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Title: QT prolongation modifies dynamic restitution and hysteresis of the beat-to-beat QT-TQ-interval relationship during normal sinus rhythm under varying states of repolarization.

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Running Title: QT prolongation modifies restitution and hysteresis

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Abbreviations: APD-action potential duration; DI-diastolic interval; LQT-Long QT syndrome; L-768,673 and E-4031 are both experimental class III antiarrhythmics.

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ABSTRACT

The analysis of cardiac electrical restitution (the relationship between an action potential duration and its preceding diastolic interval) has been used to predict arrhythmia liability. However, the procedure to measure restitution is invasive and disrupts normal physiologic autonomic balance. Dynamic analysis of sequential beat-to-beat ECG data was used to study restitution under normal sinus rhythm and quantify changes in temporal hysteresis with heart rate acceleration/deceleration during QT prolongation. Congenital LQT1 and LQT2 syndromes during sympathetic stimulation were modeled because of their association with increased risk of ventricular arrhythmia. Temporal heterogeneity and hysteresis of restitution were examined in the conscious dog under varying conditions of delayed repolarization using either the selective inhibitors of the slowly activating delayed rectifier potassium current, L-768,673 ((R) –2-(,4-trifluoromethyl)-N-[2-oxo-5-phenyl-1-(2,2,2-trifluoroethyl)-2,3-dihydro-1H-benzo[e][1,4]diazepin-3-yl]acetamide); the rapidly activating delayed rectifier potassium current, E-4031 ((1-[2-(6-methyl-2-pyridyl)ethyl]-4-methyl-sulfonylaminobenzoyl)-piperidine); or a combination of both at rest and during heart rate acceleration with sympathetic stimulation using isoproterenol challenges. Impaired repolarization with the combination of E-4031 and L-768,673 increased heterogeneity of restitution at rest 55 to 91%; increased hysteresis during heart rate acceleration after isoproterenol challenge by approximately 40 – 60%; and dramatically reduced the minimum TQ interval by 72% to only 28 ms. Impaired repolarization alters restitution during normal sinus rhythm and increases hysteresis/heterogeneity during heart rate acceleration following sympathetic stimulation. Thus, dynamic beat-to-beat measurements of restitution could lead to

clinically applicable ECG obtained biomarkers for assessment of changes associated with arrhythmogenic risk.

INTRODUCTION

The duration of the cardiac action potential is largely dependent on the duration of the preceding diastolic interval (DI) (Bass, 1975). This relationship is referred to as action potential duration (APD) restitution and is an important determinant of the beat-to-beat cardiac dynamics at all heart rates or RR intervals. The *restitution hypothesis* (Karagueuzian and Chen, 1999) infers if during any DI the kinetics of ion channels (Fox et al., 2002) or intracellular calcium handling (Chudin et al., 1999; Pruvot et al., 2004) have not reached steady-state, an oscillation of short-long DI intervals of each beat followed by a subsequent short-long APD, known as alternans, ensues to maintain stability at a given cycle length or heart rate. However, when the magnitude of these oscillations is increased to the point where the DI is extinguished (i.e. due to refractoriness), the synchronous pattern is broken leading to a triggered unstable reentry and arrhythmia (Karma, 1994; Qu et al., 1999). Therefore, drugs or conditions that affect APD (or DI) may increase arrhythmogenic risk by prolonging APD directly to the described triggering point. This could reduce the threshold heart rate at which instability is more likely to occur or allow the heart to be more susceptible to autonomic states that cause rapid changes in heart rate to trigger arrhythmias like Torsade de Pointes. Sudden cardiac death in congenital long-QT syndromes LQT1 and LQT2 is consistent with this hypothesis and is usually associated with sympathetic-mediated accelerations of heart rate (Schwartz et al., 2001). Increases in hysteresis of the QT-RR interval relationship have been reported in these patients (Krahn et al., 2002). In vitro, modeling of these syndromes with inhibitors of the slowly activating delayed rectifier potassium current (IKs), as in LQT1, combined with either adrenergic stimulation (Shimizu and

Antzelevitch, 1998) and/or the rapidly activating delayed rectifier potassium current (IKr), as in LQT2, produces early afterdepolarizations indicative of the liability for arrhythmia triggering (Volders et al., 2003; Aiba et al., 2005).

Cardiac restitution and extrapolation to arrhythmia liability has been confounded by pacing protocols (Otani and Gilmour, 1997; Kalb et al., 2004) and varying autonomic states that are necessitated by anesthesia in the patient or animal. In order to determine if heterogeneity and hysteresis of restitution could be examined under normal sinus rhythm conditions as biomarkers associated with arrhythmia liability, dynamic sequential beat-to-beat analyses were employed as previously reported by this lab (Fossa et al., 2005). To mimic the conditions of risk for arrhythmia as in patients with LQT1 and LQT2 syndromes, conscious dogs were evaluated under varying conditions of delayed repolarization using either selective inhibitors of the IKs current, L-768,673 ((R) –2-(,4-trifluoromethyl)-N-[2-oxo-5-phenyl-1-(2,2,2-trifluoroethyl)-2,3-dihydro-1H-benzo[e][1,4]diazepin-3-yl]acetamide) (Salata et al., 2004); the IKr current, E-4031-(1-[2-(6-methyl-2-pyridyl)ethyl]-4-methyl-sulfonylaminobenzoyl)-piperidine) (Fujiki et al., 1994); or a combination of both at rest and during heart rate acceleration with sympathetic stimulation using isoproterenol challenges. Our results indicate that dynamic beat-to-beat restitution measurements are assessable in the normal conscious state and could lead to clinically applicable ECG obtained biomarkers for assessment of changes associated with arrhythmogenic risk.

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METHODS

Surgical and Experimental Protocol

The investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH publication No.85-23, revised 1996) and the Declaration of Helsinki. All surgeries and experimental procedures were performed in agreement with an Animal Care and Usage Protocol approved by the Institutional Animal Care and Use Committee, and similar to those described elsewhere (Raunig et al., 2001; Fossa et al., 2002). Briefly, sling-trained dogs (males and females between 10 and 15 kg) were initially monitored to ensure no gross cardiac arrhythmias or behavioral irregularities. Dogs that met this criterion had a telemetry device (Data Sciences International, St. Paul, MN) surgically implanted to measure arterial blood pressure (BP) via a femoral artery, and the Lead II electrocardiogram (ECG) was used. On the day of each study, dogs were placed individually in an isolation room and two cephalic venous catheters were put in place for intravenous administration of test compounds via remote-controlled infusion pumps (Harvard Apparatus, Cambridge, MA). The digitized signals from the telemetry device were converted back to analog, sampled, and saved to a computer at 1000 Hz using a Po-Ne-Mah Data Acquisition and Analysis system (Gould Instrument Systems, Valley View, OH). Baseline data were recorded for at least 20-minutes prior to infusion of any test substance.

