Comparison of different proarrhythmia biomarkers in isolated rabbit hearts

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THE THESIS IS BASED ON THE FOLLOWING PAPERS

I. The role of the Na+/Ca2+ exchanger, I(Na) and I(CaL) in the genesis of dofetilide-induced torsades de pointes in isolated, AV-blocked rabbit hearts.

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II. The assessment of efficacy of proarrhythmia biomarkers in isolated rabbit hearts with attenuated repolarization reserve.

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ACRONYMS AND ABBREVIATIONS

APD	action potential duration
AV	atrioventricular
BG	bigeminy
EAD	early afterdepolarization
DAD	delayed afterdepolarization
DMSO	dimethyl sulfoxide
ECG	electrocardiogram
I_{CaL}	inward L-type calcium current
I_K	delayed rectifier potassium current
I_{Kr}	the rapid component of the delayed rectifier potassium current
I_{K1}	inward rectifier potassium current
I_{Na}	inward sodium current
LQTS	long QT syndrome
LTV	long-term variability
NCX	Na^+/Ca^{2+} exchanger
QTc	heart rate corrected QT interval
SCD	sudden cardiac death
STV	short-term variability
TdP	Torsades de Pointes
TDR	transmural dispersion of repolarization
TWLI	T-wave lability index
VF	ventricular fibrillation
VPB	ventricular premature beat
VT	ventricular tachycardia
vs	versus

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1 INTRODUCTION

1.1 Background of the study

The cardiovascular diseases accounted for more deaths than any other single cause of death in the United States since 1900[1]. More than 450,000 sudden cardiac death (SCDs) event occurs in the United States annually[2]. Pharmaceutical companies are really interested in the development of great number of antiarrhythmic drugs, since tachyarrhythmias are the most frequent causes of SCD. However, some clinical trials, such as CAST[3] and SWORD[4] found evidence that both Vaughan-Williams Class IC Na^+-current (I_{Na}) blockers and Class III. rapid delayed-rectifier K^+-current (I_{Kr}) blockers induce significant arrhythmia risk-enhancement, so called "proarrhythmia", which is defined as the production of de novo arrhythmias or aggravation of existing arrhythmias.

Dessertenne was the first who recognized a specific ECG pattern with twisting points and rotating peaks, described as "Torsades de Pointes" (TdP), which often associated with proarrhythmia by drugs that cause QT-prolongation[5]. TdP is considered a life-threatening form of polymorphic ventricular tachycardia, which can be evoked by several cardiac or non-cardiac drugs that disturb the process of repolarization of the myocytes. TdP has a characteristic pathophysiology (Figure 1, discussed in detail below) and can manifest as acutely decreased pump function and haemodynamic instability, leading to syncope (sometimes with seizure-like activity), or via transformation to ventricular fibrillation, causing SCD.

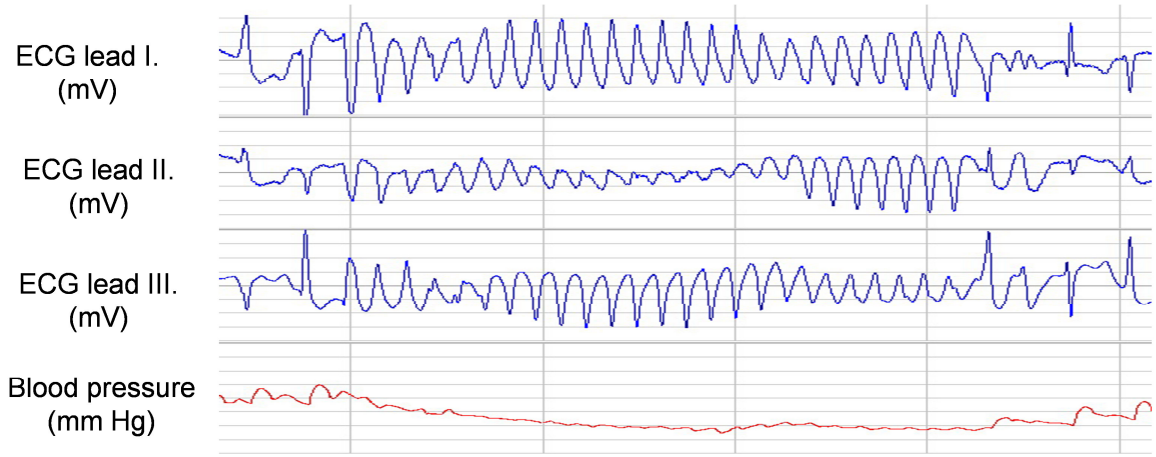


Figure 1 A representative recording of a TdP from an anaesthetized rabbit experiment

1.2 Development of Torsades de Pointes

According to the most widely accepted theory, the mechanism of TdP initiation (trigger) involves an early (EAD) or delayed (DAD) afterdepolarization-induced ectopic beat, while the mechanism of the arrhythmia maintenance (substrate) involves re-entry circuits produced by an increase in spatial dispersion of the repolarization of the ventricular wall[6]. The TdP-maintaining mechanism includes the '4 dimensions' of dispersion of cardiac repolarization in space and time: (1) transmural dispersion of repolarization (TDR); (2) apico-basal dispersion; (3) interventricular dispersion; and (4) temporal dispersion. Temporal dispersion includes instability that can be a consequence of the above-mentioned initiating mechanisms[7]. For example, dofetilide, an inhibitor of the rapid component of the delayed rectifier potassium current (I_{Kr}), prolongs repolarization, induces EAD, increases dispersion of repolarization and can evoke TdP[8]. Numerous cardiac and non-cardiac drugs have been withdrawn from the clinical use or relabeled due to their documented proarrhythmic potential[9]. The development of TdP depends on several factors. Serum electrolyte levels, disturbance in autonoumos nervous system, cardiac functional and structural diseases, the cooperation of potassium channels during repolarization etc[7].

1.3 Biomarkers in prediction of Torsades de Pointes

As the overall incidence of TdP is very low[10, 11], there is a great need to find highly sensitive and specific surrogate biomarkers of TdP. Several surrogate biomarkers were proposed recently. An index of ventricular transmural dispersion of repolarization, the interval between the peak and the end of T wave (Tpeak-Tend) has been suggested, which may have prognostic value for proarrhythmic risk[12]. Beat-to-beat non- alternating T-wave lability, which was measured by the T wave lability index (TWLI) occurred in patients with inherited LQTS was associated with a history of prior cardiac events[13]. Hondeghem et al. established a proarrhythmia screening system (SCREENIT), which assess the triangulation, reverse use dependence, instability and dispersion (TRIaD) as predictors of drug-related proarrhythmia[14]. Since, spatial and temporal inhomogeneity of repolarization, annotated "4 dimensions of dispersion"[7], have a crucial role in the development of TdP, parameters that quantify dispersion[15-18] have been reported to have better predictive value for TdP development.

However, the above mentioned parameters have not been fully confirmed. To date, among many suggested biomarkers, still the ECG QT and heart rate corrected QT (QTc)

intervals are the most accepted and the most frequently-used TdP surrogates[7, 19, 20]. However, the predictive power of these parameters has been questioned[8, 21, 22].

Recently, Thomsen et al. have proposed beat-to-variability of the QT intervals as a parameter of temporal inhomogeneity of repolarization, which may determine the proarrhythmic outcome in a dog *in vivo* proarrhythmia model[23]. It has been shown that beat-to-beat variability of the repolarization measured in regular rhythm predicted TdP in clinical[24] and experimental[16, 25, 26] settings. However, beat-to-beat variability of repolarization measured in regular rhythm did not predict drug-induced TdP in anaesthetized rabbits[27-30] and guinea pigs[31]. One of the possible reasons is that TdP is almost always preceded by simple, drug-induced arrhythmias which do not allow the measurement of the beat-to-beat variability of repolarization in sinus or regular rhythm. These 'preceding' arrhythmias can occur well before temporal instability of repolarization could be detected. In order to overcome this problem, we developed a method to allow ECG intervals to be measured during disorganized non-sinus rhythm before TdP occurrence[28, 32]. This allowed established beat-to-beat variability parameters to be derived irrespective of rhythm[28]. To differentiate from published beat-to-beat variability parameters described by others, all of which are derived during stable rhythm (*sinus* beat-to-beat variability parameters), we coined the term *absolute* to describe the derived beat-to-beat variability parameters of the ECG intervals[28]. *Absolute* beat-to-beat variability parameters of the ECG intervals accurately predicted drug-induced TdP[28] and also the development of ischaemic VF[33], whereas equivalent variables measured in sinus rhythm failed to predict TdP and VF liability[27, 28].

1.4 Preclinical proarrhythmia assays

Most pharmaceutical companies conduct cardiovascular safety studies before selection of candidate drugs for development. Table 1. summarizes the available preclinical models for TdP risk assessment[7]. Since the *in vitro*, isolated, rabbit heart model is one of the most frequently used preclinical model for testing the proarrhythmic liability of drugs, relatively simple and does not require high amount of drug, we chose this model for our studies (see the paragraph 1.5 and 1.6)

Preclinical proarrhythmia assays
***In vitro* assays** 1. Human Ether-a`-Go-Go (hERG) subunit assay (isolated cardiac ventricular myocytes or cell lines expressing hERG) 2. Purkinje fibre and papillary muscle action potential duration assays 3. Arterially perfused wedge preparations 4. Langendorff heart (SCREENIT) ***In vivo* assays in normal animals** 1. α1-adrenoceptor-sensitized anaesthetized rabbit model 2. Conscious ECG telemetry in dogs and monkeys 3. Slow delayed-rectifier potassium current-inhibited 4. Anaesthetized rabbit and canine models **Pathological in vivo TdP models** 1. Chronic atrioventricular-block animal models 2. Failing rabbit heart model

Table 1. Preclinical proarrhythmia based on the classification of the review of Farkas&Nattel[7]

1.5 The in vitro, isolated, AV ablated rabbit heart model

The in vitro, isolated, AV ablated rabbit heart model is frequently used for testing the proarrhythmic liability of drugs[34]. The primary objective of this study was to investigate the role of Na+/Ca2+ exchanger (NCX) in the development of TdP in AV ablated isolated rabbit hearts. Interestingly, the specific NCX blocker (SEA0400) could not decrease the incidence of TdP in this model questioning the pivotal role of NCX in the formation of TdP. As the ability of other pharmacological interventions to reduce the incidence of TdP induced by K+ channel blocking drugs has not been fully examined in this model, a second series of experiments was conducted to validate the model with drugs known to alleviate TdP in other experimental models. Thus, the anti-arrhythmic effect of the inhibition of the L-type Ca2+ current (I_{CaL}) by verapamil and the inhibition of the I_{Na} by lidocaine was tested against dofetilide-induced TdP in isolated, Langendorff-perfused, AV-ablated rabbit hearts. Also, an attempt was made to determine whether the occurrence of dofetilide-induced TdP was related to the prolongation of the rate corrected QT (QTc) interval or the beat-to-beat variability of the QT interval in the applied model.

