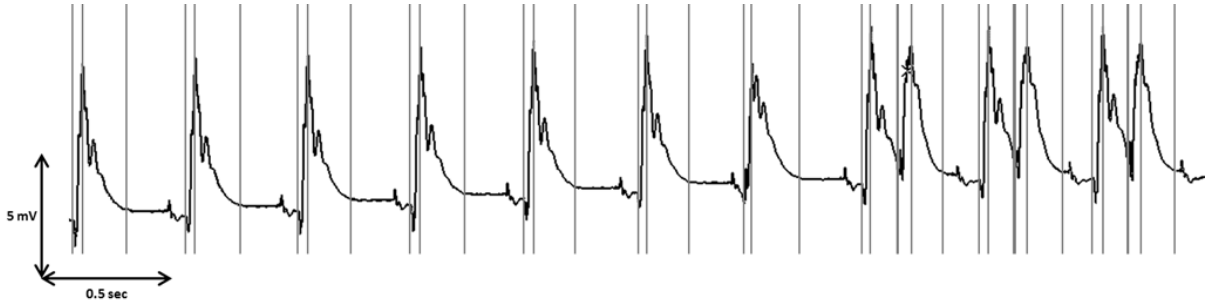

Figure 1. The vertical marker lines applied to measure ECG intervals in an ECG section recorded in an isolated rat heart.

On the surface ECG, the QT wave is the global manifestation of the ventricular action potential duration (APD). The JT interval (from the J point to the end of the T wave) represents the ventricular repolarization, where the J point is defined as the junction at which the QRS complex abruptly gives way to the ST segment [16]. In large mammals and humans, the depolarization and repolarization intervals are unequivocally separable by the J point. However, in small rodents, including rats and mice, this point is hardly determinable because the prominent I_{to} K^+ current causes rapid, phase 1 ventricular repolarization that merges into the depolarization [17]. Thus, the QRS and T waves are not separable, but incorporate into one QRT wave. The QT interval, defined as the time from the first deviation from the isoelectric line during the PQ interval until the end of the T wave (when the curve returns to the isoelectric line), is commonly used to describe the length of repolarization but also includes depolarization. To avoid this problem, we also determined the RT interval from the R peak to the end of the T wave. In our opinion, the RT interval better estimates the real repolarization as it does not contain the majority of the depolarization (QR interval) in rat hearts.

Table 1. Definition of the measured ECG intervals.

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
QTx	Interval between the first deviation of the QRT complex from the isoelectric line and the end of the extrapolated T wave
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.

During ventricular arrhythmias, the T wave frequently overlapped the QRT wave of the following beat. The QRT complex that interrupts the T wave of the preceding beat is called an ‘R on T’ ventricular premature beat (VPB). In these cases, the extrapolation method was used to describe the repolarization [18]: the end of the T wave was extrapolated from the curve of the T wave to the isoelectric line under the QRT complex. This extrapolated QT interval was labeled as QTx. The ventricular DI was defined as the time from the end of the T wave until the beginning of the following QRT complex. When the end of the T wave was chopped off by the subsequent QRT complex, the DI was arbitrarily set to 0.000001 ms (not 0 ms). (*Figure 2*)

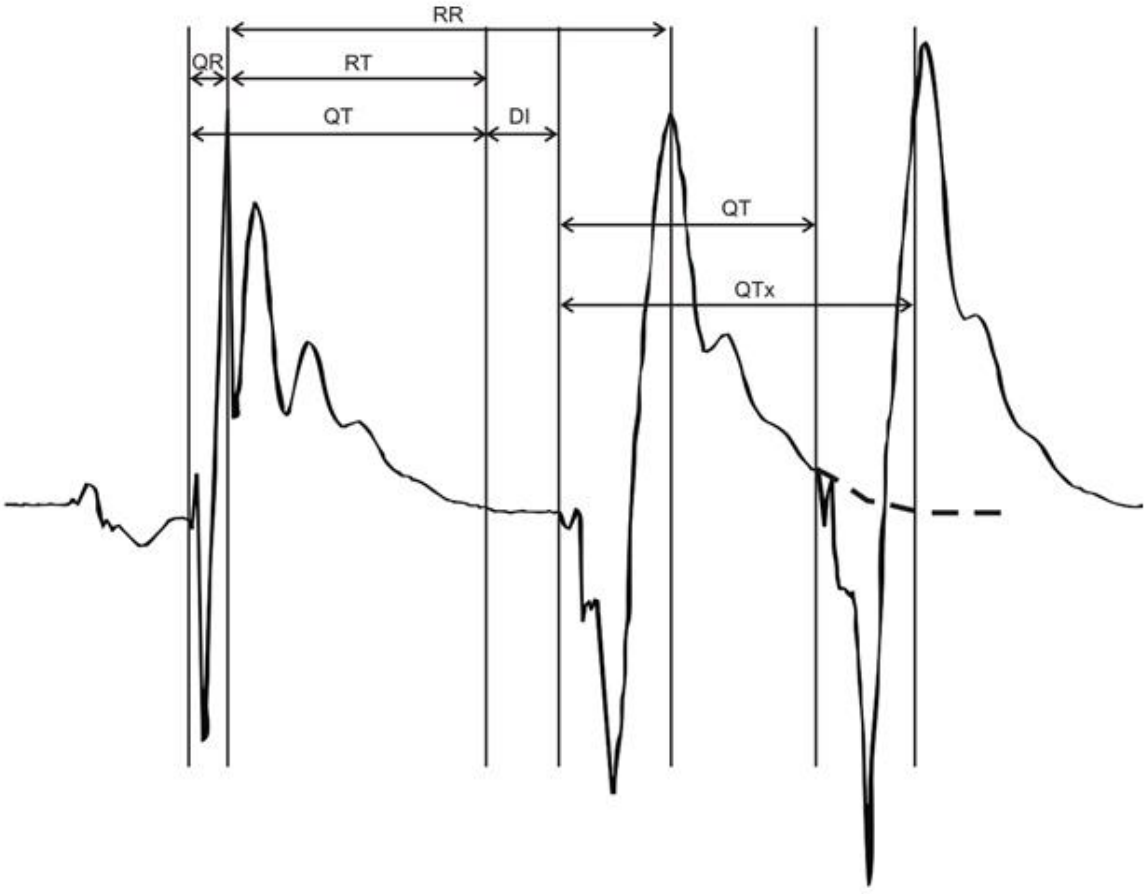

Figure 2. The measured ECG intervals in a short ECG section recorded in an isolated rat heart. The first beat is a normal sinus QRT complex with complete repolarization. The second beat is a ventricular premature beat that does not interrupt the previous repolarization process. The third beat is an ‘R on T’ ventricular premature beat that interrupts the repolarization curve of the second beat. The RR, QR, RT, QT, and DI intervals are labeled. The extrapolation method is shown at the second beat, where the T wave is interrupted by the third QRT complex. The T wave was extrapolated from the curve of the T wave to the isoelectric line under the QRT wave. The extrapolated QT interval was labeled QTx.