Dosing Protocol

The experiment was a crossover design in which five dogs received each of the following five treatments in random order: 1) L-768,673+E-4031+isoproterenol, 2)

vehicle+E4031+isoproterenol, 3) L-768,673+vehicle+isoproterenol, 4) vehicle+vehicle+isoproterenol, 5) vehicle+vehicle+saline. Studies in individual animals were separated by at least 7-days to allow for washout of compounds. A timeline that illustrates the infusion of compounds is shown in Figure 1.

L-768,673 was synthesized at Pfizer (Sandwich, UK), dissolved in 100% ethanol and then suspended in Intralipid 20% fat emulsion (Baxter Healthcare Corp, Deerfield, IL). Final vehicle solution was 5% ethanol and 95% Intralipid. L-768,673 was administered at 100 μg free base/kg as a constant infusion over 15 minutes. This dose was shown by Salata et al (2004) to achieve rate-dependent refractoriness associated with QTc prolongation in conscious dogs of about 10% and a plasma concentration of approximately 100 ng/mL.

E-4031 was synthesized at Pfizer (Sandwich, UK) and dissolved in saline. The clinically relevant free drug concentration of E-4031 to produce a 10-15% increase in QT prolongation is between 3 and 4 nM (Fujiki et al., 1994). In this study, three ascending doses were administered as previously described and measured (Fossa et al., 2002) to achieve clinically relevant concentrations of 0.6, 1.7 and 8.8 nM free drug. Infusion of E-4031 began 5-minutes after the end of the infusion of L-768,673 or vehicle.

±Isoproterenol hydrochloride was purchased from Sigma-Aldrich (St. Louis, MO) and dissolved in saline. The experimental data was assessed separately during each Period as (A) before isoproterenol and (B) during isoproterenol (Figure 1). Four separate infusions of isoproterenol, 0.2 μg free base/kg, were given over 1-minute in each study: one infusion during Period 1B, and one infusion at 5-minutes of each maintenance dose of E-4031 or vehicle (Periods 2B, 3B and 4B). This dose of isoproterenol causes an

increase heart rate of approximately 180 BPM over the 1-minute infusion, followed by a gradual decrease in heart rate back to the pre-isoproterenol level within 10 minutes.

Review of Dynamic Beat-to-beat Data

ECG waveforms were replayed on the Po-Ne-Mah system and each cardiac cycle was reviewed for accurate detection of the beginning of the Q-wave and the end of the T-wave as described previously (Fossa et al., 2005). All computer-generated validation marks were visually inspected. QT intervals that could not be measured due to motion artifact or electrical noise of the ECG were removed from the dataset and if necessary the QT interval was manually measured using a digital measure command on the Po-Ne-Mah system. Similarly, the computer generated validation marks for PR and QRS intervals were also reviewed as described above. In some instances not all ECG intervals from a given ECG complex could be measured. For example, at rest and during the isoproterenol response a T-wave may have coincided with a preceding P-wave or there was ventricular depolarization without atrial depolarization (ectopic beat or PVC). If the T-wave morphology was preserved and the end of the T-wave could be identified, the QT interval was recorded but the PR interval was not measured. Therefore, rather than exclude all interval measurements from that waveform, we included those measurements that were valid. In those cases the number of data points that were incorporated in the analyses were not the same (varied as much as 7%) for QT, PR and QRS, but resided in the same sequence of beats.

The raw signals were reviewed continuously for the last 10-minutes of Period 1 and throughout the remaining treatment periods including the infusions of isoproterenol

(Periods 2 to 4) as shown in Figure 1. The TQ interval of the ECG was calculated as the RR-interval minus the QT-interval of the previous beat. The replayed data files containing the ECG intervals and blood pressure measurements were imported into EXCEL (Microsoft, Inc., Redmond, WA) and transformed into a format that was read by programs written in MATLAB (MathWorks, Inc., Natick, MA) and R (Ihaka and Gentleman, 1996).

Statistical Analysis

The experiment was designed as a randomized block study with dog as the random block. Figure 1 shows the data analyses periods. Data from each period were broken down into two separate datasets for analyses: before isoproterenol (A) and during isoproterenol (B).

To examine changes in blood pressure and ECG intervals, data were collected from each dog for a 10-minute baseline period prior to initial treatment (Period 1A) and for 5-minutes during the maintenance dose of each treatment (Periods 2A, 3A and 4A). The change in QT interval from baseline Period 1A was estimated using the heart-rate-adjusted bootstrap method. The method discussed in our previous work (Fossa et al., 2005) estimated an uncorrected change in QT intervals from sequential sets of beat-to-beat ECG cycles. This value was adjusted for heart rate by estimating the distance to a typical baseline QT value within a similar 50 ms RR bin. The bootstrap technique was used to estimate the center of the distances because distribution of these distances does not resemble a standard normal distribution.

To examine the changes in hysteresis under delayed repolarization, data were collected for a maximum of 5-minutes after administration of the isoproterenol challenge. A baseline isoproterenol response prior to treatment was obtained (Period 1B) and compared to isoproterenol responses after treatments (Periods 2B, 3B and 4B). These data were separated into an acceleration and deceleration phase by fitting a segmented regression function to RR interval vs. time (sequential cardiac cycles). To assess the arrhythmia liability related to a diminishing diastolic interval, the minimum TQ value observed after isoproterenol challenge was used. Although the minimum is not considered a robust statistic, the data from this experiment suggested that the lower TQ bound was well defined and reliable, especially since the data were verified by manual over-reading.