1.6 The in vitro, isolated, reduced repolarization reserve rabbit heart model

According to the theory of repolarization reserve, there is some redundancy in repolarizing currents in the myocardium. Inhibition of a single repolarizing current can still allow sufficient repolarization via other repolarizing currents. However, concomitant inhibition of two or more repolarizing ion channels reduces repolarization reserve and potentiates inhibition of cardiac repolarization, which can result in TdP development[35-37]. In human and also in many mammals applied for laboratory experiments (e.g. guinea pig, rabbit, dog) the main outward repolarizing current is the rapid component of the I_{Kr}, that is a common target of drugs with proarrhythmic side effects[38]. However, under normal conditions, I_{Kr} block just rarely evokes serious arrhythmias (e.g. TdP) because another repolarizing current, e.g. the slow component of the delayed rectifier K^+ current (I_{Ks}), becomes more prominent and compensates the repolarization lengthening and decreases the arrhythmogenic effect of the I_{Kr} blocker[39-42]. However, the concomitant block of I_{Kr} and I_{Ks} currents constitutes an increased risk to TdP development[25, 30, 41-45].

The aim of this study was to investigate which TdP biomarker (QTc, *sinus* or *absolute* beat-to-beat variability of the QT interval) can adequately indicate the increased risk of proarrhythmia in the setting of reduced repolarization reserve in an *in vitro*, spontaneous beating rabbit heart proarrhythmia model. Reduced repolarization reserve was achieved by concomitant pharmacological inhibition of I_{Kr} and I_{Ks}.

2 MATERIALS AND METHODS

2.1 Animals in the study

Female New Zealand white rabbits weighing 2.5±0.24 kg were used for the experiments. Animals were obtained from WOBE Ltd. (Budapest, Hungary) and acclimatized at the site for at least 3 to 4 days before any experiments started. Animals were kept under standard conditions (temperature 21°C; relative humidity 55–65%; 12:12 h dark/light cycle) on commercial laboratory chow and tap water ad libitum. Animal maintenance and research were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the Local Ethical Committee of Gedeon Richter Plc. (RG-MÁB No. 44/2001).

2.2 Langendorff perfusion of isolated rabbit hearts

The animals were anticoagulated with sodium heparin (1000 International Units) injected into the marginal ear vein and stunned by a blow to the neck. The heart was rapidly removed via thoracotomy and rinsed in ice-cold modified Krebs–Henseleit buffer solution containing (in mM): NaCl 118.5, CaCl2 1.8, glucose 11.1, MgSO4 0.5, NaH2PO4 1.2, NaHCO3 25, and KCl 3. A K+ concentration of 3 mM was chosen, as the proarrhythmic action of dofetilide tended to be exacerbated by perfusion with 3 mM K+ versus (vs) 4 mM K+ in isolated, AV-blocked rabbit hearts[46]. The aorta was cannulated and hung on a Langendorff apparatus and hearts were retrogradely perfused at a constant temperature of 37°C with the modified Krebs–Henseleit buffer solution described above. A mixture of 95 % O2 and 5 % CO2 was bubbled through the buffer, which was equilibrated to pH 7.4. All solutions were filtered (5 μm pore size filter) before use. The perfusion pressure was maintained constant at 80 mmHg. Volume conducted electrocardiogram (ECG) was recorded by using National Instruments data acquisition hardware (PC card, National Instruments, Austin, TX, USA) and SPEL Advanced Haemosys software (version 2.76, Experimetria Ltd. and Logirex Software Laboratory, Budapest, Hungary). Coronary flow was measured with a glass flowmeter (Cole-Parmer Instrument Company, Vernon Hills, Illinois, USA) positioned immediately above the retrogradely perfused aorta and was later corrected for the weight of each heart to give values in ml min 1 g-1.

In AV ablated rabbit hearts an incision was made in the right atrium and the AV node was ablated using forceps two minutes after mounting the heart. AV ablation was regarded

successful when the P wave was dissociated from the QRS complex on real-time ECG recording. After AV ablation the hearts were allowed to beat in their own spontaneous rhythm. Since a single ventricular pacing stimulus can initiate TdP in the presence of a Class III antiarrhythmic drug (e.g. E-4031) in isolated, AV-blocked rabbit hearts[47], ventricular pacing was not applied in the present experiments in order to avoid the occurrence of electrical pacing induced arrhythmias. Hearts were allowed 15 min to equilibrate before starting the experimental protocol. At the end of each experiment, the atria were removed from the heart and the ventricles were weighed.

2.3 The in vitro, isolated, AV ablated rabbit heart model

2.3.1 Experimental protocol in isolated AV ablated rabbit heart study

The experiments were design to examine whether it is possible to reduce dofetilide-induced TdP with other drugs in the applied model. Lidocaine and verapamil were chosen as test drugs as they could successfully reduce the incidence of drug-induced TdP in other experimental models[48, 49]. The experiments comprised three groups of hearts: 1.) 100 nM dofetilide (DOF), 2.) 30 μM lidocaine+100 nM dofetilide (LID+DOF), 3.) 750 nM verapamil+100 nM dofetilide (VER+DOF). Each group contained n=8 hearts. The administration of lidocaine or verapamil or the solvent DMSO was started at the beginning of the 'pretreatment' period, whereas dofetilide was added to the perfusion solution at the beginning of the 'treatment' period (Table 2).

Group	n	Pretreatment (20 min)	Treatment (30 min)
Dofetilide 100 nM	8	DMSO	DMSO+dofetilide
Lidocaine 30 μM+Dofetilide 100 nM	8	DMSO+lidocaine	DMSO+lidocaine+dofetilide
Verapamil 750 nM+Dofetilide 100 nM	8	DMSO+verapamil	DMSO+verapamil+dofetilide

Table 2. The experimental protocol applied in isolated, Langendorff-perfused, AV-blocked rabbit hearts. The groups of hearts, the periods of the experiments and the applied drugs are summarized. In the baseline period of the experiments, all hearts were perfused with Krebs buffer (not signed); the perfusion solution was then switched to the test drug in the experiments (pretreatment); in the last period dofetilide was added to the perfusion (treatment). Group size is indicated by n.

2.3.2 ECG analysis and coronary flow measurement in AV ablated rabbit heart study

ECG intervals and coronary flow were measured at predetermined time points. After completion of the experiments, the data were replayed and the QT and RR intervals were measured by manual positioning of on-screen markers. The QT interval was defined as the

time between the first deviation from the isoelectric line of QRS complex until the end of the TU wave. In the rabbit hearts, where the T or U wave overlapped the following QRS complex of the subsequent ventricular beat, the extrapolation method was used to measure the length of the QT (or QU) interval[50].

As QT interval is influenced by the heart rate, baseline data for ventricular heart rates and QT intervals were used to determine the relationship between the RR interval and the QT interval in sinus rhythm before AV ablation and in idioventricular escape rhythm after AV ablation according to Batey et al.[51] and Farkas et al.[52]. These data were obtained from 56 isolated, Langendorff-perfused rabbit hearts. Forty consecutive QT intervals were measured together with the corresponding RR intervals in each hearts immediately before and 10 min after the mechanical AV ablation. Simple linear regression revealed a positive correlation between QT and RR intervals in sinus rhythm (QT_{Sinus}=0.326RR + 103.4) as well as in idioventricular escape rhythm ($QT_{AV\text{-}block}$=0.081RR + 186.8), though the regression coefficient (R) and the slope of the regression line were greater during sinus rhythm as compared with the values calculated in idioventricular escape rhythm (Figures 2a and 2b). As there was a difference between the slope of the regression line of the sinus and the idioventricular rhythm, a QT correction was calculated for either rhythm in a manner similar to that described by Batey et al.[53]. The equations were rearranged to allow the calculation of the rate-corrected QT interval in sinus rhythm (QTc-Sinus) at an RR interval of 300 ms (i.e. a ventricular rate of 200 beats min^{-1}) using the formula QTc-Sinus=QT_{Sinus} - 0.326(RR-300) and in idioventricular escape rhythm (QTc-AV) at an RR interval of 500 ms (i.e. a ventricular rate of 120 beats min^{-1}) using the formula QTc-AV=$QT_{AV\text{-}block}$ - 0.081(RR-500). With these equations, plotting QTc-Sinus and QTc-AV against the corresponding RR interval produces a regression line with a slope of zero (Figure 2c and 2d) indicating that these corrections remove the influence of heart rate. As we examined drug effects after AV ablation in idioventricular escape rhythm, all QTc intervals in this study were corrected according to the QTc-AV.

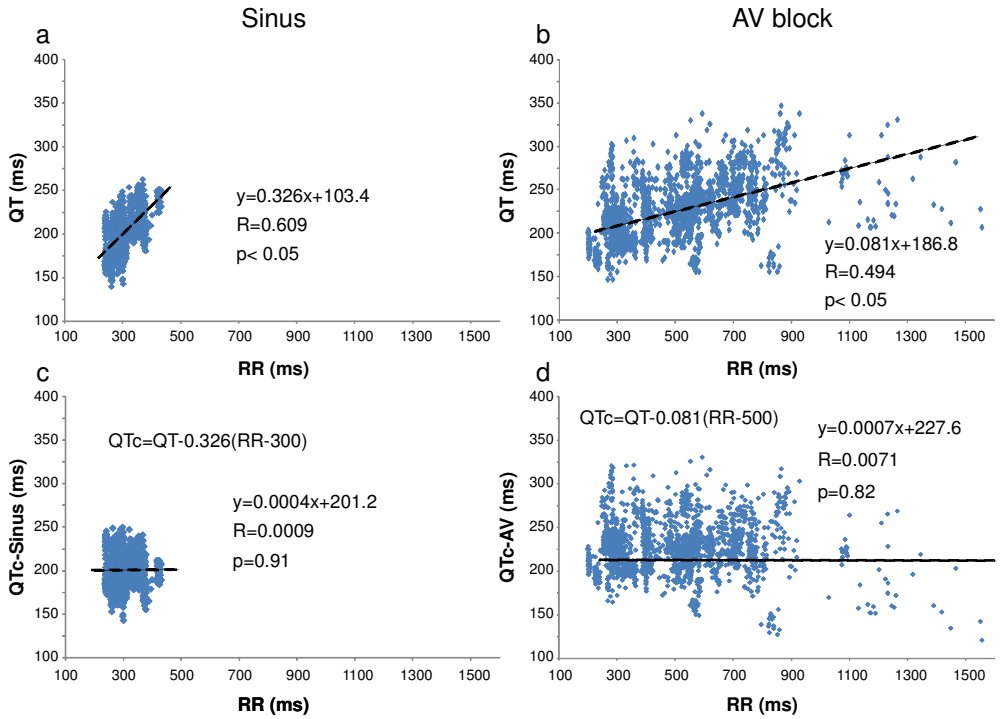


Figure 2. Correlation between individual values of the QT and RR intervals in sinus rhythm (A) and after AV ablation (B) in isolated Langendorff-perfused rabbit hearts. Correlation between individual values of the 'rate-corrected' QT intervals and the RR intervals in sinus rhythm (C) and after AV ablation (D) in isolated Langendorff-perfused rabbit hearts. QTc-Sinus, the heart rate-corrected QT interval during sinus rhythm; QTc-AV, the ventricular heart rate-corrected QT interval after AV nodal ablation. AV, atrioventricular; QTc, heart rate-corrected QT interval.