3.5. 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, QTx and DI intervals were measured. Then, the mean and the standard BVI parameters were derived as previously described. [19], [3], [20], [21] (*Table 2*). The following BVI parameters were calculated.

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

Root mean square (RMSSD) and standard deviation (SDSD) of successive differences

One approach to characterise 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 [19]: $\mathrm{RMSSD} = \sqrt{E([\Delta d]^2)},$ 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 visualise 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. [3]: $\mathrm{STV} = \frac{1}{N\sqrt{2}} \sum_{i=0}^{N-2} (d_{i+1} - d_i)$, 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 above, long-term variability 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. [3]: $\mathrm{LTV} = \frac{1}{N\sqrt{2}} \sum_{i=0}^{N-2} (d_{i+1} + d_i - 2E(d))$.

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 the paper of Van der Linde et al. [21].

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) [20].

The BVI parameters were defined as *sinus* when the 40 consecutive QRTs for analysis were selected to be all in sinus rhythm, and *absolute* when they were selected irrespective of the rhythm at predetermined time points [2]. *Sinus* BVI parameters were determined for the last arrhythmia-free period, either immediately before VF in the 'VF+' group or before the 15th minute of ischemia in the 'VF-' group. *Absolute* BVI parameters were determined during the last minute before coronary occlusion, the 7th minute of the ischemia, 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 the number per 40 beats times 100.

3.6. Morphological characterization of the arrhythmic beats before VF

The multiplicity of the arrhythmic beats was quantified by measuring the R voltage and the QR, QT, QTx, RT, and coupling intervals (CI) from manual measurement data on the last 40 arrhythmic beats immediately before VF occurrence (or at an equivalent time point in the 'VF-' group of hearts). The R wave of the QRT complex was defined as the wave with the greatest deflection from the isoelectric line within the QRT complex. The R wave was regarded as a negative wave when the greatest deflection of the QRT complex was negative. R voltage was defined as the voltage of the peak of the R wave from the isoelectric line. Coupling interval (CI) was defined as the distance between the peak of the R wave of the arrhythmic beat and the peak of the R wave of the preceding QRS complex. Mean and standard deviation of the ECG intervals were determined and compared.

3.7. Statistics

Continuous data were expressed as mean ± standard error of the mean (SEM). All data from independent samples were compared using Mann-Whitney tests. VF frequencies were compared using Fisher's exact probability test. Differences were considered statistically significant when $P<0.05$. Receiver operating characteristic (ROC) curve analysis [22] was performed to determine the predictive power of ECG variables, derived BVI parameters, and percent frequencies of arrhythmic beats and 'R on T' VPBs. Area under the ROC curve (AUC) and confidence interval for AUC were calculated using IBM SPSS 20 software (IBM Corporation, Armonk, New York, U.S.A.). Parameters with an AUC above 0.8 were considered to have a validated predictive value for VF occurrence. The optimal cut-off points were determined using Youden indexes [22]. The sensitivity of a parameter was estimated as the fraction of 'VF+' hearts that had a greater value (or in the case of the absolute mean ECG interval parameters, a smaller value) than the cut-off value. Specificity was estimated as the fraction of 'VF-' hearts that had a smaller value (or in the case of the absolute mean ECG interval parameters, a greater value) than the cut-off value [22].

RESULTS

4.1. Comparison between the ‘VF+’ and ‘VF-’ groups

4.1.1. Absolute mean ECG intervals

Before the coronary occlusion, there was no significant difference in the mean RR, QR, RT, QT, QTx, and DI intervals between the ‘VF+’ and ‘VF-’ groups of hearts. In the 7th minute of ischemia, the mean QR interval was significantly longer in the ‘VF+’ group as compared with the value measured in the ‘VF-’ group. There was no other difference between the ‘VF+’ and ‘VF-’ groups at this time-point. However, at the time point of ‘before VF’, the mean RR interval was significantly shorter in the ‘VF+’ group as compared with the value measured in the ‘VF-’ group. Furthermore, the QT and RT intervals were shorter in the ‘VF+’ group than the respective values in the ‘VF-’ group, but the QTx did not differ between the ‘VF+’ and the ‘VF-’ groups at the ‘before VF’ time point (*Figure 3*). This indirectly shows that the number of interrupted T waves (because of ‘R on T’ VPBs) was greater in the ‘VF+’ group than in the ‘VF-’ group at this time point (see ‘R on T’ data later).

ROC analysis showed that among the derived *absolute* mean ECG interval parameters, only the absolute mean RR and the *absolute* mean DI parameters 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*). Our analysis found that when the mean RR interval was shorter than 193 ms or the mean DI interval was shorter than 94 ms, there was a high probability of VF occurring in isolated rat hearts subjected to local ischemia.