The measure of heterogeneity used in the analyses was the area between the upper and lower 98% confidence bounds of the QT intervals across a specified TQ interval range. This calculation represents the average length of the QT confidence bounds, which is a standard measure of variability. The upper confidence bounds of the data were estimated by binning the TQ interval and fitting a spline through the 98% QT quantiles in each TQ bin (similarly for the lower confidence bounds). Analyses of data at rest prior to isoproterenol (A) encompassed all TQ intervals. Analyses of data during isoproterenol (B), used all beats occurring within the 98% QT interval confidence bounds below TQ intervals of 1000 ms to calculate total area of temporal heterogeneity. The total area of the heterogeneity for hysteresis was further broken down and standardized for dogs with different rates by utilizing beats occurring during the timeframe when TQ intervals were

reducing from 1000 ms to 150 ms (acceleration) and increasing after peak response from 150 ms to 1000 ms (deceleration).

To determine which variables differed significantly from baseline (Period 1A or 1B) and time-matched vehicle (Periods 2-4; A or B), the data from each dataset were fit to a mixed effects repeated measures model in SAS V8.2 (SAS Institute Inc., Cary, NC).

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RESULTS

Effect of impaired repolarization on the QT-RR and QT-TQ interval relationships prior to isoproterenol challenge (Periods 1A, 2A, 3A and 4A).

Two vehicle treatments (vehicle/saline and vehicle/isoproterenol) were conducted as controls and determined to be statistically similar in relation to baseline QT, RR, TQ intervals and BP. Therefore, all further statistical comparisons were made to the vehicle/isoproterenol treatment and will be referred to as the control treatment data.

Representative examples of the beat-to-beat relationships between QT-RR and QT-TQ intervals (restitution) are illustrated in Figure 2. The QT-RR interval and QT-TQ interval relationships appear very similar under normal conditions. However, as QT varies more with impaired repolarization, the restitution becomes more perturbed because changes in TQ interval take into account the increased short-long variability of QT interval also occurring on the adjacent cardiac cycle. By fixing TQ interval on the horizontal axis (restitution panel), assessing where the diastolic period approaches zero for individual cardiac cycles is more apparent than when plotted on the QT-RR axes (see diagonal line where TQ=0 when RR =QT). Restitution responses to all treatments in the same dog are illustrated in Figure 3.

No changes in QT, RR, and TQ intervals and temporal heterogeneity were observed during the control treatment as displayed in Table 1. An increase in baseline blood pressure of 6 to 10 mmHg did occur throughout the course of the study (Periods

2A, 3A and 4A) with vehicle controls compared to baseline values (Period 1A). This response was consistent with no differences observed between vehicle treatments.

L-768,673 significantly increased QT interval 27 ms during Period 2A compared to baseline (Period 1A). This was accompanied by a reduction of the TQ interval of 26 ms. These changes, however, did not remain constant throughout the study despite a reported duration of activity for L-768,673 of greater than 15 hours (Salata et al., 2004; also see discussion). Whereby Periods 3A and 4A, the QT interval only increased 11 and 6 ms, respectively, which were not statistically different from controls (Period 1A for L-768,673 and Periods 3A and 4A for vehicle). RR interval and heterogeneity showed no differences during any period with L-768,673.

E-4031 caused dose-dependent increases in QT and reductions in TQ intervals with no changes in RR interval. The respective QT and TQ interval changes were statistically significant at Period 3A (17 and -18 ms) and Period 4A (24 and -28 ms). No statistically significant effect on heterogeneity was observed.

The combination of IKs and IKr inhibition with L-768,673 plus E-4031 produced statistically significant increases in QT interval and decreases in TQ interval at all dose levels (see example in Figure 3). The largest changes in QT (+ 50 ms) and TQ (-46 ms) intervals occurred during Period 2A but remained approximately 38 ms for QT and -41 ms for TQ during Periods 3A and 4A. No changes in RR interval were observed during any period. Heterogeneity was increased approximately 47 to 79% during Periods 2A, 3A, and 4A compared both to baseline (Period 1A) and 55 to 91% when compared to the time-matched vehicle responses. Compared with responses of L-768,673 and E-4031 alone, it would appear that the magnitude of the effects on QT, TQ and particularly

heterogeneity, are more than additive with the combination (i.e. Period 4A heterogeneity; L-768,673 (-2718 ms^2) plus E-4031 (492 ms^2) < 15352 ms^2 with combination).

Since QT-TQ interval relationship was most affected during the combination of E-4031 and L-768,673, the effect on conduction velocity was assessed as well. The combination produced no changes in either PR or QRS intervals during Period 4A when compared to baseline (Period 1A) or the time-matched vehicle control response during Period 4A (Table 1).

Effect of isoproterenol on restitution hysteresis (heterogeneity and minimum TQ analyses) before and after impaired repolarization (Periods 1B, 2B, 3B and 4B)

The changes in hysteresis as measured by heterogeneity of the plotted temporal response during acceleration, deceleration and total response as well as minimum TQ interval with isoproterenol challenges are represented in Table 2. As expected isoproterenol reduced blood pressure and PR interval while increasing heart rate (as RR interval), these parameters were not statistically assessed across treatments. Figure 4 illustrates the response to isoproterenol and how heterogeneity was calculated for each phase in a representative single animal. Also see a supplemental dynamic beat-to-beat movie of the data within Figure 4 (Supplemental Data:IKs+IKr block with iso.AVI)

Total Heterogeneity - Significant increases in total heterogeneity of 25% were obtained with L-768,673 in Period 2B and with E-4031 (30 and 53%) in Periods 3B and 4B, respectively, when compared to pretreatment baseline responses to isoproterenol (Period 1B). The combination of L-768,673 plus E-4031 increased total heterogeneity during all three treatment periods between 41 and 89%. These data seem to indicate that

total heterogeneity follows closely the magnitude of the effect on the resting TQ interval (refer to Table 1).

Acceleration phase - No changes were observed in acceleration heterogeneity after L-768,673 or E-4031 alone (TQ interval reducing from 1000 to 150 ms). The combination L-768,673 plus E-4031 statistically increased acceleration heterogeneity 41% during Period 3B and 57% during Period 4B.

Deceleration phase - No statistically significant changes were observed with vehicle or treatment during the deceleration phase after isoproterenol for any period. From this, it would appear that changes in total heterogeneity after impaired repolarization were determined primarily by changes in acceleration heterogeneity only. It should be mentioned here, although not noted in Table 2, analyses of the isoproterenol responses at each period after saline showed statistically significant decreases in the total heterogeneity at each period indicating that some attenuation of the isoproterenol response occurred across all groups with time.