Coronary flow values were read directly from the flowmeter every 5 min during the experiment and 1 min before and after switching to a different perfusion solution and normalized to heart weight.

2.3.3 Measurement of the absolute variability of the QT intervals and the RR intervals

The absolute variability of the QT and the RR intervals was determined from the manual measurement data on 40 consecutive QT intervals and the corresponding RR intervals at predetermined time points, i.e. in the last min before AV ablation, in the last min of drug-free state after AV ablation, in the last min of the 'pretreatment' period before switching to the dofetilide perfusion, and in the 5th min of the dofetilide infusion or before the onset of TdP. A computer program was developed in a .NET environment to obtain the following parameters of the absolute variability of the QT and RR intervals: For a sequence of RR or QT interval durations, the 'root mean square' (RMS) was calculated according to the following definition: where represents the sequence of RR or QT interval durations and is the total number of intervals. Another approach for characterizing the absolute variability of RR and QT intervals is to take their successive differences (where represents the RR or QT

interval durations and is the total number of intervals) and calculate the root mean square of these differences: where denotes the mean value. The percentage of successive QT intervals that differ by more than 8 ms (PNN8), the 'instability' of the RR and QT intervals, the 'short-term variability' (STV) and the 'long-term variability' (LTV) of the RR and QT intervals were calculated as described earlier[27].

2.3.4 Exclusion criteria in AV ablated rabbit heart study

Any heart with a coronary flow < 3 ml $min^{-1}g^{-1}$ or asystole longer than 20 s during the whole experimental protocol was excluded. Longer than 20 s asystole was found after the start of dofetilide perfusion in 6 hearts pre-treated with verapamil and in 3 hearts pre-treated with lidocaine, these hearts were excluded. All excluded hearts were replaced to maintain equal group sizes.

2.4 The in vitro, isolated, reduced repolarization reserve rabbit heart model

2.4.1 Experimental protocol in isolated, reduced repolarization reserve rabbit heart study

In the first set of experiments three groups of hearts were compared: 1) control group of hearts perfused with dimethyl sulphoxide (DMSO), that is the solvent of dofetilide and HMR-1556 ('Control A' group; n=6); 2) hearts perfused with dofetilide at 15 nM ('Dof 15' group; n=8); 3) hearts perfused with HMR-1556 at 460 nM + dofetilide at 15 nM ('HMR + Dof 15' group; n=6)(Table 3).

In the first set of experiments there were only few drug-induced arrhythmias, therefore we performed a second set of experiments, in which we intended to increase the incidence of arrhythmias. In the second set of experiments four groups (each contained 8 hearts) were compared: 1) control group of hearts perfused with DMSO and water, the latter is the solvent of catecholamines ('Control B' group); 2) hearts perfused with catecholamines (epinephrine 25 nM + norepinephrine 100 nM) + DMSO ('Cat Control' group); 3) hearts perfused with catecholamines + dofetilide at 50 nM ('Cat + Dof 50' group); 4) hearts perfused with catecholamines + HMR-1556 at 460 nM + dofetilide at 50 nM ('Cat + HMR + Dof 50' group)(see Table 4).

The administration of the I_{Ks} inhibitor HMR-1556 or its solvent (DMSO) was started at the beginning of a 30 min pretreatment period. Dofetilide or its solvent (DMSO) was added to the perfusion solution after the pretreatment period, at the beginning of a 30 min treatment

period. In the second set of experiments catecholamines or their solvent were administered from the beginning of the pretreatment period (Tables 3 and 4).

PERIOD GROUP	Pretreatment (30 min)	Treatment (30 min)
Control A (n=6)	vehicle	vehicle
Dof 15 (n=8)	vehicle	Dofetilide 15 nM
HMR + Dof 15 (n=6)	HMR	HMR + Dofetilide 15 nM

Table 3. Experimental protocol in the first set of experiments. 'Dof 15': group perfused with 15 nM dofetilide; 'HMR + Dof 15': group perfused with 460 nM HMR-1556 and 15 nM dofetilide. HMR: HMR-1556 at 460 nM, vehicle: DMSO (dimethyl sulphoxide), the common solvent of the dofetilide and HMR-1556

PERIOD GROUP	Pretreatment (30 min)	Treatment (30 min)
Control B (n=8)	vehicles	vehicles
Cat control (n=8)	Cat. + vehicle	Cat. + vehicle
Cat + Dof 50 (n=8)	Cat. + vehicle	Cat. + Dofetilide 50 nM
Cat + HMR + Dof 50 (n=8)	Cat. + HMR	Cat. + HMR + Dofetilide 50 nM

Table 4. Experimental protocol in the second set of experiments. 'Cat + Dof 50': group perfused with catecholamines and 50 nM dofetilide; 'Cat + HMR + Dof 50': group perfused with catecholamines, 460 nM HMR-1556 and 50 nM dofetilide. Cat.: catecholamines (epinephrine 25 nM + norepinephrine 100 nM). Vehicles in the 'Control B' group are water acidified with ascorbic acid (the solvent of the catecholamines) and DMSO (the common solvent of the dofetilide and HMR-1556). Vehicle in the other groups is only DMSO. For further details see Table 3.

In the first set of experiments we aimed to achieve a sufficient impairment of the repolarization without provoking a prominent arrhythmic activity. Thus, the selective I_{Kr} blocker dofetilide was applied at a concentration of 15 nM, which inhibited approximately 50% of the I_{Kr} K^+ current[54, 55] and led to QTc prolongation without significant arrhythmic activity in our pilot study.

In the second set of experiments we intended to increase the incidence of arrhythmias by further repolarization impairment. The concentration of dofetilide was increased to 50 nM in order to achieve complete inhibition of I_{Kr}. Since I_{Ks} blockade does not significantly increased repolarization instability unless β-adrenergic stimulation is added in canines *in vivo*[56-58], catecholamines were added to the perfusion solution to mimic the sympathetic activity and to boost the function of I_{Ks} (and the effect of I_{Ks} inhibition) in the isolated rabbit hearts.

Since the I_{Ks} inhibitor HMR-1556 may adsorbs to glass or plastic surfaces, leading to reduced effective drug concentrations in the heart, the concentration of HMR-1556 was

measured in the perfusate right above the heart by the means of spectrophotometry (Hitachi F-4000, Hitachi Ltd. Tokyo, Japan) with an excitation and emission wavelength of 295 nm and 325 nm, respectively (data not shown). Since the IC_{50} of HMR-1556 ranged between 10.5 nM[59] and 65 nM[44], our target concentration of HMR-1556 was 250 nM in order to achieve a significant inhibition of the I_{Ks}. However, it was found in the pilot experiments that the concentration of HMR-1556 in the perfusate was lower at the level of the aorta than in the working buffer. Thus, we increased the concentration of HMR-1556 to 460 nM in the working buffer, which resulted in a stable concentration of ~250 nM HMR-1556 in the perfusate at the level of the hearts in our perfusion setup.

2.4.2 Measurement of the ECG intervals and QTc in, reduced repolarization reserve rabbit heart study

ECG intervals were measured in sinus rhythm at predetermined time points (14, 25, 35, 44, 50, 55 min). After the completion of experiments, the data were replayed with the SPEL Advanced Haemosys software (version 2.76, Experimetria Ltd. and Logirex Software Laboratory, Budapest, Hungary). The software averaged the ECG signal from 20 consecutive sinus beats at the predetermined time-point. If there were not 20 consecutive sinus beats at the predetermined time point or in the preceding or subsequent 30 sec, then the measurement was not performed at the time point. The RR and QT intervals were measured by manual positioning on screen markers in the signal averaged ECG. Heart rate was calculated from the RR interval.

The QT interval is influenced by the heart rate, thus rate corrected QT interval (QTc) was calculated with a correction method described earlier[51, 60]. Baseline data in sinus rhythm for QT intervals together with the corresponding RR intervals were obtained from pooled data of our laboratory (from 100 isolated, Langendorff-perfused rabbit hearts prepared as described above). The linear regression between QT and RR intervals was: QT = 0.3381RR + 89.85. This equation was rearranged to allow the calculation of the rate-corrected QT interval at a mean RR interval of 340 ms (i.e. a ventricular rate of ~177 beats min^{-1}) using the formula QTc = QT - 0.3381(RR-340). With these equations, plotting QTc against the corresponding RR interval produces a regression line with a slope of zero (data not shown), indicating that these corrections successfully removed the influence of heart rate.

2.4.3 *Measurement of the absolute and sinus beat-to-beat variability of the QT and RR interval in reduced repolarization reserve rabbit heart study*

To determine beat-to-beat variability of the RR and QT intervals, all analyses were based on samples of 40 consecutive ventricular complexes (RR intervals) at the predetermined time points, that is at baseline (1 min before the pretreatment period), in the last min of the pretreatment period and in the 5th min of the treatment period. From these samples, each of the 40 RR and QT intervals were measured manually, then the short-term variability (STV) and the long-term variability (LTV) of the RR and QT intervals were derived as described earlier[15, 16, 27, 32, 61].

The derived beat-to-beat variability parameters described above are referred to *absolute* beat-to-beat variability parameters when the raw ECG interval data were taken irrespective of the rhythm i.e., regardless of whether rhythm was sinus or non-sinus at the time-point of the measurement. The derived beat-to-beat variability parameters described above are referred to *sinus* beat-to-beat variability parameters when the raw ECG interval data were taken from samples of 40 consecutive complexes strictly in sinus rhythm at the predetermined time-point. The sample of 40 consecutive sinus complexes was measured only if it was preceded by at least 10 arrhythmia-free beats in order to avoid any effect of preceding arrhythmias on the ECG intervals.

In the first set of experiments arrhythmias were infrequent, therefore the measurement of the ECG intervals could be done in stable sinus rhythm at all of the predetermined time points. Thus, only *sinus* beat-to-beat variability parameters were determined. However, in the second set of experiments, hearts usually experienced arrhythmias during drug perfusion at the predetermined time points. Thus, the measurement of the ECG intervals in sinus rhythm must have been done before the predetermined time point; if there were not 50 consecutive sinus beats at the predetermined time point or in the preceding 2 min, then the measurement was not performed and *sinus* beat-to-beat variability parameters were not determined at the time point.