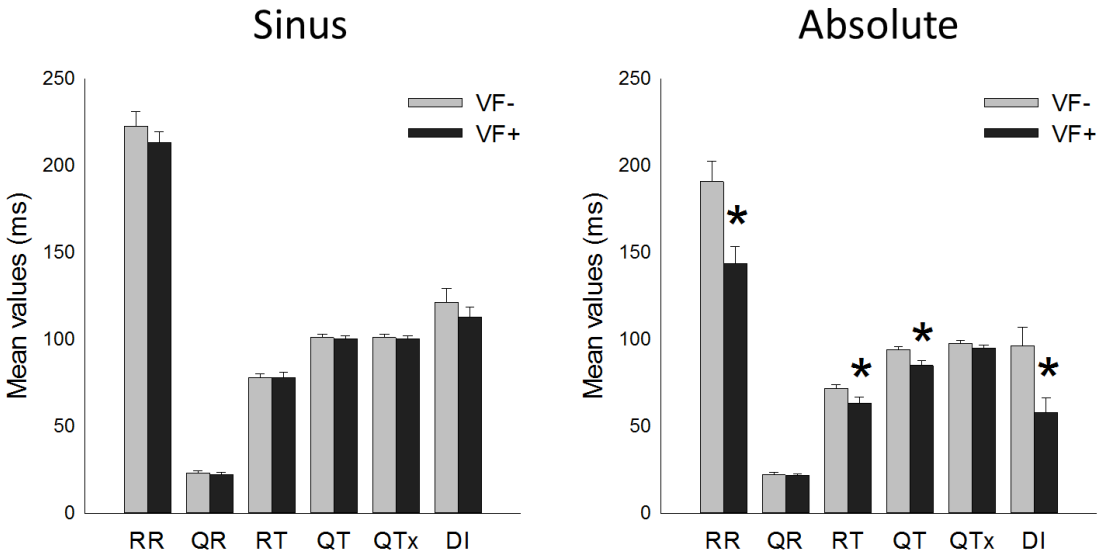

Figure 3. The *sinus* and *absolute* mean ECG intervals. Part A: Values were measured in sinus rhythm in the last arrhythmia-free period either before VF in the 'VF+' group or before the end of 15th minute of ischemia in the 'VF-' group. Part B: Values were measured irrespective of the rhythm immediately 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 vs. the 'VF-'.

Table 3. Power of the *absolute* mean ECG intervals to predict VF.

Parameter	AUC	p-value	95% CI		Cutoff (ms)	Sensitivity	Specificity
			Lower Bound	Upper Bound			
Mean RR	0.814	0.010	0.631	0.998	193	93%	70%
Mean DI	0.807	0.012	0.628	0.986	94	86%	70%
Mean RT	0.764	0.030	0.571	0.957	76	93%	50%
Mean QT	0.746	0.043	0.547	0.946	95	86%	60%
Mean QTx	0.632	0.279	0.395	0.870	97	64%	70%
Mean QR	0.557	0.639	0.315	0.799	23	64%	70%

Parameters (derived from 40 consecutive ventricular complexes irrespective of the rhythm immediately before VF or at an equivalent time point in the 'VF-' group of hearts) were arranged in decreasing order of AUC value. For the p value, the null hypothesis was: true area=0.5. A 95% confidence interval (CI) was determined for the AUC. Sensitivity and specificity values were calculated for the given cut-off value.

4.1.2. Absolute BVI parameters of the ECG intervals

Before coronary occlusion (‘base’) and in the 7th minute of ischemia, the derived *absolute* BVI parameters were low and did not differentiate between the ‘VF+’ and ‘VF-’ groups of hearts. *Absolute* BVI parameters increased during ischemia in both the ‘VF+’ and ‘VF-’ groups, but the increment was significantly greater in the ‘VF+’ group (*Tables 4 and 5*; *Figures 4 and 5*). The instability of the repolarization was prominent because all the *absolute* BVI parameters that represent the repolarization (QT, RT, and QTx), except LTV of QTx, were significantly greater in the ‘VF+’ group than the respective values in the ‘VF-’ group (*Table 5*). Additionally, AUC values of all *absolute* BVI parameters of QT and RT intervals were greater than 0.8 (*Table 6*). STV, RMSSD, SDSD, and STI parameters of RT and QT intervals had very high predictive power for VF with 93% sensitivity and 80% specificity. The lower AUC values of the *absolute* BVI parameters of the extrapolated QT interval (QTx) compared with the non-extrapolated QT interval indicated a relatively lower predictive power of the *absolute* BVI parameters of QTx interval (*Table 6*). Furthermore, three instability parameters (TI, LTI, and STI) of the cycle length (RR interval) were also significantly greater in hearts that experienced VF as compared with the respective values in the ‘VF-’ group of hearts (*Tables 4 and 5*; *Figures 4 and 5*). Although the predictive power values (AUC values) of the *absolute* BVI parameters of the RR interval were unequivocally weaker than the predictive power of the QT and RT interval parameters, the AUC values of LTI RR and STI RR were greater than 0.8 (*Table 6*).

Table 4. The *absolute* BVI parameters of the ECG intervals.

	RR		QR		RT		QT		QTx		DI	
	VF-	VF+	VF-	VF+	VF-	VF+	VF-	VF+	VF-	VF+	VF-	VF+
SD (ms)	51±11	75±7	3.2±0.7	3.8±0.4	10.1±2.5	17.3±1.3*	9.7±2.4	16.9±1.4*	7.2±1.5	10.3±0.8*	47±9	66±8
RMSSD (ms)	63±14	99±14	3.8±0.6	5.1±0.5	10.3±2.1	23.1±2.4*	9.9±2.0	22.2±2.4*	7.8±1.6	13.1±1.5*	59±13	89±15
SDSD (ms)	64±14	100±14	3.9±0.6	5.2±0.6	10.4±2.2	23.4±2.4*	10.0±2.0	22.5±2.4*	7.9±1.6	13.3±1.5*	60±13	90±15
STV (ms)	27±7	48±8	1.6±0.3	2.6±0.3	5.2±1.2	12.4±1.5*	5.0±1.1	11.9±1.5*	4.0±0.9	7.2±0.9*	25±6	40±8
LTV (ms)	42±11	64±7	2.7±0.7	3.1±0.3	9.1±2.6	13.7±1.4*	8.7±2.6	13.5±1.5*	6.3±1.6	8.3±0.7	38±9	56±6
TI (ms)	50±15	86±10*	2.8±0.7	4.2±0.5	10.2±3.0	20.1±1.9*	9.5±2.8	19.5±1.8*	7.5±1.9	11.5±1.2*	45±13	75±12
LTI (ms)	35±12	61±7 *	2.2±0.6	2.8±0.5	7.8±2.6	12.6±1.7*	7.1±2.6	12.4±1.7*	5.0±1.5	7.2±0.7*	31±9	53±6 *
Inst. (ms)	62±21	110±16	3.6±1.1	4.9±0.8	11.7±3.6	24.9±2.9*	11.5±3.3	23.6±2.7*	9.1±2.8	13.0±1.4*	54±17	93±19

Values were measured immediately before VF occurred in the 'VF+' hearts (n=10) or at an equivalent time point in the 'VF-' hearts (n=14). All values are mean±SEM. *P<0.05 vs. 'VF-'.

Table 5. Statistical significance of differences in the *absolute* BVI parameters of the ECG intervals between the 'VF+' and 'VF-' groups.

	RR	QR	RT	QT	QTx	DI
SD			X	X	X	
RMSSD			X	X	X	
SDSD			X	X	X	
STV			X	X	X	
LTV			X	X		
TI	X		X	X	X	
LTI	X		X	X	X	X
STI	X	X	X	X	X	
Inst.			X	X	X	

X means that the parameter for the ECG interval is significantly increased (P<0.05) in the 'VF+' group as compared with that in the 'VF-' group. Values were determined immediately before VF occurred in the 'VF+' hearts or at an equivalent time point in the 'VF-' hearts. For the numerical data see Table 4, Figure 4 and 5.