TQ minimum - As TQ approaches zero, theoretically, arrhythmia liability will increase and thus the minimum TQ interval was examined. No significant effects on minimum TQ were observed with L-768,673 or E-4031 alone. A statistically significant decrease to a mere 28 ms was observed only with the combination L-768,673 plus E-4031 during Period 4B. This decrease of 73 ms represented a 72% change from the Period 1B baseline response.

DISCUSSION

In this study we examined the dynamic QT-RR interval relationship and restitution function by the QT-TQ interval relationship in the conscious canine under varying conditions of delayed repolarization that utilizes an adapted bootstrap sampling method, previously described by our lab (Fossa et al., 2005), to compare sequential series of cardiac beats that when plotted are referred to as "clouds". Increases in heterogeneity of restitution at rest and during acceleration of heart rate were observed after isoproterenol challenge following the combination of IKr and IKs inhibition (E-4031 plus L-768,673, respectively).

Treatment with the selective IKr blocker, E-4031, produced a dose-related prolongation in the QT interval consistent with previous studies (Fossa et al., 2002). Selective blockade of IKs current with L-768,673, on the other hand, produced a much larger increase in the QT interval upon initial dosing during Period 2A, but attenuated during Periods 3A and 4A. This effect that diminished with time may have resulted from additional non-selective IKr block reported for L-768,673 by Salata et al. (2004) with high concentrations that were most likely achieved upon initial iv dosing prior to complete drug distribution. It should be noted that upon initial dosing, QT prolongation of greater than 70 ms was observed in all dogs, and as much as 120 ms for some, without incidence of arrhythmia (data not shown). Humans, on the other hand, may not be able to tolerate such changes without producing arrhythmia for the reasons discussed below.

The combination of IKr and IKs inhibition with E-4031 plus L-768,673 produced greater QT prolongation than the apparent additive responses to the individual agents.

This effect could not be attributed to any changes in conduction velocity as measured by the PR and QRS intervals. Thus, it is most likely attributed to more complete inhibition of the *repolarization reserve* as described by Roden (1998). Isoproterenol challenge during combined IKr and IKs inhibition caused a marked increase in hysteresis primarily due to increased heterogeneity of the cardiac cycles upon acceleration. Since isoproterenol increases cardiac intracellular calcium release through beta-adrenergic stimulation (Steinberg et al., 2002), it is speculated that a lack of repolarization capacity amplifies the heterogeneity (Katra et al., 2004; O'Rourke et al., 1999). QT interval at rest was ominously increased with decreasing TQ (Figure 3) and a transient sharp increase in QT variability (as evidenced by an increase QT width of cloud) was apparent in three of the five dogs at higher heart rates.

Although increases in beat-to-beat variability have been reported in other models of reduced repolarization reserve, our findings appear to differ with respect to heart rate. Thomsen et al. (2004, 2005) showed, in chronic AV blocked anesthetized dogs given sotalol, that short-term variability of the left ventricular monophasic action potential duration was dose-dependently related to Torsades de Pointes incidence at slow but not fast heart rate pacing cycles. Further, reverse use dependence has been used as a primary indicator of arrhythmia liability in the isolated rabbit heart (Hondeghem et al., 2001). The rate-dependent differences from our results may be attributed to a lack of intact autonomic influences in both of these other models. It is possible that different heart rate dependencies result in mechanistically different arrhythmogenic liabilities (Gilmour et al., 1997). Therefore, other autonomic manipulations to examine the impact of vagal tone would be insightful. However, one must also consider that the rapidity of onset and

biological half-life of the physiological maneuver (i.e., exercise) or pharmacological agent (i.e. atropine) will affect the ability to assess beat-to-beat hysteresis if both acceleration and deceleration components are to be studied together.

The ability of our model to assess arrhythmia liability is based on the restitution principal that each working cycle of the heart (QT interval) is determined partly by the preceding resting diastolic phase (TQ interval). To conceptualize this model, Figure 5 is a hand-drawn cartoon to illustrate the impact of heart rate on the QT-TQ relationship with QT prolongation, and why it may be associated with ventricular arrhythmia. At a normal human resting heart rate of 60 bpm (RR interval = 1000; QT interval = 430 ms), the heart has roughly half the time between heart beats (RR-QT = TQ of 570 ms) to get its nutrients and for the ionic kinetics of each cell to return to steady state. QT interval decreases with increasing heart rate (Fridericia, 1920) so that on the average a heart rate of 150 BPM, the QT interval would be 320 ms. TQ interval is reduced at this rate 7-fold to 80 ms. Coincidentally, it is generally regarded that healthy humans transition from ventricular tachycardia to fibrillation around 250 BPM (where TQ = zero when RR = QT). Torsades de Pointes arrhythmia theoretically could be caused by conduction block in the same way when heart rate is accelerated to the point where TQ approaches zero (i.e. RR = QT) as well. QT prolongation may be associated with Torsades de Pointes because as the QT interval is prolonged at high heart rates, a disproportionate amount of the TQ interval is reduced. TQ interval is reduced from 570 ms at rest to 10 ms with prolongation and tachycardia (e.g. a 57-fold reduction in TQ vs. a 20% increase in QT interval) thus providing much less time for the heart to recover between beats. Concurrent with the reduction of the diastolic interval, it has been demonstrated that accumulation of

intracellular calcium occurs which is associated with the generation of alternans (a measure of temporal heterogeneity; Chudin et al., 1999). This may lead to increases in spatial heterogeneity (Katra et al., 2004) and triggering of arrhythmias (Karma, 1994). Luo-Rudy equations of ion kinetics would predict that this response would be further amplified with sudden changes from bradycardia or a long pause (Viswanathan and Rudy, 1999).

Generation of arrhythmias to confirm the relationship of biomarkers such as heterogeneity and TQ minimum to outcome may be more difficult in the dog than humans. Dogs have a profound sinus arrhythmia as part of their normal cardiac physiology (Hariman et al., 1980) that is compensated by ionic mechanisms that may adapt more quickly to heart rate changes than humans. Interestingly, even though the resting heart rate of the dog and human is almost the same (i.e. 60 BPM), the average dog QT interval (240 ms) is approximately 200 ms shorter than humans at the same heart rate. This may allow an additional 200 ms during heart rate acceleration or QT interval prolongation for the TQ interval to provide recovery of the heart between beats. This could explain why we did not observe arrhythmias in our study even when transient increases of >100 ms of the QT interval were obtained with the combination of L-768,673 plus E-4031.