2.4.4 *Arrhythmia diagnosis in reduced repolarization reserve rabbit heart study*

The incidence and the time to onset of arrhythmias were determined. Ventricular premature beat, bigeminy, salvo and ventricular fibrillation were defined according to the Lambeth Conventions[62]. TdP was defined as a polymorphic ventricular tachycardia consisting

of four or more ventricular complexes where clear twisting of the QRS complexes around the isoelectric axis could be seen in at least one ECG lead[32]. Runs of 4 or more ventricular premature beats without the TdP-like twisting QRS morphology were differentiated from TdP and were defined as ventricular tachycardia. Blocks in the conduction system were also monitored. Conduction disturbances included atrioventricular (AV) blocks and intraventricular conduction defects (right or left bundle branch blocks)[28].

2.4.5 Exclusion criteria in reduced repolarization reserve rabbit heart study

Any heart with a sinus rate < 120/min or a coronary flow > 10 ml/min/g or < 3 ml/min/g 5 min before the start of the 60 min drug perfusion protocol, or not in a constant sinus rhythm before the start of the 60 min drug perfusion, was excluded. In the first set of experiments, excluded experiments were not replaced as they may not have influenced the main results. In the second set of experiments all excluded hearts were replaced to maintain equal group sizes.

2.5 Study designs

The choice of drug solution was made by reference to a randomization table in each in vitro study. Randomization was achieved by coding each group with a letter whose meaning was unknown to the operator. Blinded analysis was achieved by using stock solutions prepared by a second operator, who did not participate in the heart perfusion or data analysis. The protocol of studies involved a randomized design with a time-matched control group, and blinded experimentation, data collation and analysis. For the assessment of the vehicle control or each individual concentration of the drug, each heart was used only once.

2.6 Drugs and materials

Perfusion solutions were prepared fresh each day. Dofetilide and HMR-1556 were synthesized at Gedeon Richter Plc. and were dissolved in DMSO. DMSO, lidocaine, ascorbic acid, epinephrine, norepinephrine and verapamil were purchased from Sigma-Aldrich, Inc., St. Louis, MO, U.S.A. The components of modified Krebs–Henseleit buffer solution (NaCl, CaCl2, MgSO4, NaH2PO4, NaHCO3, and KCl) were purchased from Molar Chemical Ltd., Budapest, Hungary. Water for the preparation of perfusion solution was obtained from a reverse osmosis system (Milli-Q RG, Millipore Ltd., Billerca, MA, U.S.A.) fed by distilled water, and had a specific resistivity of >18 MΩ.

2.7 Test solutions

DMSO control solution and all final test solutions for heart perfusion contained 0.08 ml DMSO in 1 l of modified Krebs-Henseleit solution in the AV ablated rabbit heart study.

All final test solutions for heart perfusion contained 0.045 mL DMSO in 1 L of modified Krebs–Henseleit solution. Catecholamines (epinephrine and norepinephrine) were dissolved in water acidified with ascorbic acid; all final test solutions for heart perfusion contained 0.2 ml water and 25 µmol ascorbic acid in 1 L of modified Krebs–Henseleit solution in the second set of experiments in the reduced repolarization reserve rabbit heart study.

2.8 Statistics

Continuous data were expressed as mean ± standard error of the mean (S.E.M.). All data from independent samples, except arrhythmia incidences, were compared with Kruskal-Wallis tests. The incidences of arrhythmias were compared by using Fisher's exact probability test with the Bonferoni correction, i.e. the P values of Fisher's exact probability test were multiplied by the number of comparisons between the groups to allow multiple comparisons [63]. $P<0.05$ was taken as indicative of a statistically significant difference between values.

3 RESULTS

3.1 The in vitro, isolated, AV ablated rabbit heart model

3.1.1 The impact of AV node ablation on heart rate, QT intervals and on ECG absolute variability

Mechanical AV block significantly decreased the ventricular heart rate in all groups (Figure 3).

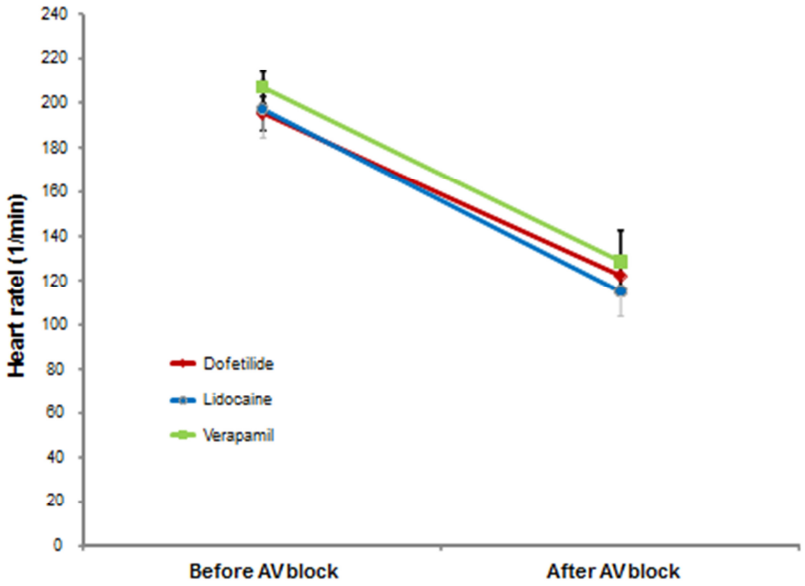
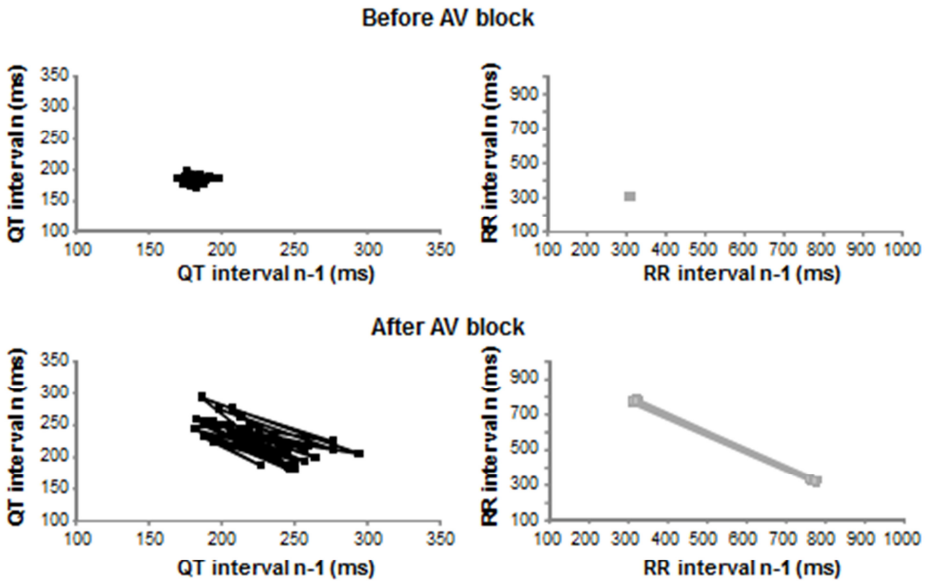


Figure 3. The heart rate before and after AV block in the Dofetlide, Lidocaine and Verapamil groups.

Although, heart decreasing effect of AV node ablation did not lead to a regular and slower, spontaneous ventricular rhythm in the majority of isolated hearts, but caused a chaotic and *irregular* spontaneous ventricular rhythm (Figure 4).



Figue 4. Poincare´ plot of before and after AV block showing the relationship of successive QT and RR intervals to each other

The average QT interval also lengthened significantly due to the increased RR intervals. As AV node ablation triggered chaotic heart rhythm, the measurement of ECG variability parameters was only possible during arrhythmias in many experiments. To differentiate from published beat-to-beat variability parameters derived during arrhythmia-free rhythm (*sinus* beat-to-beat variability parameters), we coined the term *absolute* to describe the beat-to-beat variability parameters derived irrespective of rhythm. The measured absolute QT and RR variability data also significantly increased and sensitize the hearts for the genesis of arrhythmias in the presence of proarrhythmic drugs (LTV: 6.1±0.6 ms to 9.8±0.9 ms; STV: 5.7±0.7 ms to 9.9±1.0 ms, before AV block and after AV block, respectively) (Figure 4).

3.1.2 The RR and the QTc intervals

Pretreatment period

After the exclusion of all hearts that experienced asystole longer than 20 s from the experiments (see exclusion criteria in *2.3.4*) the mean RR interval and the mean QTc interval (Figure 5) did not significantly differ between the lidocaine, verapamil and the control groups before dofetilide perfusion.

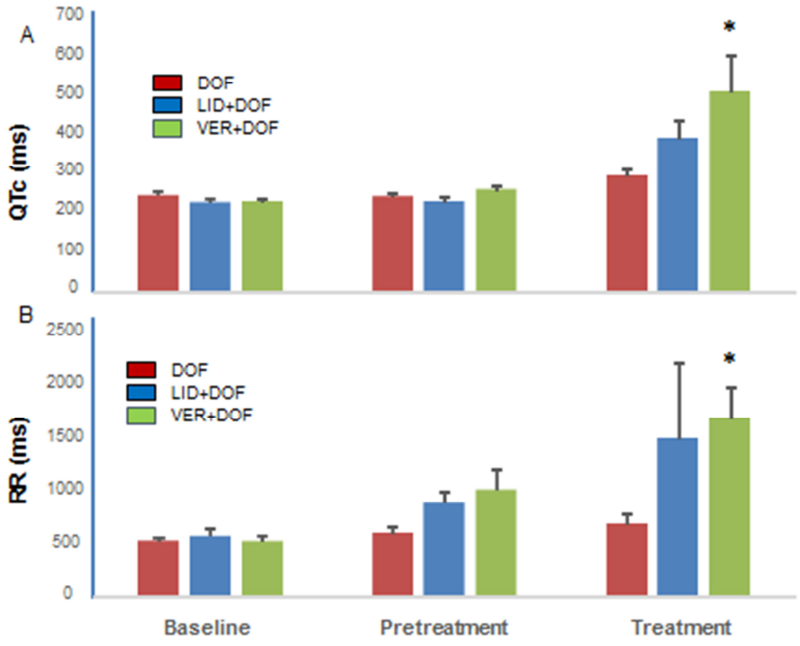


Figure 5. The heart rate-corrected QT (QTc) intervals (A) and the RR intervals (B) during the pretreatment and the treatment periods in AV-ablated isolated rabbit hearts

Treatment period

When dofetilide was added to the perfusate verapamil further increased the dofetilide-induced QTc prolongation leading to significant QTc lengthening. However, lidocaine did not affect the mean QTc interval significantly when compared with that of the DOF group (Figure 5).

Verapamil on top of dofetilide perfusion significantly increased the mean RR interval i.e. decreased the ventricular heart rate as compared with that of the DOF group (Figure 5).