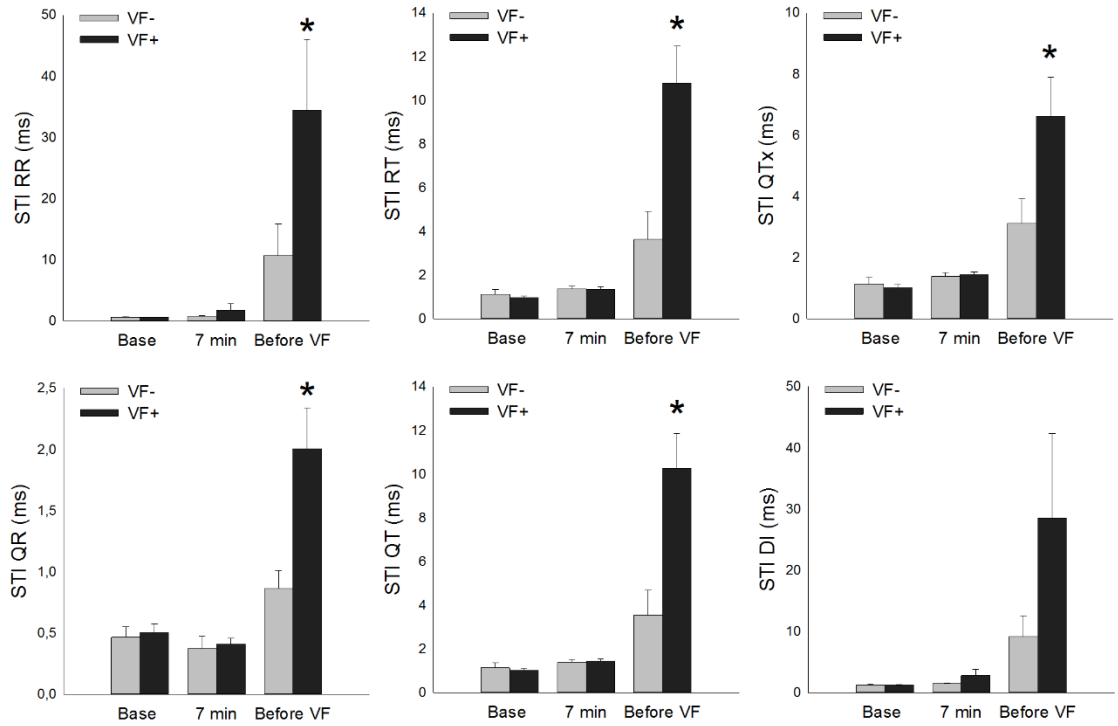

Figure 4. The *absolute* short-term instability (STI) parameters of the ECG intervals in the last minute before coronary occlusion (Base), in the 7th minute of the ischemia (7 min), and immediately before VF occurred in the 'VF+' group or at an equivalent time point in the 'VF-' group (Before VF). All values are mean ± SEM. *P < 0.05 vs. the 'VF-'.

Table 6. Predictive power of the *absolute* BVI parameters of the ECG intervals

Table A

Absolute BVI parameters	AUC	p-value	95% CI		Cutoff	Sensitivity	Specificity
			Lower Bound	Upper Bound			
STV RT	0.886	0.002	0.744	1.000	6.5 ms	93%	80%
STV QT	0.886	0.002	0.744	1.000	6.6 ms	93%	80%
RMSSD QT	0.879	0.002	0.736	1.000	13.3 ms	93%	80%
SDSD QT	0.879	0.002	0.736	1.000	13.4 ms	93%	80%
STI QT	0.879	0.002	0.735	1.000	3.3 ms	93%	80%
STI RT	0.871	0.002	0.715	1.000	3.0 ms	93%	80%
RMSSD RT	0.864	0.003	0.710	1.000	14.9 ms	93%	80%
SDSD RT	0.864	0.003	0.710	1.000	15.1 ms	93%	80%
TI QT	0.843	0.005	0.649	1.000			
SD QT	0.843	0.005	0.647	1.000			
STI QR	0.836	0.006	0.673	0.998	1.1 ms	79%	70%
SD RT	0.836	0.006	0.637	1.000			
STV QTx	0.832	0.006	0.650	1.000	4.9 ms	86%	80%
STI QTx	0.821	0.008	0.640	1.000	3.2 ms	86%	80%
Inst. QT	0.821	0.008	0.627	1.000			
LTI QT	0.814	0.010	0.613	1.000			
TI RT	0.814	0.010	0.610	1.000	12.1 ms	86%	80%
Inst. RT	0.814	0.010	0.621	1.000			
LTI RT	0.807	0.012	0.605	1.000			
LTV RT	0.807	0.012	0.605	1.000			
LTI RR	0.800	0.014	0.592	1.000	35.4 ms	86%	80%
LTV QT	0.800	0.014	0.597	1.000			
LTI QTx	0.800	0.014	0.596	1.000			
STI RR	0.800	0.014	0.609	0.991	12.7 ms	79%	80%

Table B

Absolute BVI parameters	AUC	p-value	95% CI	
			Lower Bound	Upper Bound
RMSSD QTx	0.786	0.019	0.584	0.987
SDSD QTx	0.786	0.019	0.584	0.987
LTI DI	0.779	0.022	0.558	1.000
SD QTx	0.764	0.030	0.544	0.985
TI QTx	0.757	0.035	0.534	0.981
Inst. QTx	0.757	0.035	0.519	0.995
TI RR	0.743	0.046	0.525	0.961
Inst. RR	0.736	0.053	0.523	0.949
LTV RR	0.736	0.053	0.519	0.953
LTV QTx	0.736	0.053	0.513	0.959
STV QR	0.729	0.061	0.525	0.932
TI QR	0.729	0.061	0.494	0.963
LTV DI	0.721	0.069	0.502	0.941
TI DI	0.707	0.089	0.480	0.934
Inst. QR	0.707	0.089	0.470	0.945
SD RR	0.700	0.101	0.479	0.921
LTI QR	0.693	0.114	0.453	0.933
STV RR	0.679	0.143	0.455	0.902
RMSSD QR	0.671	0.160	0.443	0.900
SDSD QR	0.671	0.160	0.443	0.900
Inst. DI	0.657	0.198	0.429	0.885
RMSSD RR	0.657	0.198	0.423	0.891
SDSD RR	0.657	0.198	0.423	0.891
LTV QR	0.657	0.198	0.406	0.909
RMSSD DI	0.650	0.219	0.413	0.887
SDSD DI	0.650	0.219	0.413	0.887
SD QR	0.650	0.219	0.401	0.899
SD DI	0.650	0.219	0.418	0.882
STV DI	0.629	0.292	0.388	0.869
STI DI	0.614	0.349	0.379	0.849

Parameters were arranged in decreasing order of AUC and were determined immediately before VF (or at an equivalent time point in the 'VF-' group of hearts). AUCs in Table A and Table B are above and below 0.8, respectively. Sensitivity and specificity values were calculated for some of the cut-off values if the AUC was higher than 0.8.