The choice for autonomic challenge, in our case isoproterenol (1 minute infusion), is very important for determining the analysis range and the hysteresis profile. Unlike humans, the sinus arrhythmia in dogs causes RR intervals to oscillate between approximately 400 and 1600 ms with every respiratory cycle at rest. To standardize the heterogeneity boundary during acceleration and deceleration, TQ intervals above 1000

ms were omitted from the analyses even though they temporally occurred with isoproterenol when the sinus arrhythmia was maintained early in the infusion. Likewise at very fast heart rates individual beats oscillated below TQ values of 150 ms making the true nadir of the response difficult to determine. Even though humans exhibit much less sinus arrhythmia, the rapidity and direction of the RR or TQ interval change will always need to be considered in the analyses. TQ interval is not equivalent to RR interval and thus should not be extrapolated to mean heart rates. TQ interval takes into account the high short-long variability of QT interval also occurring on the adjacent cardiac cycle. To visualize the sequential beat-to-beat dynamics during heart rate changes, a supplemental movie of the response has been appended (Supplemental Data: IKs+IKr block with iso.AVI).

Limitations of study

We did not compare our restitution findings to direct measurements of APD/DI. Although this would be of interest in future studies, we would expect differences. Regional events (e.g. apical vs. base; LV vs. RV, endocardial vs. epicardial, etc) may not be recognized by the ECG restitution because it represents a summation of electrical activity. Systematic study will probably be required to determine which cardiac region best coincides with surface measurements. Technically, computerized data acquisition systems for digital analyses of large volumes of high quality digital ECG data are in their early stages of development. Only recently has it been possible to begin quantitative assessment of the sequential beat-to-beat dynamics (Fossa et al., 2005; Jensen et al., 2004; Mizumaki et al., 2005). Many issues with automated systems affecting spatial

differences and T-wave morphology must be overcome to improve confidence it their use without manual oversight of all beats.

In conclusion, using dynamic beat-to-beat analyses, these parameters and hopefully others may be measured in the conscious normal autonomic state and could lead to non-invasive biomarkers for assessment of changes associated with arrhythmogenic risk. However, since arrhythmia was not produced with the treatments in this study, it cannot be determined which of the parameters best describes the absolute risk. Ethically, this will only be verifiable with these analyses in the normal conscious state when performed retrospectively with human Holter-acquired data where arrhythmias occurred.

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LEGENDS FOR FIGURES

Figure 1. Study protocol to illustrate the timeline for treatment and data analysis periods. For each Period 1 through 4, (A) designates the data analysis period prior to isoproterenol challenge and (B) designates the data analysis period during the isoproterenol response or vehicle. Period 1 represents the baseline and Periods 2 through 4 each represent escalating doses of either E-4031 or vehicle that were preceded by a single infusion dose of L-768,673 or equivalent vehicle. L-768,673 was administered as a constant infusion over 15-minutes and E-4031 was infused over 3-successive periods each containing a 5-minute loading dose followed by a 15-minute maintenance dose producing free plasma concentrations approximately 20, 50 and 250%, respectively, the clinically relevant level of 3.5 nM.

Figure 2. Comparative relationships between QT-RR interval and restitution (QT-TQ interval). Using the exact same cardiac cycles from the same individual dog, the baseline response before any treatment (Period 1A; ■ blue squares) and after either vehicle (Period 4A; ◆ turquoise diamonds) or the combination of L-768,673 plus E-4031 (Period 4A; ● black circles) are exemplified.

Figure 3. Comparison of the resting QT-TQ interval relationship when given either L-768,673, E-4031 or the combination of both (● black circles) to the time-matched vehicle responses (◆ turquoise diamonds) in the same individual dog during Periods 2A, 3A and 4A.

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Figure 4. Effect of time-matched isoproterenol challenge on the heterogeneity of restitution hysteresis after either vehicle or the combination of L-768,673 plus E-4031 treatment in the same dog during Period 4B when compared to resting state prior to isoproterenol (Period 4A; ◆ blue diamond). Areas of 98% confidence bounds for cardiac cycles after isoproterenol are separated by TQ intervals during acceleration (● red dots; cycles between 1000 to 150 ms), deceleration (● green dots; cycles between 150 to 1000 ms) and total response (● pink dots; all cycles less than 1000 ms). Only dots within specified TQ interval were used in the analyses. Also see supplemental movie IKs + IKr block with ISO.avi.

Figure 5. Relationship between heart rate (RR interval) QT interval and diastolic period (TQ interval) during rest and tachycardia in the presence and absence of QT prolongation. Please note that this is a hand-drawn illustration for conceptual purposes and does not accurately reflect interval measurements. *Top left complex*- normal resting heart rate of 60 BPM provides a TQ interval of 570 ms. *Top right complex* – Tachycardia of 150 BPM reduces TQ interval approximately 7-fold to 80 ms. *Bottom left complex* – QT prolongation of 70 ms at resting heart rate of 60 BPM has relatively little affect on TQ interval (from 570 ms to 500 ms). *Bottom right complex*- QT prolongation of 70 ms is combined with tachycardia of 150 BPM to cause dramatic decrease of TQ interval to 10 ms (57-fold reduction from rest) thus providing little time for oxygenation and return of ion kinetics to normal states for next beat. Also see discussion.

LEGENDS FOR SUPPLEMENTAL DATA

IKs + IKr block with ISO.avi: Dynamic continuous display of an individual dog beat-to-beat QT-TQ interval relationship as depicted in Figure 4. Display shows sequential beats during 10 minutes of baseline (blue), after isoproterenol bolus during acceleration phase (red) and after peak heart rate or deceleration phase (green) and total isoproterenol response (magenta)

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Table 1. Comparison of baseline blood pressure, QT, RR, TQ intervals and area of heterogeneity prior to treatment and isoproterenol challenges.