3.1.3 The absolute variability of the RR and QT intervals

The absolute variability parameters tended to increase of all groups in the treatment period, however, any of the QT or RR variability parameters did not significantly differ between the lidocaine, verapamil and the control groups before dofetilide perfusion neither during 'Pretreatment period' nor during treatment period (Figure 6).

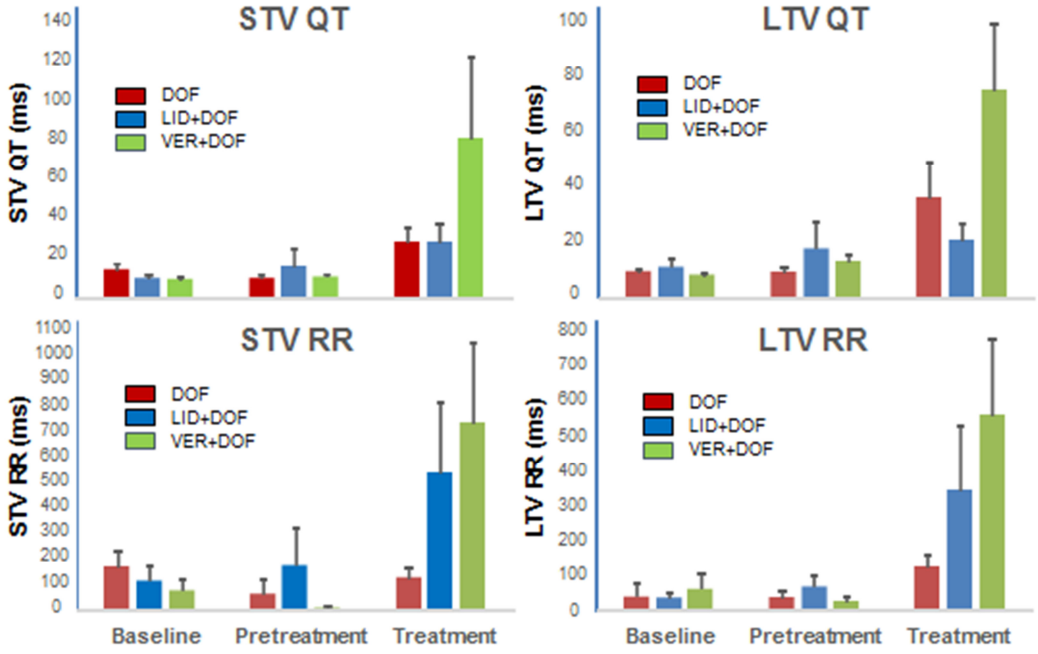


Figure 6. The short-term variability (STV) and the long-term variability (LTV) of the QT and RR interval during the pretreatment and the treatment periods in AV-ablated isolated rabbit hearts

3.1.4 TdP incidences

In the experiments, dofetilide provoked TdP in the majority of the hearts (88%). Lidocaine significantly decreased the incidence of dofetilide-induced TdP (13%), while verapamil completely prevented the development of this arrhythmia (0%). Thus, both lidocaine and verapamil could significantly decrease the incidence of TdP in this model.

3.1.5 Coronary flow

The group mean baseline coronary flows 1 min before the 'pretreatment' period ranged from 7.9±0.5 to 10.4±0.5 ml min^{-1} g^{-1} (no significant difference between the groups). Perfusion with verapamil significantly increased the coronary flow as compared with the DOF group (the maximum coronary flow values were 13.3±1.3 and 6.7±0.2 ml min^{-1} g^{-1} in the VER+DOF and the DOF group, respectively). Lidocaine did not influence the coronary flow as compared with the DOF group (Figure 7).

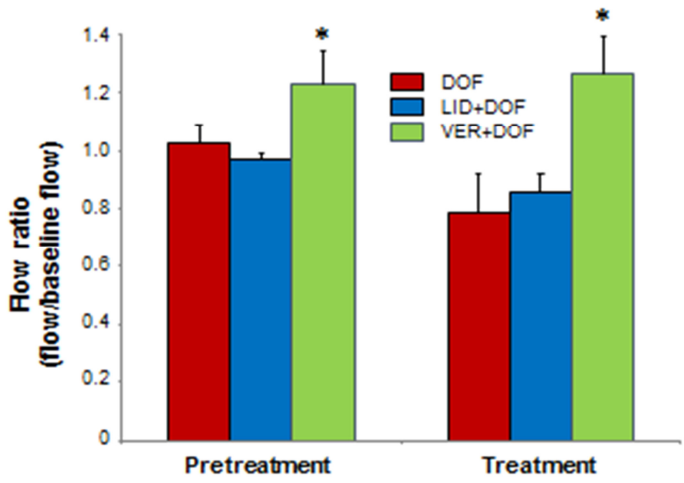


Figure 7. The coronary flow during the pretreatment and the treatment periods in AV-ablated isolated rabbit hearts

3.2 The in vitro, isolated, reduced repolarization reserve rabbit heart model

3.2.1 QTc interval and heart rate in sinus rhythm

First set of experiments

QTc did not differ between the groups in the pretreatment period (data not shown). In the treatment period, administration of dofetilide for 10 min significantly prolonged the QTc interval, however QTc did not differentiate between the 'Dof 15' and the 'HMR + Dof 15' groups (Figure 8). Occurrence of dofetilide-induced arrhythmias did not allow measurement of the QTc interval after 10 min of dofetilide perfusion. There was no significant difference in the heart rate between the groups during the whole experiment (Figure 9).

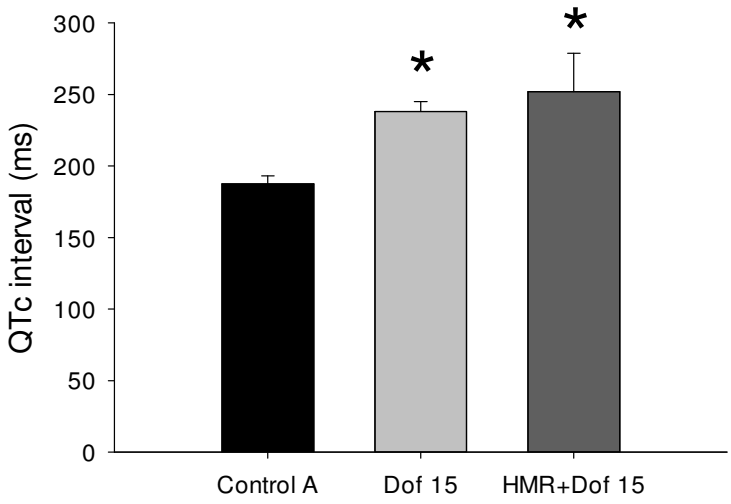


Figure 8. The QTc intervals 10 min after the start of the dofetilide (or its vehicle) perfusion in the first set of experiments. *P<0.05 vs. 'Control A' group. For further details see Table 3.

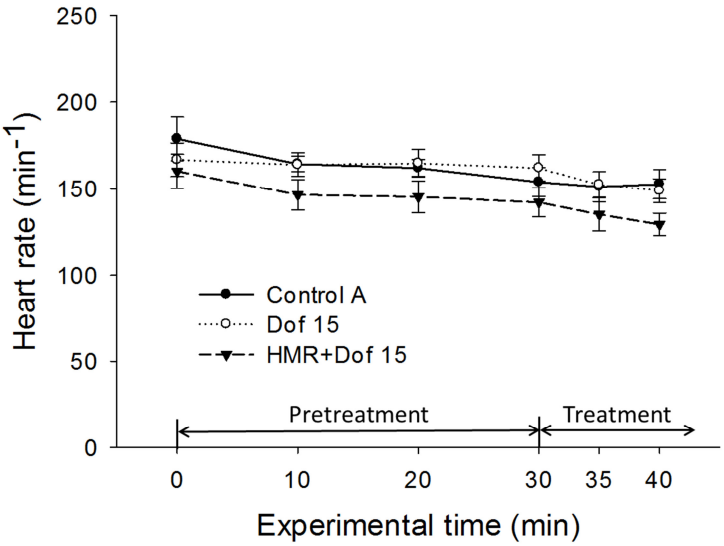


Figure 9. The heart rate in the first set of experiments. HMR-1556 (or its vehicle) perfusion commenced at 0 min (pretreatment period). Dofetilide (or its vehicle) perfusion (treatment period) began at 30 min. For further details see Table 3.

Second set of experiments

QTc did not differ between the groups at baseline and in the pretreatment period, before dofetilide perfusion (data not shown). In the treatment period, administration of dofetilide for 10 min significantly widened the QTc interval, however QTc did not differentiate between the 'Cat + Dof 50' and the 'Cat + HMR + Dof 50' groups (Figure 10); dofetilide-induced arrhythmias prevented further measurements of the biomarker at the subsequent time points. Although catecholamines tended to increase heart rate, and HMR 1556 tended to reduce heart rate, there was no significant difference in the heart rate between the groups during the whole experiment (Figure 11).

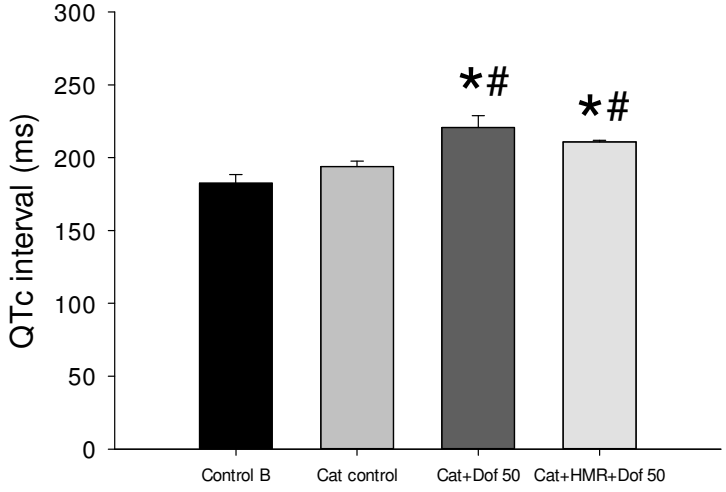


Figure 10. The QTc intervals 10 min after the start of the dofetilide (or its vehicle) perfusion in the second set of experiments. *P<0.05 vs. 'Control B' group; #P<0.05 vs. 'Cat control' group. For further details see Table 4.