4.1.3. 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 15th minute of

ischemia in the 'VF-' group, notably revealed no significant differences between the 'VF+' and 'VF-' groups (*Figures 3 and 5*).

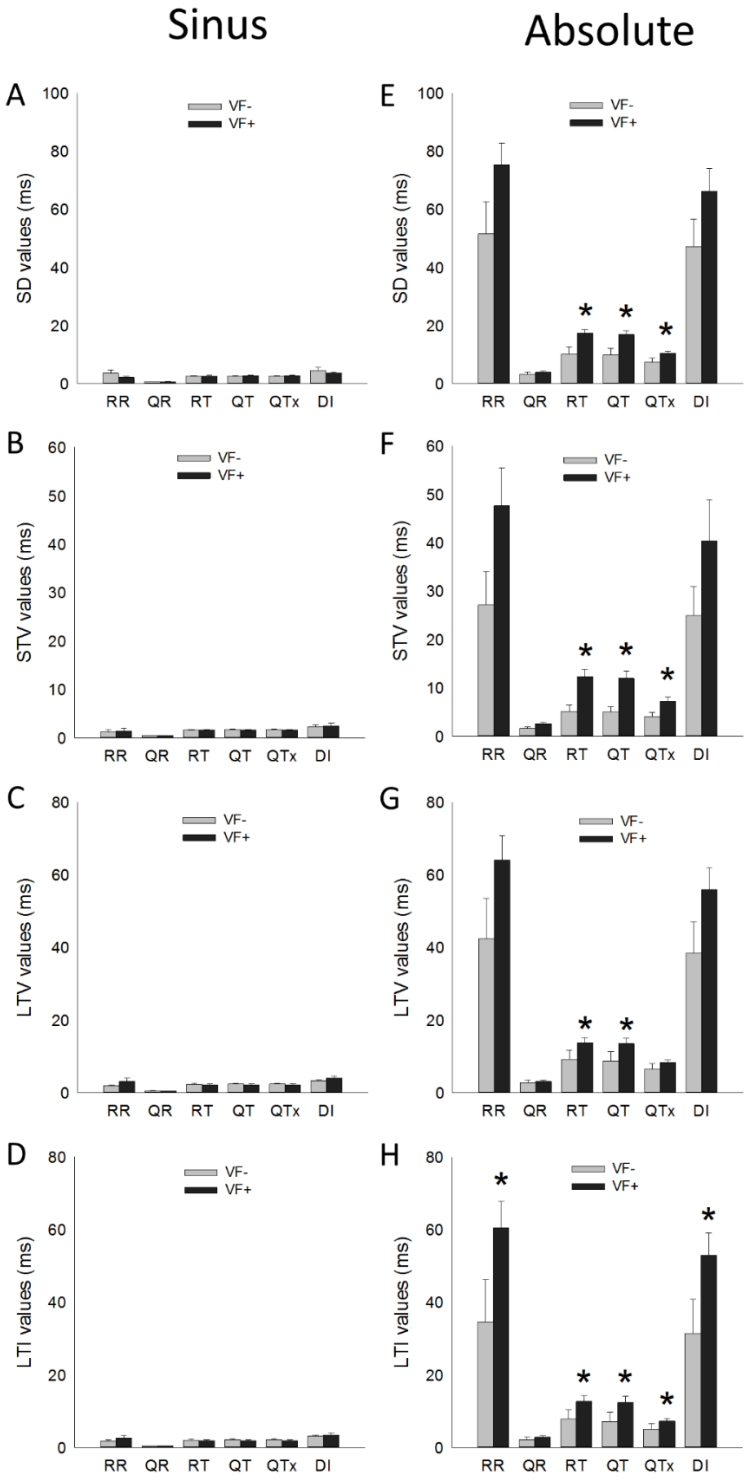

Figure 5. The *sinus* and *absolute* SD, STV, LTV and LTI parameters. Parts A-D: Values were determined in the last arrhythmia-free period either before VF in the 'VF+' group or before the 15th minute of ischemia in the 'VF-' group. Parts E-H: Values were determined irrespective of the rhythm immediately 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 vs. the 'VF-'.

4.1.4. Frequency of arrhythmic beats and R on T arrhythmic beats

Before coronary occlusion and in the 7th minute of ischemia, there were close to zero arrhythmic beats in all hearts, with 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 as compared with those in the 'VF-' group (*Figure 6*). However, the predictive power (AUC of the ROC), sensitivity, and specificity of the frequencies of arrhythmic beats and 'R on T' arrhythmic beats as predictors of VF were lower than those of many of the *absolute* BVI parameters, especially those related to ventricular repolarization (*Table 7*).