Treatment	Baseline Value (CB) Period 1A	Change from Baseline Prior to Isoproterenol[a]		
		Period 2A	Period 3A	Period 4A
Vehicle				
BP (mmHg)	98 (85-110)	6	7	10*
QT Interval (ms)	256 (247-265)	2	5	3
RR Interval (ms)	1008 (874-1141)	2	3	1
PR Interval (ms)	120 (105 –135)	ND[b]	ND	0
QRS Interval (ms)	47 (34-61)	ND	ND	1
TQ Interval (ms)	748 (611-886)	0	-2	-1
Heterogeneity (ms^2)	19699 (13807-26311)	-1502	-3419	-2453
L-768,673				
BP (mmHg)	102 (87-116)	7	7	8*
QT Interval (ms)	258 (248-268)	27#*	11	6
RR Interval (ms)	1012 (845-1179)	2	1	0
TQ Interval (ms)	754 (593-914)	-26*#	-10	-7
Heterogeneity (ms^2)	19105 (12493-25716)	1424	-3621	-2718
E-4031				
BP (mmHg)	103 (92-114)	9*	10*	7
QT Interval (ms)	256 (247-264)	10	17*	24#*
RR Interval (ms)	918 (790-1046)	0	-2	-2
TQ Interval (ms)	660 (527-792)	-10	-18*#	-28*#
Heterogeneity (ms^2)	21120 (14509-27732)	-4151	70	492
L-768,673+E-4031				
BP (mmHg)	102 (88-115)	6	6	2
QT Interval (ms)	255 (250-259)	50#*	39#*	38#*
RR Interval (ms)	1014 (940-1088)	4	-2	-3
PR Interval (ms)	119 (104-133)	ND	ND	-1
QRS Interval (ms)	48 (35-61)	ND	ND	0
TQ Interval (ms)	756 (682-830)	-46*#	-42*#	-41*#
Heterogeneity (ms^2)	19463 (12851-26074)	9098*#	7128*#	15352*#

a- Statistical significance ($P < 0.05$) from 95% confidence bounds (CB) established at baseline prior to treatment (*) or vehicle response during same period (#).

b- ND – not determined

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Table 2. Comparison of hysteresis of the QT-TQ interval restitution relationship after isoproterenol challenges.

Treatment	Baseline Isoproterenol Response (CB) in ms^2 Period 1B	Change from Baseline Isoproterenol during Treatment[a]		
		Period 2B	Period 3B	Period 4B
Vehicle				
Total Heterogeneity[b]	34372 (27492-41252)	2607	3343	1937
Acceleration [b]	16847 (11343-22351)	1250	-1004	-85
Deceleration [b]	21599(16834-26364)	395	-894	-1299
TQ minimum[c]	91 (63-118)	7	6	1
L-768,673				
Total Heterogeneity	33748 (26868-40628)	8584*	2119	1355
Acceleration	15474 (9970-20978)	2742	1394	3448
Deceleration	20541 (15776-25306)	-819	-1560	276
TQ minimum	93 (66-120)	4	-2	2
E-4031				
Total Heterogeneity	31760 (24880-38640)	3630	9684*	16749*#
Acceleration	17366 (11862-22870)	-481	3275	5089
Deceleration	21379 (16614-26144)	144	3411	1737
TQ minimum	98 (70-125)	-13	-7	-21
L-768,673+E-4031				
Total Heterogeneity	35917 (29037-42797)	18010*#	14634*#	31812*#
Acceleration	18854 (13350-24359)	3166	7673*#	10674*#
Deceleration	21640 (16875-26405)	1648	745	2534
TQ minimum	101 (73-128)	-17	-7	-73*#

a- Statistical significance ($P < 0.05$) from 95% confidence bounds (CB) established at baseline prior to treatment (*) or vehicle response during same period (#).

b- Units expressed as ms^2 .

c- Units expressed as ms.