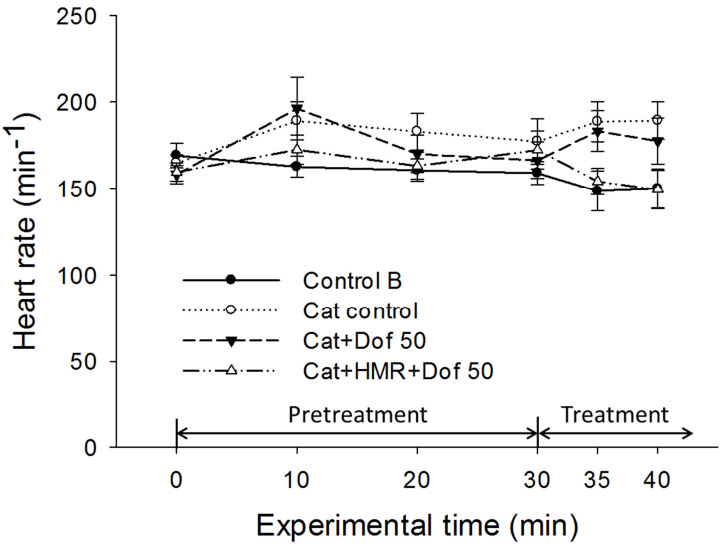


Figure 11. The heart rate in the second set of experiments. HMR-1556 (or its vehicle) and catecholamine perfusion commenced at 0 min (pretreatment period). Dofetilide (or its vehicle) perfusion (treatment period) began at 30 min. For further details see Table 4.

3.2.2 Arrhythmia incidences and onset time of arrhythmias

First set of experiments

Hearts were in sinus rhythm during the 'pretreatment' period; HMR-1556 (without dofetilide) did not evoke arrhythmias. Dofetilide tended to increase arrhythmia incidences in the 'Dof 15', and 'HMR + Dof 15' groups in the 'treatment' period, but there were no significant differences in the arrhythmia incidences among the groups (data not shown). Dofetilide induced TdP in one heart (13%) in the 'Dof 15' group; the drug did not evoke ventricular fibrillation. There were no significant differences in the onset times of arrhythmias between the groups (data not shown).

Second set of experiments

As expected, catecholamines increased arrhythmic activity. In the pretreatment period, catecholamine perfusion evoked mostly VPBs, the incidences of other types of arrhythmias (bigeminy, salvo, VT) were low. HMR-1556 on top of catecholamines did not increase the incidence of any arrhythmia; there were no significant differences in the incidences of arrhythmias between the groups (data not shown). TdP and VF did not occur in this period.

In the treatment period, addition of dofetilide on top of HMR-1556 and catecholamines increased the incidence of bigeminy, salvo, VT and VF, but the effect was not

significant as compared with control (Table 5). TdP occurred in one heart in both dofetilide-perfused groups. Co-perfusion of dofetilide, HMR-1556 and catecholamines significantly increased the incidence of conduction blocks as an indirect sign of markedly prolonged repolarization (Table 5). There were no significant differences in the onset times of arrhythmias between the groups (data not shown).

Incidences of arrhythmias (%)							
Group	VPB	BG	Salvo	VT	Block	TdP	VF
Control B	13	13	13	0	0	0	0
Cat control	88#	50	63	25	0	0	0
Cat+Dof 50	75#	63	63	38	50	13	25
Cat+HMR+Dof 50	88#	75	75	63	75*#	13	38

Table 5. Percent incidences of arrhythmias during the treatment period in the second set of experiments. VPB: ventricular premature beat; BG: bigeminy; VT: ventricular tachycardia different from torsades de pointes; Block: conduction block of any kind; TdP: torsades de pointes; VF: ventricular fibrillation. *P<0.05 vs. 'Cat control' group; #P<0.05 vs. 'Control B' group. For further details see Table 2.

3.2.3 Temporal inhomogeneity of the repolarization and the cycle length

First set of experiments

The short-term variability (STV) and long-term variability (LTV) parameters of the RR and QT intervals were determined in sinus rhythm (*sinus* variability parameters). The *sinus* STV QT and the *sinus* LTV QT parameters did not differ between the groups at baseline and during the pretreatment period (data not shown). In the treatment period, dofetilide perfusion for 5 min increased the *sinus* STV QT and the *sinus* LTV QT values. Importantly, both *sinus* STV QT and *sinus* LTV QT increased significantly in the 'HMR + Dof 15' group as compared with the values in the 'Dof 15' and the 'Control A' groups (Figure 12). The *sinus* variability parameters could not be determined at subsequent time points due to frequent occurrence of dofetilide-induced arrhythmias. There was no significant difference in any of the variability parameters of the RR interval at any time points between the groups (data not shown).

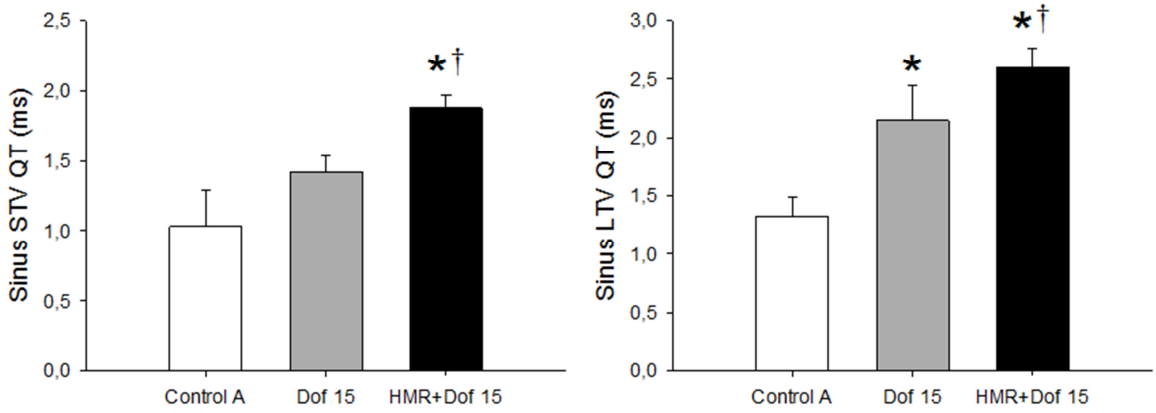


Figure 12. The short-term variability (STV) and the long-term variability (LTV) of the QT interval 5 min after the start of the dofetilide (or its vehicle) perfusion in the first set of experiments. *P<0.05 vs. 'Control A' group; †P<0.05 vs. 'Dof 15' group. For further details Table 3.

Second set of experiments

The short-term variability and long-term variability of the RR and QT intervals were determined in sinus rhythm (*sinus* variability parameters) and also irrespective of the rhythm (*absolute* variability parameters).

The sinus variability parameters of the ECG intervals

The *sinus* variability parameters of the QT and RR intervals did not differ significantly between the groups at any predetermined time points (Figures 13 and 14). Neither catecholamines nor HMR-1556 influenced the *sinus* variability parameters during the pretreatment period (data not shown). Dofetilide co-administered with HMR-1556 for 5 min increased the *sinus* STV QT and LTV QT in the 'Cat + HMR + Dof 50' group as compared with the values of the 'Cat + Dof 50' and the control groups. However, the effect was not significant, most probably because the parameter could be determined in only 5 hearts in the 'Cat + HMR + Dof 50' group due to frequent occurrence of dofetilide-induced arrhythmias (Figure 13). These arrhythmias did not allow measurement of the *sinus* variability parameters at subsequent time points.

The absolute beat-to-beat variability of the ECG intervals

Unlike *sinus* variability parameters, *absolute* variability parameters could be determined in all hearts, as measurement of the *absolute* variability parameters are not restricted to sinus rhythm. None of the *absolute* variability parameters of the RR and QT intervals differed between the groups at baseline and during the pretreatment period (data not shown). In the treatment period, co-perfusion of dofetilide with HMR-1556 for 5 min

increased the *absolute* variability parameters of the QT and RR intervals (Figures 13 and 14). Importantly, the *absolute* STV QT and the *absolute* LTV QT parameters differentiated between the 'Cat + Dof 50' and 'Cat + HMR + Dof 50' groups; that is the *absolute* variability parameters of the QT interval were significantly increased in the 'Cat + HMR + Dof 50' group as compared with the values in the 'Cat + Dof 50' group (Figure 13).

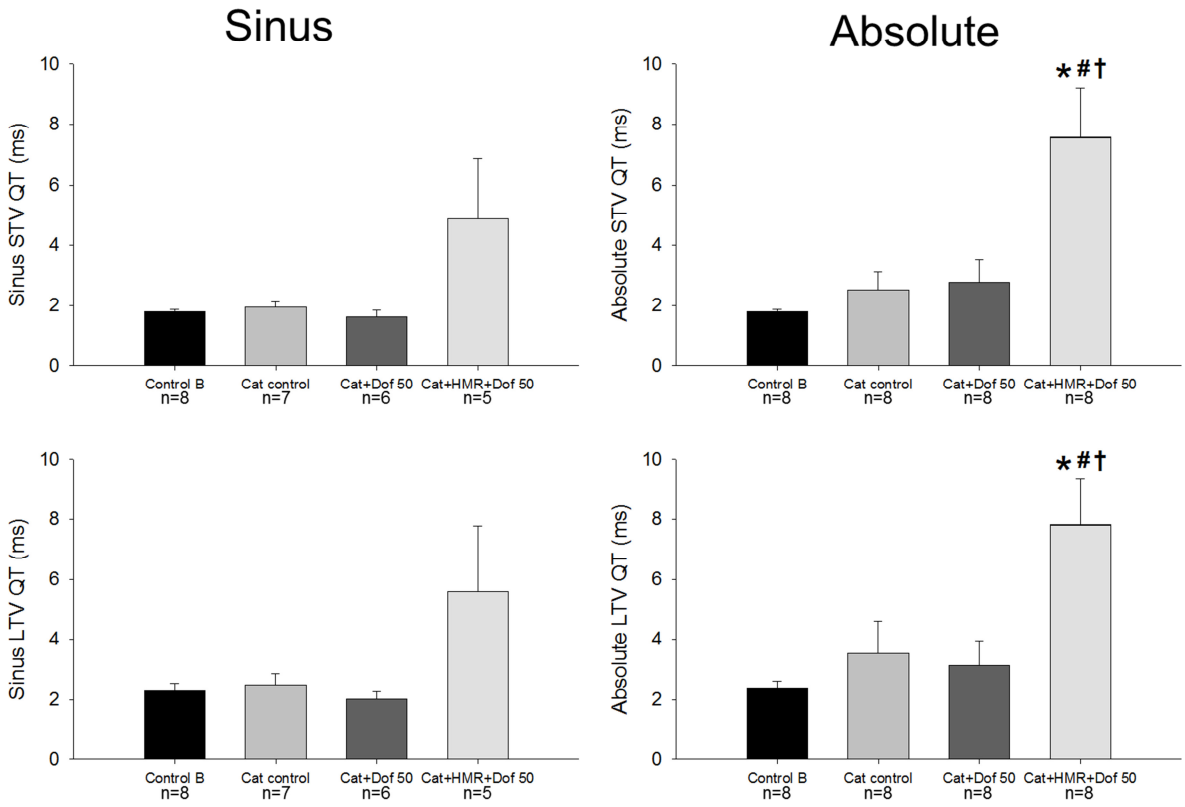


Figure 13. The *sinus* and *absolute* short-term variability (STV) and long-term variability (LTV) parameters of the QT interval in the 'Treatment' period, 5 min after the start of the dofetilide (or its vehicle) perfusion in the second set of experiments. *P<0.05 vs. 'Control B' group; #P<0.05 vs. 'Cat control' group; †P<0.05 vs. 'Cat + Dof 50' group. For further details Table 4.