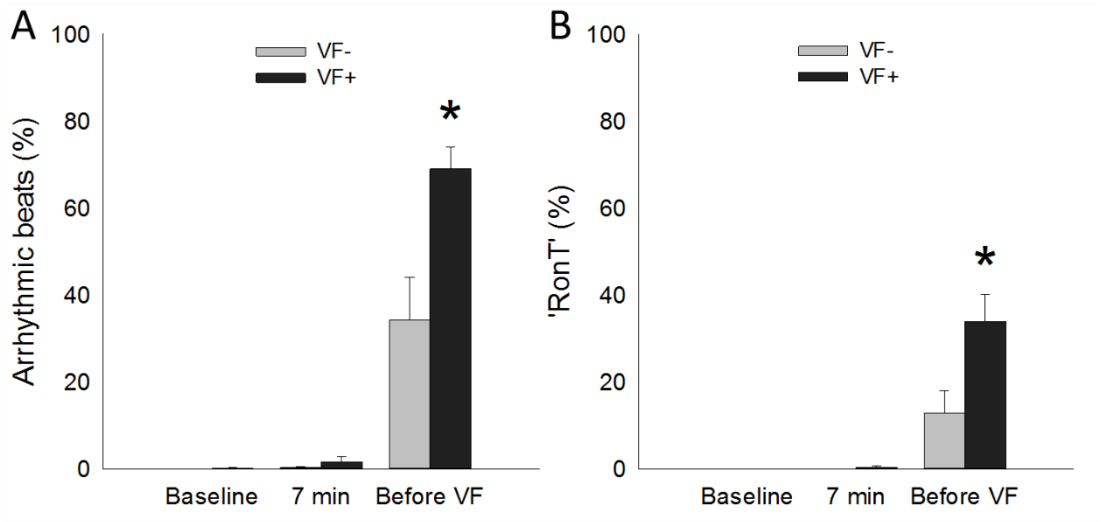

Figure 6. Percent frequency of arrhythmic beats (part A) and 'R on T' arrhythmic beats (part B) in 40 consecutive ventricular beats. All values are shown as mean ± SEM. *P < 0.05 vs. the 'VF-'.

Table 7. 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 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.

4.1.5. Morphological characterization of arrhythmic beats

The morphology of a VPB depends on its origin. The higher the number of regions the VPBs come from, the larger the morphological variability the VPBs show. To quantify this multiplicity, we characterized the arrhythmic beats. R voltage, as well as the QR, QT, QTx, RT, and coupling intervals (CI) of the last 40 arrhythmic beats were measured immediately before VF occurrence in the 'VF+' group and at an equivalent time point in the 'VF-' group. Comparing the 'VF-' and 'VF+' groups, we did not notice any significant difference in the R wave amplitude, ECG intervals, and coupling intervals of the arrhythmic beats between the groups (data not shown).

4.2. Self-controlled comparison of the *absolute* BVI parameters

To examine the changes in the *absolute* BVI parameters from baseline to VF occurrence, we conducted a self-controlled within-group comparison in the 22 control hearts that experienced VF during the entire 30-minute ischemic period. There was an immense increase in all *absolute* BVI parameters from the baseline ('base') to VF occurrence ('30 min VF') (*Table 8*).

Table 8. Within-group comparison of the *absolute* variability and instability parameters of the ECG intervals between the 'base' and '30 min VF' time points.

	RR			QR			RT		
	Base	30 min VF		Base	30 min VF		Base	30 min VF	
SD	2.3 ± 0.7	68.2 ± 6.9	*	0.8 ± 0.1	3.4 ± 0.3	*	1.9 ± 0.2	15.8 ± 1.1	*
RMSSD	2.7 ± 0.9	90.8 ± 13.4	*	1.0 ± 0.1	4.5 ± 0.5	*	2.3 ± 0.2	20.5 ± 1.9	*
SDSD	2.7 ± 0.9	91.9 ± 13.6	*	1.0 ± 0.1	4.5 ± 0.5	*	2.4 ± 0.2	20.7 ± 1.9	*
STV	1.1 ± 0.3	45.4 ± 8.4	*	0.5 ± 0.0	2.2 ± 0.3	*	1.2 ± 0.1	11.0 ± 1.2	*
TI	1.9 ± 0.6	76.9 ± 10.1	*	0.8 ± 0.0	3.6 ± 0.4	*	2.0 ± 0.2	18.1 ± 1.5	*
LTI	1.6 ± 0.5	49.2 ± 6.0	*	0.5 ± 0.0	2.4 ± 0.3	*	1.2 ± 0.1	11.0 ± 1.4	*
Inst.	2.0 ± 0.5	96.1 ± 15.5	*	0.7 ± 0.1	4.1 ± 0.6	*	2.1 ± 0.3	22.4 ± 2.6	*

	QT			QTx			DI		
	Base	30 min VF		Base	30 min VF		Base	30 min VF	
SD	1.8 ± 0.2	15.4 ± 1.1	*	1.8 ± 0.2	8.9 ± 0.7	*	3.2 ± 0.7	59.2 ± 6.9	*
RMSSD	2.3 ± 0.2	19.9 ± 1.9	*	2.3 ± 0.2	11.0 ± 1.2	*	3.8 ± 0.8	80.3 ± 13.2	*
SDSD	2.3 ± 0.2	20.1 ± 2.0	*	2.3 ± 0.2	11.2 ± 1.2	*	3.8 ± 0.9	81.4 ± 13.4	*
STV	1.3 ± 0.1	10.8 ± 1.3	*	1.3 ± 0.1	6.0 ± 0.7	*	1.8 ± 0.3	38.4 ± 8.1	*
TI	2.1 ± 0.2	17.6 ± 1.5	*	2.1 ± 0.2	9.8 ± 1.0	*	3.1 ± 0.5	65.3 ± 10.6	*
LTI	1.5 ± 0.2	10.6 ± 1.4	*	1.5 ± 0.2	6.2 ± 0.6	*	2.4 ± 0.5	42.5 ± 5.2	*
Inst.	2.3 ± 0.2	21.7 ± 2.5	*	2.3 ± 0.2	11.5 ± 1.1	*	3.5 ± 0.5	80.6 ± 16.2	*

The ECG tracings of the 22 control hearts that experienced VF were examined at two predetermined time points; *absolute* beat-to-beat variability parameters were compared between the last minute before coronary occlusion ('base') and immediately before VF occurred in the 30-minute long ischaemia after coronary occlusion ('30 min VF'). *P < 0.05 '30 min VF' vs. 'base', Wilcoxon test. For further details, see Tables 1-2.

DISCUSSION

According to our results, there was a strong relationship between instability of repolarization and VF occurrence in isolated rat hearts subjected to local ischemia. *Absolute* BVI parameters of ECG intervals that refer to repolarization had very high predictive power with high sensitivity and specificity for VF occurrence. In contrast, variability and instability parameters measured in sinus rhythm did not differentiate between the 'VF+' and 'VF-' groups of hearts and did not predict VF.

An increased number of preceding VPBs and 'R on T' VPBs were observed just before VF in hearts that subsequently experienced VF, but the characteristics of the preceding arrhythmic beats in terms of coupling interval and shape did not differ between the 'VF+' and 'VF-' groups. 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. The following sections consider the significance of the predictive properties of absolute BVI parameters.