Figure 1

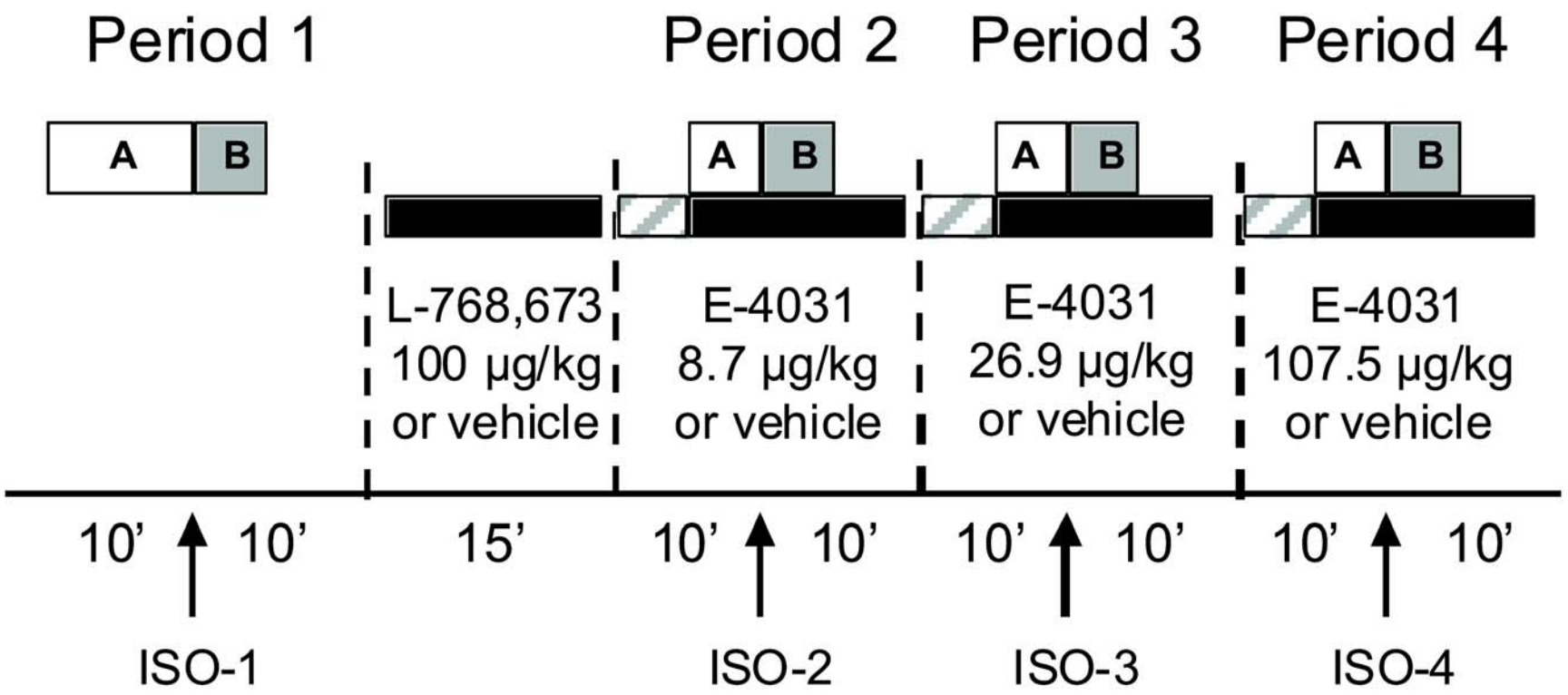

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Figure 2

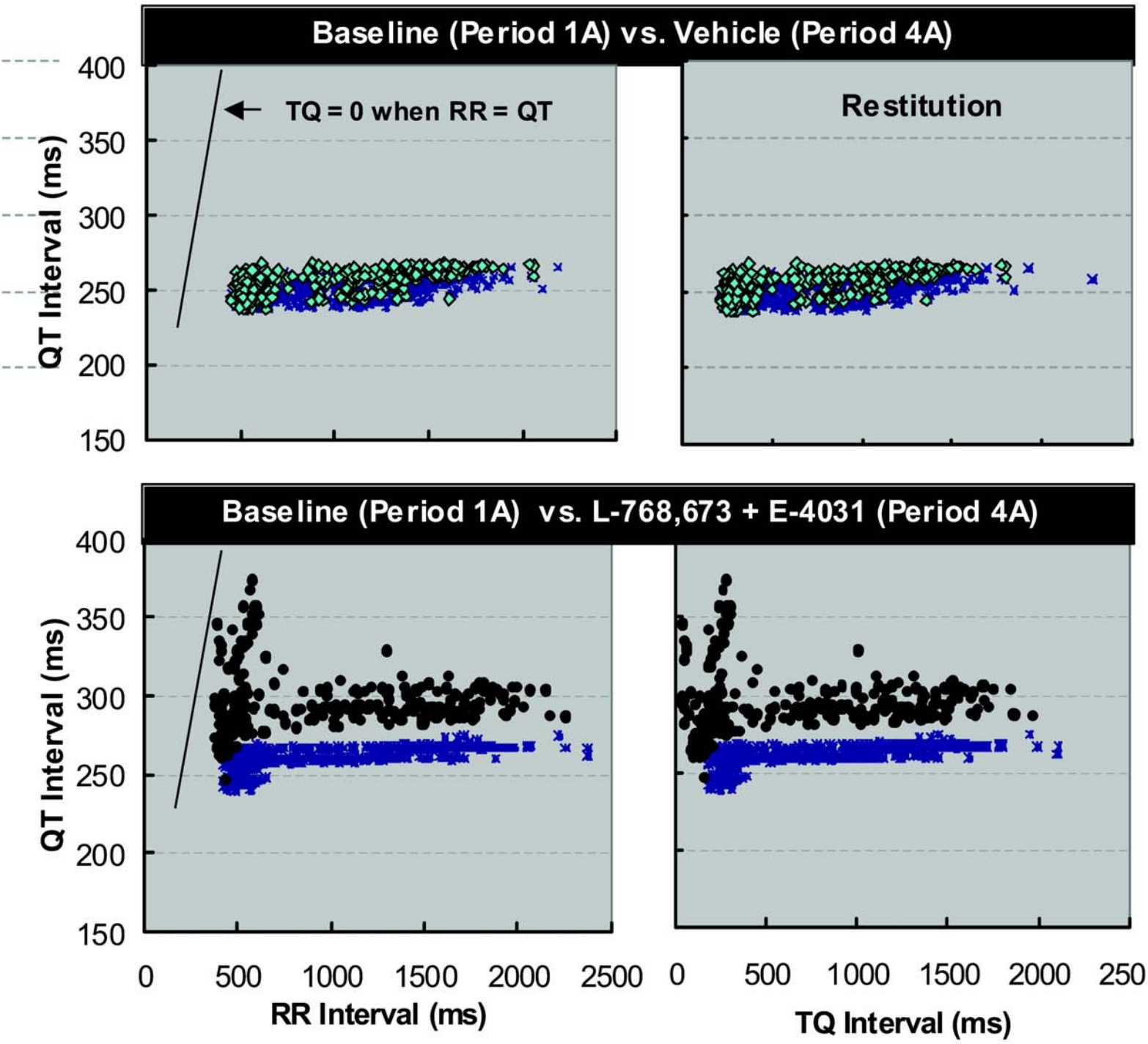