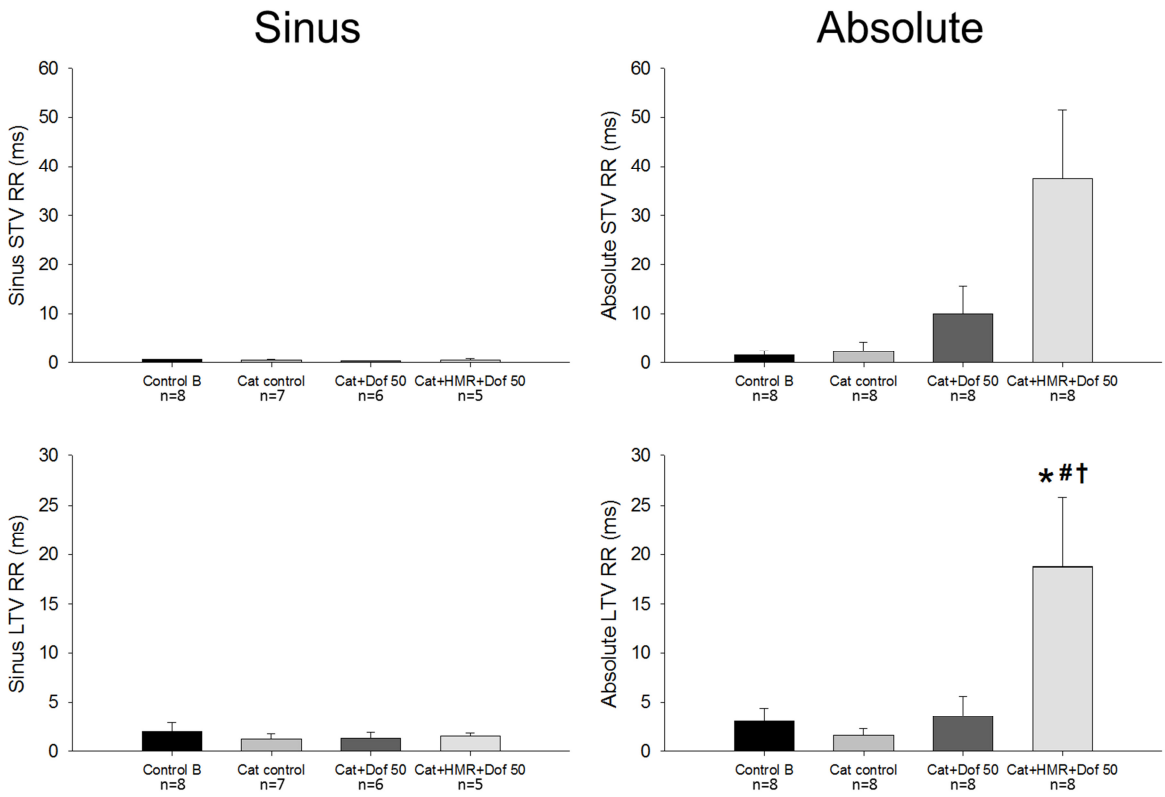


Figure 14. The *sinus* and *absolute* short-term variability (STV) and long-term variability (LTV) parameters of the RR interval in the 'Treatment' period, 5 min after the start of the dofetilide (or its vehicle) perfusion in the second set of experiments. *P<0.05 vs. 'Control B' group; #P<0.05 vs. 'Cat control' group; †P<0.05 vs. 'Cat + Dof 50' group. For further details Table 4.

3.2.4 Coronary flow

The mean baseline coronary flows ranged from 4.1±0.3 to 7.3±0.5 ml min^{-1} g^{-1}.in the first set of experiments and from 4.2±0.3 to 7.4±0.7 ml min^{-1} g^{-1} in the second set of experiments. There was a small time-dependent fall in the coronary flow values during the experiments in all groups.

In the first set of experiments there was no significant difference in the coronary flow between the groups. In the second set of experiments, catecholamine perfusion increased the flow slightly but there was no significant difference in the coronary flow between the groups (data not shown).

4 Discussion

AV block led to a chaotic idioventricular rhythm under drug-free conditions which was accompanied by an elevated beat-to-beat variability of the RR and QT intervals. The inhibition of the Na^+ channels by lidocaine as well as the block of the L-type Ca^{2+} channels by verapamil significantly antagonized the genesis of dofetilide-induced Torsades de Pointes. However, verapamil further increased the dofetilide-induced QTc prolongation and neither verapamil nor lidocaine reduced the dofetilide-induced increase in the beat-to-beat variability of the QT interval. Thus, neither QTc prolongation nor an increase in the beat-to-beat variability of the QT interval is a sufficient prerequisite of TdP genesis in rabbit hearts.

QTc failed to predict the known increased risk of TdP in the setting of reduced repolarization reserve. However, a significant elevation in the *sinus* beat-to-beat variability values of the QT interval (*sinus* STV QT and LTV QT) indicated the increased probability of proarrhythmia, when drug-induced arrhythmias were infrequent during administration of a low concentration of dofetilide. In contrast, when catecholamines and an elevated concentration of dofetilide was applied, *sinus* beat-to-beat variability parameters of the QT interval could not forecast an increased hazard to proarrhythmia occurrence, since frequent drug-induced arrhythmias precluded measurement of the beat-to-beat variability parameters in sinus rhythm. However, increased *absolute* beat-to-beat variability parameters of the QT interval (*absolute* STV QT and LTV QT), which are determined irrespective of the rhythm even during arrhythmias, were indicatives of the increased risk of TdP in the setting of reduced repolarization reserve even when frequent arrhythmias compromised the measurement of the *sinus* STV QT and LTV QT.

4.1 The in vitro, isolated, AV ablated rabbit heart model

4.1.1 AV block induced a chaotic rhythm and an electrical instability

In our study, acute AV ablation was performed in order to decrease the ventricular heart rate since bradycardia has been reported to improve the proarrhythmic activity of I_{Kr} inhibitors in isolated rabbit hearts[64]. However, AV block did not lead to regular bradycardic escape rhythm, but a chaotic idioventricular rhythm in almost all hearts under drug-free conditions. This chaotic idioventricular rhythm is characterized by an elevated beat-to-beat variability of the RR and QT intervals. Since these beat-to-beat variability parameters were measured under dysrhythmia (other rhythm than sinus rhythm) these parameters were

described as absolute variability parameters. Interestingly, similar electrical instability was reported in isolated mouse hearts after AV ablation, and the level of instability (i.e. the incidence of baseline arrhythmias after AV block) inversely correlated with the K+ concentration of the perfusion solution65. Thus, baseline electrical instability in the AV-blocked rabbit hearts perfused with 3 mM K+ in the present study might contribute to the high sensitivity of these hearts to the proarrhythmic activity of dofetilide.

4.1.2 The applied repolarization related biomarkers did not correlated with the incidence of Torsades de Pointes

Large amount of experimental and clinical data suggest that QT prolongation alone is an unreliable predictor of drug-induced TdP[34]. In our study, to get the best QT correction formula matching the applied AV ablation circumstances, all QTc intervals in this study were corrected according to the QTc-AV (see Methods). Interestingly, verapamil exaggerated the dofetilide-induced QTc prolongation while it prevented the formation of TdP. Likewise, lidocaine could decrease significantly the incidence of TdP, but did so without affecting the QTc interval. These results corroborate earlier reports showing that QT prolongation did not correlate to the proarrhythmic liability of repolarization-prolonging drugs in isolated, AV-ablated rabbit hearts[66, 67].

Temporal dispersion is characterized as an important mechanism of the maintenance of comlex proarrhythmias, e.g. Torsades de Pointes[7]. The temporal dispersion of repolarization defined as the beat-to-beat variability of the monophasic action potential duration has been shown to predict the proarrhythmic activity of repolarization-prolonging drugs in isolated rabbit hearts[15] and in chronic AV-blocked dogs *in vivo*[16]. In accordance with this, Lengyel et al. found that the beat-to-beat variability of the QT interval predicted the occurrence of drug-induced TdP in anaesthetized rabbits[68]. In contrast, the beat-to-beat variability of the QT interval failed to predict repolarization-prolonging-drug-induced TdP in two recent investigations with adrenergically stimulated, anaesthetized rabbits[27, 69]. Thus, the predictive power of an increase in the beat-to-beat variability of the QT interval is still debated. In our AV ablated isolated heart study, 100 nM dofetilide perfusion increased each QT variability parameter, and the drug induced TdP incidence reproducibly in nearly all hearts. On the other hand, neither verapamil nor lidocaine decreased any of the QT variability parameters, nevertheless, they successfully prevent the development of TdP. Thus, according to our results, the predictive value of an increase in beat-to-beat variability of the QT interval in

isolated, AV ablated, non-paced rabbit hearts is questionable. Further investigation is needed to clarify whether it is because an increase in the beat-to-beat variability of the action potential is not a contributor to TdP or rather it is because an increase in the beat-to-beat variability of the QT interval is only one out of several contributing factors needed to be present at the same time to allow generation of TdP in the model.

4.1.3 The antitorsadogenic effect of verapamil and lidocaine

The I_{CaL} channel blocker verapamil significantly prolonged the QT interval in the presence of dofetilde and prevented the development of TdP. Verapamil has never been reported to cause TdP and indeed has been proposed as a therapy for long QT–related arrhythmias, so verapamil is considered as a relatively safe antiarrhythmic drug. However, verapamil can inhibit I_{Kr} in the same concentration range as quinidine and amiodarone[70]. This may explain why the drug prolonged further the QT interval in our study, when the repolarization reserve was very small as a result of dofetilide perfusion. Verapamil also decreased the heart rate (prolonged the mean RR interval), i.e. reduced the number of idioventricular beats mostly when it was co-perfused with dofetilide. Therefore, this effect of verapamil might reduce the number of trigger beats for the initiation of TdP. Since lidocaine tended to have the same effect on the ventricular rate, this may also explain its antiarrhythmic effect in our study. Shimizu et al. reported that verapamil suppressed spontaneous or epinephrine-induced EADs and TdP in patients with congenital LQTS[71]. Milberg et al. found, that the same concentration of verapamil we used (750 nM) prevented TdP via the reduction of EAD and ventricular transmural dispersion of repolarization in an isolated rabbit heart model of the acquired LQT3 syndrome[49]. Likewise, lidocaine suppressed the I_{Kr} blocker almokalant-induced dispersion of repolarization and the development of EADs in rabbit Purkinje fibres *in vitro*[72]. Thus, the antiarrhythmic effect of verapamil and lidocaine in our experiments may also be related their direct effect on the development of EAD and/or dispersion of the repolarization.