5.1. Functional re-entry is the common mechanism of phase I VF and TdP; absolute BVI parameters quantify substrate instability contributing to functional re-entry

Recently, we developed new biomarkers, the *absolute* BVI parameters of the ECG intervals, which quantify the beat-to-beat electrical instability of the myocardium [2]. Beat-to-beat electrical instability (temporal instability) results in spatial electrical inhomogeneity, which is a key contributing factor to functional re-entry [3]. *Absolute* BVI parameters strongly correlated with the probability of TdP occurrence in a commonly used, *in vivo* proarrhythmia rabbit model [2]. Earlier investigations provided evidence that the maintenance mechanism of the TdP type ventricular tachycardia involves functional re-entry circuits [23], [24]. Additionally, it is widely accepted and documented that re-entrant excitation can also be the source of ischaemic ventricular fibrillation [7], [25]. A substrate (maintenance mechanism) and a trigger mechanism are required for a re-entrant arrhythmia [26]. The substrate (functional re-entry) is produced by spatial electrical inhomogeneity, though the mechanism of spatial inhomogeneity is different in TdP and phase I VF. In the case of TdP, variety of genetic mutations, electrical remodelling and numerous drugs can affect the function of cardiac ion channels *globally* eventuating spatiotemporal inhomogeneity of the repolarization [27]. During myocardial ischemia, *regional* heterogeneities in excitability, conduction and refractoriness

caused by hypoxia, acidosis, and elevated extracellular potassium levels can generate spatial inhomogeneity [26], [28]. The distinction is real, but insubstantial. Inhomogeneity is the common substrate of TdP and phase I VF. In both settings, arrhythmia triggers are VPBs that occur during the vulnerable period of the repolarization process. In the presence of electrophysiological or structural inhomogeneity these triggers may initiate re-entrant arrhythmias [29]. As functional re-entry is common to TdP and phase I ischaemia induced VF, it was hypothesized that absolute BVI parameters that quantify temporal electrical instability 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 isolated, Langendorff perfused, acute regional ischemic rat heart model has been used extensively for the study of the pathophysiology of myocardial ischemia and ischemia-induced arrhythmias, e.g. VF [8], [9], [11], [30]. Under experimental conditions, it is well established that after coronary occlusion two temporally distinct phases of the VF occurrence evolve. During the first 2 to 30 minutes (phase I) the myocardial infarction is reversible and VF appears to arise from re-entry [31], [32]. During this phase, VF susceptibility of isolated hearts is similar to *in vivo* hearts [8]. Phase II (after more than 90 minutes of ischemia) is the infarct evolution phase and hardly ever arises in isolated hearts [33]. While VF is frequently associated with acute myocardial infarction (MI), it can also occur in different heart diseases [34]. Although substrates and triggers may differ in VF associated with different diseases, experimental models or phases of MI, the main mechanism, the re-entrant activation is common. In this regard, we can expect that our findings about VF prediction with absolute BVI parameters in this experimental approach may be valid in other experimental and human settings. However, this needs further examinations to prove.

5.2. The predictive value and the role of arrhythmic beats in the development of ischaemic VF

In our study, myocardial ischemia *per se* caused a notable increase in the incidences of arrhythmic beats and ‘R on T’ VPBs. However, their frequencies were significantly greater in the ‘VF+’ group compared to the ‘VF-’ group. Additionally, the heart rate before VF increased as a result of frequent arrhythmic activity. The ‘R on T’ phenomenon was introduced in the 1940s to label early ventricular premature beats that interrupt the T wave of the preceding beat

[35]. ‘R on T’ VPBs have previously been shown to predict the occurrence of VF. ‘R on T’ VPBs were implicated as the most dangerous form of ventricular ectopic beats that could lead to lethal ventricular tachyarrhythmia and sudden cardiac death [36], [37], [38]. The increased number of ‘R on T’ VPBs was associated with VF occurring in the acute phase of myocardial ischemia. For example, the onset of VF was almost always initiated by closely coupled ‘R on T’ VPBs under conditions of ST-segment elevation in an arterially perfused canine right ventricular wedge model with regional ischemia [39]. The registry of ambulatory sudden cardiac death [40] and *in vitro* isolated heart studies [41] described a significant increase in the number of VPBs before VF. On the other hand, other experimental and clinical studies have questioned the unique value of ‘R on T’ VPBs in predicting VF [42]. It has also been proven that ‘R on T’ VPBs are not a prerequisite for VF [43].

Arrhythmic beats may not only serve as triggers for re-entrant arrhythmias, but also increase substrate inhomogeneity, facilitating development of functional re-entry circuits [23], [24]. In an earlier investigation from our study group, simply the number (frequency) of the arrhythmic beats was not predictive for TdP in a commonly used in vivo rabbit proarrhythmia model [2]. However, increased variability in the duration of the coupling interval and in the shape (origin) of the arrhythmic beats correlated with TdP occurrence. We concluded that the more chaotic the ventricular rhythm, the greater absolute BVI values measured, and the higher the probability of TdP development [2]. Our new biomarkers, the *absolute* BVI parameters, were sensitive TdP predictors and were able to quantify the beat-to-beat electrical instability irrespective of the rhythm, which provided the substrate and the trigger mechanism of re-entrant circuits responsible for TdP.

In the present study, we evaluated the predictive power of *absolute* BVI parameters for phase I ischemic VF in isolated rat hearts with regional ischaemia. Similar to our findings in the field of TdP, *absolute* BVI parameters were able to predict the occurrence of VF, while the same parameters measured in sinus rhythm were not able to do it. These findings emphasize the importance of the preceding arrhythmias in the development of both TdP and phase I ischaemic VF. However, our result show that the development of TdP and VF differs in terms of preceding arrhythmias. In contrast to our findings in the field of TdP, the frequency of arrhythmic beats and ‘R on T’ arrhythmic beats did predict VF in isolated rat hearts, whereas the variability in terms of coupling interval and shape did not have predictive power for VF occurrence. In the case of TdP, one of the sources of the increase in *absolute* BVI parameters was mainly the great variability in coupling intervals and the morphology of the preceding arrhythmic beats [2]. In contrast, present results show that one of the main sources of the

increase in absolute BVI parameters in isolated rat hearts subjected to local ischaemia was the elevated number of arrhythmic beats, not their variability in their coupling interval and morphology. Importantly, the predictive power of the frequencies of arrhythmic beats and 'R on T' arrhythmic was lower than the predictive power of most of the repolarization-related *absolute* BVI parameters. Given that inhomogeneity is influenced by numerous factors, any metric that quantifies just one contributing element (such as the frequency of arrhythmic beats) inherently has limited predictive power for VF and TdP. Conversely, absolute BVI parameters capture the cumulative impact of all factors that enhance substrate inhomogeneity, which explains their superior predictive ability for the occurrence of VF and TdP.