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Figure 3

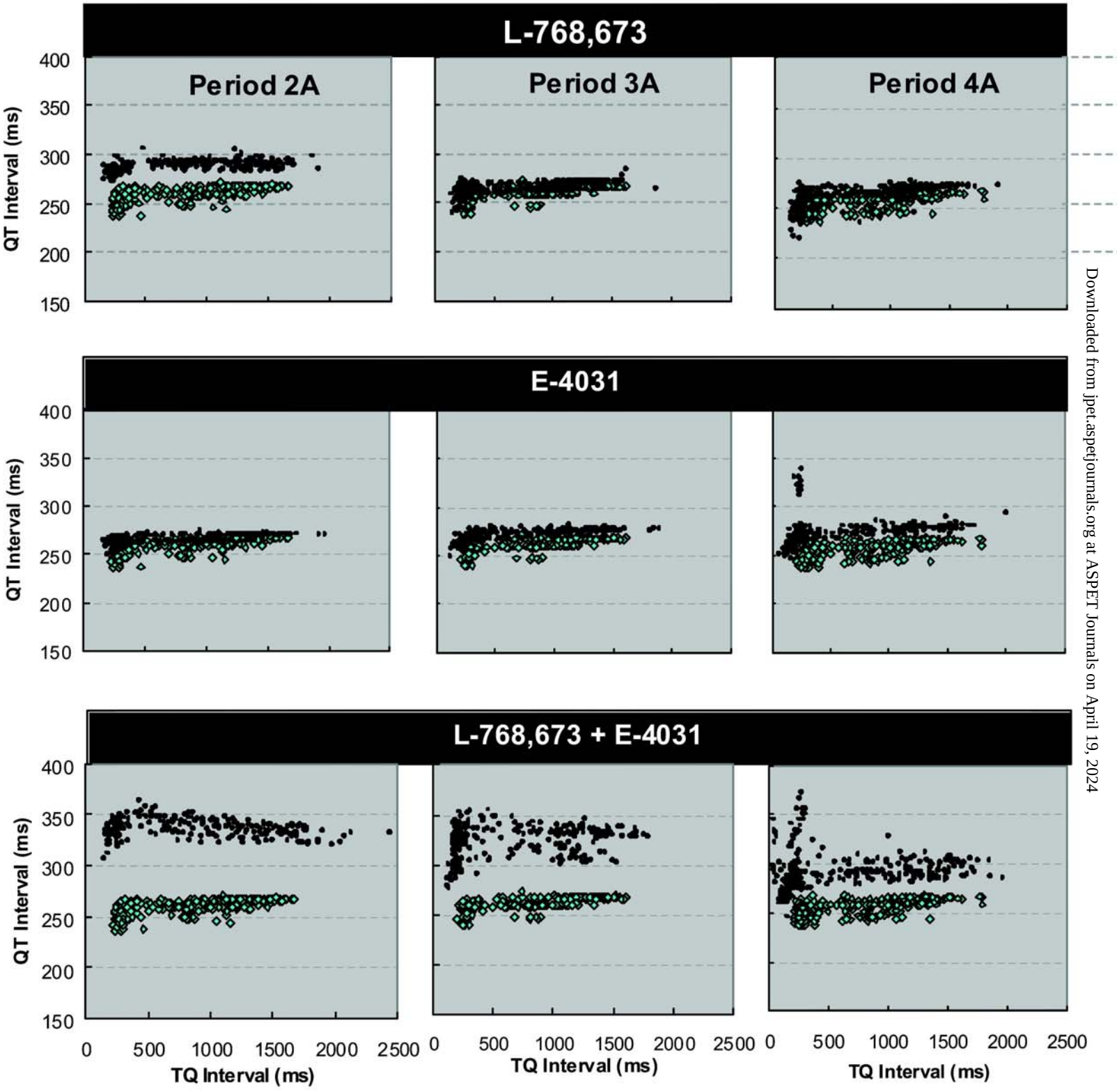

Figure 4

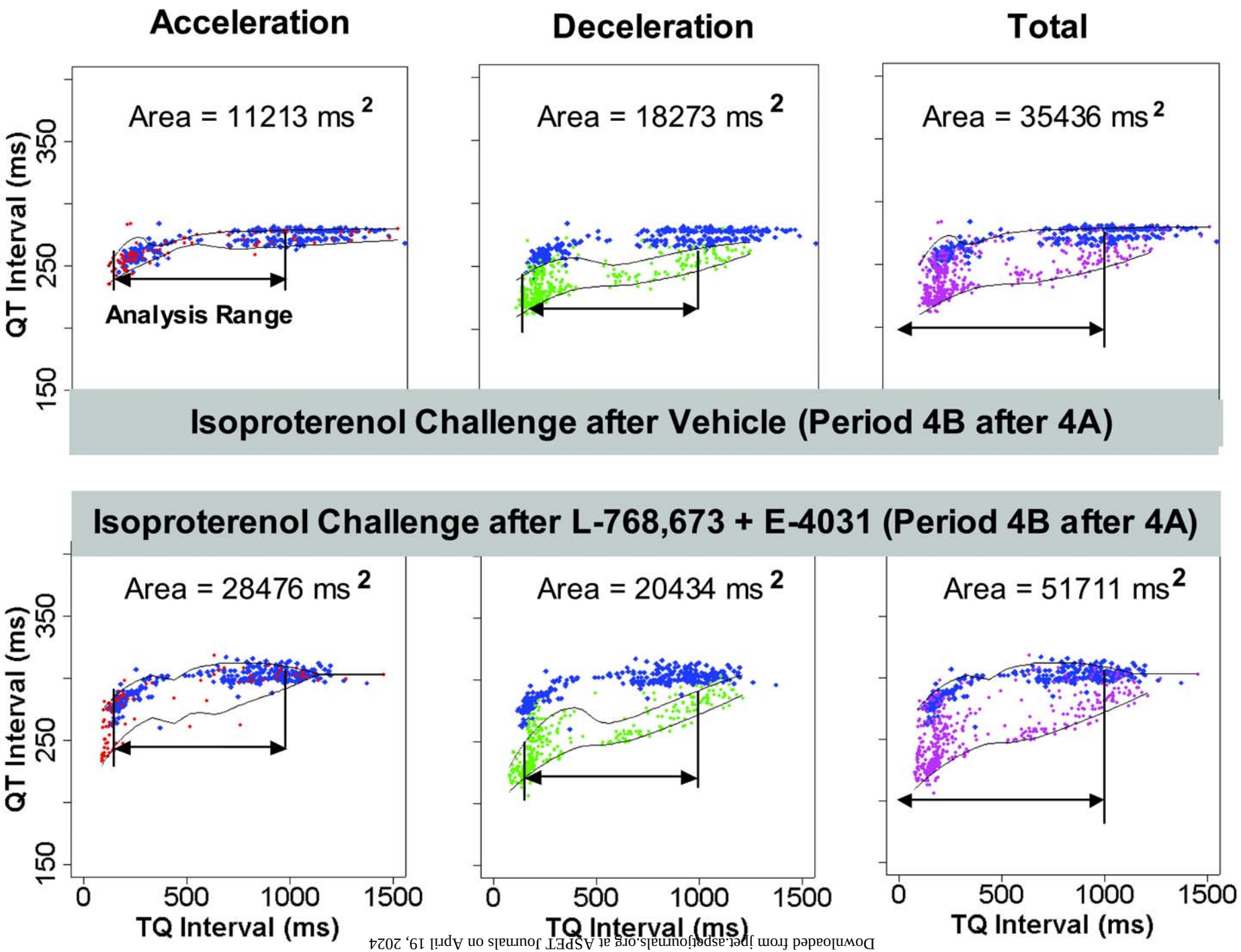

Figure 5