4.2 The in vitro, isolated, reduced repolarization reserve rabbit heart model

4.2.1 Decreased repolarization reserve augments risk of proarrhythmia, and limits the use of biomarkers

Based on the theory of repolarization reserve, the fine-tuned function of repolarization currents may compensate the lack of a repolarizing current[35], e.g. I_{Ks} may compensate the

decreased function of I_{Kr}. However, a multiple hit on repolarization might lead to an excessive APD prolongation and may render the cardiac muscle more susceptible to proarrhythmia[25, 73].

Reduction of repolarization reserve increased the incidence of dofetilide-induced TdP in dogs and rabbits *in vivo*[25]. Also, it was documented that the increased beta-adrenergic tone increased the effect of the I_{Ks} inhibitor HMR-1556 on refractoriness observed at rapid rates in canines *in vivo*[56]. Interestingly, neither increased concentration of dofetilide nor boosted HMR-1556-induced I_{Ks} inhibition due to catecholamine administration could initiate significant amount of dofetilide-induced TdP in the present investigation in isolated rabbit hearts, which highlights the importance of the surrogate biomarkers of TdP in preclinical drug-safety investigations. The exact reason why a low incidence of TdP was observed in the present investigation is not examined. The concomitant I_{Ks} and I_{Kr} block significantly increased the incidence of conduction blocks, which indirectly proves that the repolarization process was sufficiently attenuated in the present experiments[50]. The incidence of TdP would have been increased with the application of higher concentrations of dofetilde and HMR-1556 (unpublished results from our laboratory), however, this was not the primary aim of this study. The combined I_{Ks} and I_{Kr} blockade did increase the incidence of less complex arrhythmias, and thus, prevented the use of TdP biomarkers that are based on ECG interval measurement restricted to sinus rhythm. However, the occurrence of arrhythmias provided scope for testing the predictive power of the novel TdP biomarkers, the *absolute* variability parameters[28] that are not restricted to sinus rhythm.

4.2.2 QTc failed to predict increased proarrhythmia risk in reduced repolarization reserve

The QT and heart rate corrected QT (QTc) interval prolongation is the solely predictor of TdP that is unequivocally received by the authorities[19, 20], although, their sensitivity and specificity has been strongly questioned[8, 21, 22, 27-29].

In the present study the I_{Kr} blocker dofetilide in a concentration of 15 and 50 nM caused a significant QTc interval prolongation as compared with the control groups. HMR did not lengthen QTc interval. This supports the finding of recent studies where I_{Ks} did not prolong QTc interval in isolated rabbit hearts[39] and did not lengthen the APD in Langendorf-perfused rabbit hearts and in cardiac papillary muscles[39, 74] reinforcing the statement that I_{Ks} may have little role in normal action potential repolarization and it probably plays a vital role when cardiac APD is abnormally lengthened by other means[73]. Dofetilide alone and co-perfused with the I_{Ks} blocker HMR-1556 caused an equivalent degree of QTc prolongation, thus the

extent of the QTc prolongation did not differ between the presence and absence of the I_{Ks} inhibition. Similarly, Lengyel et al. found that the concomitant inhibition of I_{Ks} and I_{Kr} with dofetilide and HMR-1556 did not prolong the QTc interval significantly as compared with that achieved by either the I_{Kr} or I_{Ks} inhibition in anaesthetized rabbits and dogs *in vivo*[25]. These results emphasize that QTc interval is not a suitable biomarker to identify increased TdP liability in case of reduced repolarization reserve.

4.2.3 Sinus beat-to-beat variability of the repolarization is a better predictor of TdP in reduced repolarization reserve, but arrhythmic activity limits its use

It has been suggested that temporal dispersion of repolarization could predict proarrhythmic events[75-77]. It was found in the chronic AV node blocked dog model that a downregulation of several potassium currents, most notably the I_{Ks} channel, occurs due to the electrical remodeling[78]. Additional pharmacological block of I_{Kr} did not prolong significantly the QTc interval, but increased the beat-to-beat variability of the repolarization (STV QT), when it was measured in arrhythmia-free, regular rhythm[16]. Lengyel et al. measured STV QT in sinus rhythm in rabbits *in vivo*, and directly demonstrated that TdP evoked by combined pharmacological block of I_{Kr} and I_{Ks} can be better predicted by increased STV QT than by QTc changes alone[25]. Carlsson et al. reported that dofetilide increased STV QT in sinus rhythm prior to the occurrence of ventricular premature beats and TdP in methoxamine-sensitized, α-chloralose-anaesthetized rabbits[26]. However, *sinus* STV QT did not predict TdP in other studies utilizing a similar, anaesthetized rabbit model[27-30].

In the first set of experiments in the present investigation the measurement of the beat-to-beat variability of the QT intervals was possible in sinus rhythm as the arrhythmic activity was low during concomitant pharmacological inhibition of I_{Ks} and I_{Kr} in isolated rabbit hearts. In the group perfused with dofetilide together with HMR-1556 the beat-to-beat variability parameters of QT interval were significantly increased as compared with those in the control group, and more importantly, compared with those in the group perfused only with dofetilide. Accordingly, STV and LTV QT could differentiate the combined ion-channel block from the solely I_{Kr} block identifying the increased TdP liability during decreased repolarization reserve.

However, in the second set of experiments, catecholamines and elevated concentration of dofetilide increased the arrhythmic activity, which precluded the measurement of the *sinus* beat-to-beat variability parameters of the QT intervals, thus these parameters failed to indicate

the increased proarrhythmic liability of the drugs during reduced repolarization reserve. These results show that s*inus* beat-to-beat variability parameters of the repolarization (*sinus* STV QT and LTV QT) are better predictors of TdP than QTc in reduced repolarization reserve, although the application of these parameters is limited when arrhythmia occurs.

4.2.4 Absolute beat-to-beat variability of the repolarization is the best proarrhythmia predictor in reduced repolarization reserve

In the presence of reduced repolarization reserve ectopic beats can render the APD unstable for many beats[15] and thus produce large beat-to-beat irregularity[79]. However, increased variability of repolarization may remain latent until the heart is disturbed by an ectopic beat[80]. In a previous *in vivo* study, most TdP events were preceded by less complex arrhythmias (e.g. VPBs, salvos, bigeminy, etc.) in an anesthetized rabbit proarrhythmia model[28]. Similar non-complex arrhythmias occurred frequently in the second set of experiments in the present *in vitro* investigation, when hearts were sensitized to arrhythmia development by the co-perfusion of catecholamines and an elevated concentration of dofetilide. Since TdP rarely occurs spontaneously without preceding arrhythmias, however the number of arrhythmic beats was not a precise predictor of drug-induced TdP[28], new TdP biomarkers that can be measured irrespective of the rhythm even during arrhythmias are needed. The *sinus* beat-to-beat variability of the repolarization can predict TdP development, but its predictive power is not consistent especially when frequent arrhythmias preclude its measurement. However, the newly developed *absolute* beat-to-beat variability parameters of the ECG intervals seem to be more reliable surrogate biomarkers of TdP[28]. Indeed, in the present study, where the *sinus* beat-to-beat variability was not predictive in the second set of experiments, the *absolute* beat-to-beat variability parameters of the repolarization still indicated the increased proarrhythmic liability of dofetilide in the setting of decreased repolarization reserve.

	QTc	*sinus* variability	*absolute* variability	simple arrhythmia	complex arrhythmia
AV ablation study					
DOF	↑vs. control[#]	not evaluable	↑ vs. control[#]	not evaluable	↑TdP vs. control[#]
LID+DOF	↑ vs. DOF	not evaluable	-	not evaluable	↓TdP
VER+DOF	↑ vs. DOF*	not evaluable	↑ vs. DOF	not evaluable	↓TdP
Repol. reserve study 1. series					
DOF15	↑ vs. control A*	↑ vs. control A*	not evaluable	↑ vs. control A	↑ vs. control A
HMR+DOF15	↑ vs. control A*	↑ vs. control A/DOF15*	not evaluable	↑ vs. control A	-
Repol. reserve study 2. series					
CATcontrol	-	-	↑ vs. control B	↑ vs.control B*	↑ vs. control B
CAT+DOF50	↑ vs. control B/CAT control*	-	↑ vs. control B	↑ vs.control B*	↑ vs. control B
CAT+DOF50+HMR	↑ vs. control B/CAT control*	↑ vs. each group	↑ vs. each group*	↑ vs.control B*	↑ vs. control B

Table 6. Summarizing table of the assessed proarrhythmia biomarkers of the two studies. See the difference between decreased repolarization reserve group vs. simple IKr inhibited group, highlighted with red; not evaluable: the parameter cannot have been measured/evaluated [#] $p<0.05$ vs. control group in first series of experiments in AV ablation study (not detailed); *$p<0.05$, significant change

4.2.5 The efficacy of the applied proarrhythmia biomarkers in TdP forecast

Summarizing our assessment of TdP predictors in the two similar Langendorff TdP model, we can conclude that beat-to-beat variability parameters could reliably forecast the higher chance of TdP development (Table 6). The applied absolute variability parameter allow the analysis of ECG segments during arrhythmia, therefore, the beat-to-beat ECG analysis is not only restricted to sinus or stable, regular rhythm. The beat-to-beat variability parameters tended to be more sensitive than QTc, or simple arrhythmia analysis alone in the TdP prediction. However, the specificity of variability parameters is still merit further assessment.

5 Conclusion

AV ablation resulted in a chaotic idioventricular rhythm, which might make the hearts more sensitive to the proarrhythmic activity of dofetilide in the applied isolated rabbit heart model. In our AV ablated model, the inhibition of the I_{Na} and I_{CaL} currents successfully antagonized the genesis of this arrhythmia. Neither QTc prolongation nor an increase in the beat-to-beat variability of the QT interval is a sufficient prerequisite of TdP genesis in this model.

QTc prolongation did not identify the higher proarrhythmic risk of dofetilide in the setting of reduced repolarization reserve in isolated rabbit hearts. In contrast, the *sinus* beat-to-beat variability of QT interval as a TdP biomarker indicating temporal dispersion of repolarization was a sensitive biomarker of proarrhythmia in the setting of decreased repolarization reserve. However, when measurement of the *sinus* variability parameters was hindered by arrhythmias caused by addition of catecholamines and an increased concentration of dofetilide, only *absolute* beat-to-beat variability parameters of the repolarization could reveal the higher TdP liability in the setting of reduced repolarization reserve. This confirms that the *absolute* beat-to-beat variability parameters of the repolarization (*absolute* STV QT and LTV QT) are very sensitive and useful biomarkers of proarrhythmia.

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