5.3. Cycle length variability

To the best of our knowledge, this is the first study that could quantify the electrical and mainly the repolarization instability before phase I ischemic VF in isolated hearts. Electrical instability is generally measured in sinus rhythm and ectopic beats are examined separately. Our newly developed *absolute* beat-to-beat variability parameters make it possible to evaluate them together.

Previously Lemmert et al. [43] measured the RR interval instability irrespective of the rhythm among patients with acute ST elevation myocardial infarction. Although, they found that VF patients had an elevated RR interval instability and also more ventricular ectopic beats, the multivariate analysis revealed that only the RR interval instability was independently associated with an increased chance of ischemic VF. Their findings are in agreement with our results, as the RR instability measured by STI, LTI and TI parameters of the RR interval were significantly increased before VF in our investigation in isolated rat hearts. The analogy between the two approaches and findings shows that irregularities in cycle length caused by ventricular arrhythmic activity may contribute to the initiation of VF.

One can confuse heart rate variability (HRV) with *absolute* BVI of the RR interval. HRV strictly takes account only the sinus beats derived from 24 hours Holter ECG and is considered to be a marker of vagal nerve activity and the parasympathetic tone [44]. In contrast, *absolute* BVI parameters are measured *irrespective of rhythm*. Thus, *absolute* BVI parameters are affected by various factors including the frequency, coupling interval and shape of the arrhythmic beats. Furthermore, *absolute* BVI parameters were measured in isolated, denervated hearts in the present study; therefore, these parameters had absolutely no relation to the

autonomic nervous system. Decreased HRV is a long-term predictor of sudden cardiac death in patients with chronic heart disease [44]. In contrast, *absolute* BVI parameters and RR interval instability (see above) were tested as short term predictors of ischemic VF in the acute phase of myocardial ischemia. Furthermore, *absolute* BVI parameters and also the RR interval instability take account every beat independently of their origin, therefore evaluate the real electrical instability caused by every contributing factor e.g., arrhythmic activation. Thus, HRV and *absolute* BVI parameters cannot be compared with each other.

5.4. The variability of the repolarization measured regardless of the rhythm predict VF

Our novel method developed in order to evaluate the electrical instability considers not only the rhythm irregularity but also the instability of the ventricular repolarization irrespective of the rhythm. Recently, there has been increased interest in validating changes in repolarization dynamics and variability to quantify myocardial vulnerability and to predict sudden cardiac death, and many different algorithms have been examined under both clinical and experimental conditions [45], [46], [47]. Almost all of the methodologies consider only sinus rhythm or use a pacing protocol to determine the repolarization instability, although it is well known that ectopic beats can influence electrical instability and play a role in arrhythmogenesis [48]. The constant-rate pacing protocol eliminates the effect of short-term memory on QT interval dynamics, which is the dependence of QT duration on the sequence of the QT intervals and DIs of the preceding beats [49]. On the other hand, it was shown that instability in action potential duration (APD) after premature beats leads to heterogeneous distribution of DIs in the heart, and consequentially, to the induction of conduction blocks needed for the development of functional re-entry circuits [50]. Thus, as QT interval is the global manifestation of ventricular APD, the instability of the QT interval caused by premature beats can lead to development of re-entrant arrhythmias e.g. TdP [2], and VF [48].

Chen et al. included VPBs in their assessment of QT instability prior to the onset of ventricular tachycardia (VT) [48]. They demonstrated that a higher frequency of VPBs and increased QT interval dynamics instability, measured regardless of the rhythm, preceded sustained VT onset in patients with acute myocardial infarction [48]. Their approach evaluated the QT interval dynamics not only from RR intervals but also from prior QT intervals that contributed to arrhythmia initiation. Ectopic beats, either atrial or ventricular premature beats, were included in their evaluation to capture the effective arrhythmogenic unstable

repolarization dynamics. In our study, the instability of the repolarization measured irrespective of the rhythm by the *absolute* BVI parameters was most prominent before VF, and had the greatest predictive power for phase I ischaemia-induced VF. The findings of Chen et al. [48] and our data on absolute and sinus BVI parameters highlight that methods for predicting severe ischaemia-induced arrhythmias should not solely focus on sinus or regular rhythm. Instead, repolarization instability, when assessed irrespective of rhythm, has a strong predictive power for the occurrence of ischaemic VT or VF.

5.5. Analysis of ECG intervals during irregular rhythm elucidates the mechanism of phase I ischaemia-induced VF

The analysis of ECG intervals during irregular rhythms revealed that a significant increase in the frequency of arrhythmic beats and 'R on T' VBPs, a significant shortening of the mean cycle length and electrical DIs, and an increased *absolute* BVI of cycle length and repolarization predicted phase I ischemia-induced VF. These findings elaborate on the mechanism of ischemia-induced VF and align with previous research results. Laurita et al. [51] demonstrated that both single and multiple VPBs increase spatial inhomogeneity of repolarization. Ischemia itself, even in the absence of arrhythmic beats, results in significant beat-to-beat variability of repolarization in the ischaemic area, while repolarization remains stable in non-ischaemic myocardium. This discrepancy leads to significant spatial inhomogeneity between ischemic and non-ischemic myocardium [52]. The spatial inhomogeneity of repolarization induced by arrhythmic beats and ischemia can create a substrate for re-entry, with arrhythmic beats also acting as triggers [48]. A high frequency of arrhythmic beats shortens the average cycle length, and shorter cycle lengths facilitate the generation of delayed afterdepolarization-induced VPBs [53]. Additionally, a shorter cycle length results in shorter DIs. Greater variability in repolarization duration, combined with short DIs, promotes re-entry.

CONCLUSIONS

The new *absolute* BVI parameters that predicted TdP risk in rabbits also predict VF risk during regional ischemia in rat hearts, showing a diagnostic and mechanistic similarity. Repolarization inhomogeneity seems crucial for ischemic VF induction, as absolute BVI parameters measuring repolarization variability showed excellent predictive power with high sensitivity and specificity. These newly validated biomarkers could act as substitutes for VF in preclinical drug studies.

LIMITATIONS

Given that repolarization in rat ventricles differs significantly from that in humans [17] [54], these results may not be directly applicable to humans. Accurately predicting VF in the minute before its occurrence may not have clear clinical value. However, the *absolute* BVI parameters we have validated could serve as surrogates for VF in preclinical drug research, aligning with the 3Rs principle (refinement). Additionally, these validated biomarkers would be valuable in later translational research to identify potential benefits of new drugs against VF in a broader, low-risk human population.

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