Title: Transmural unipolar electrogram change occurs within 7s at the left atrial posterior wall during pulmonary vein isolation

Short title: VISITAG™ Module-based atrial lesion assessment

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Abstract

Background To assess occurrence of a histologically validated measure of transmural (TM) atrial ablation – pure R unipolar electrogram (UE) morphology change – at first-ablated left atrial posterior wall (LAPW) sites during contact force (CF)-guided pulmonary vein isolation (PVI).

Methods Objectively annotated VISITAG™ Module and CARTOREPLAY™ (Biosense Webster Inc.) UE morphology data was retrospectively analysed in 23 consecutive patients undergoing PVI under general anesthesia.

Results PVI without spontaneous / dormant recovery was achieved in all, employing 16.3[3.2] minutes of radiofrequency (RF, 30W) energy. All first-ablated LAPW sites demonstrated RS UE morphology pre-ablation, with RF-induced pure R UE morphology change in 98%. Time to pure R UE morphology was significantly shorter at left-sided LAPW sites (4.9[2.1] s versus 6.7[2.5] s; p=0.02), with significantly greater impedance drop (median 13.5Ω versus 9.9Ω; p=0.003). Importantly, neither first-site RF duration (14.9 versus 15.0s) nor maximum ablation catheter tip distance moved (during RF) were significantly different, yet the mean CF was significantly higher at right-sided sites (16.5g versus 11.2g; p=0.002). Concurrent impedance and objectively annotated bipolar electrogram (BE) data demonstrated ~6-8Ω impedance drop and ~30% BE decrease at the time of first pure R UE morphology change.

Conclusions Using objective ablation site annotation, UE morphology evidence of TM RF effect was demonstrated far sooner than considered biologically possible according to the “conventional” 20-40s RF per-site approach, with significantly greater ablative effect evident at left-sided sites. This novel methodology represents a scientifically more rigorous foundation towards future research into the biological effects of RF ablation in vivo.
Keywords

Atrial fibrillation; contact force catheter ablation; pulmonary vein isolation; unipolar electrogram; VISITAG™ Module
Introduction

Radiofrequency (RF) catheter ablation for atrial fibrillation (AF) is a complex undertaking, since in order to achieve electrical isolation of the pulmonary veins, encircling lesions must be transmural (TM) yet without causing extra-cardiac thermal trauma via inadvertently excessive local RF energy delivery.\(^1\) Considerable research efforts have focussed on the identification of optimal RF targets for lesion creation during pulmonary vein isolation (PVI); following the development of contact-force (CF) sensing catheters, recommendations for the CF “working range” and force time integral (FTI, area under the force-time curve) were derived.\(^2,3\) However, recent reports of an increased association of CF sensing catheter use with atrioesophageal fistula (AEF)\(^1,4\) – the most feared complication following PVI due to its high mortality\(^5\) – suggests that these recommendations may be inappropriate and highlights the risk of conducting PVI with an imperfect understanding of when TM ablation has been achieved.

When considering the tissue response to RF application, electrogram changes indicating TM atrial ablation effects are well described in animal studies. Otomo et al identified that an ablation catheter tip unipolar electrogram (UE) morphology transition from RS to “pure R” was 100% predictive of histologically proven TM lesions in porcine atria, and was catheter-tissue angle independent.\(^6\) Using a contact-force (CF) sensing catheter in canines, Bortone et al demonstrated that 95% of atrial lesions were histologically TM following immediate RF termination at the first occurrence of a pure R morphology UE, with no evidence of extra-cardiac thermal trauma. However, continuous RF delivery for 10 or 20s beyond this time (and indeed a “conventional” 30s duration application) consistently resulted in 100% TM lesions yet with 11-17% lesions associated with extra-cardiac thermal trauma.\(^7\) Considering the employed power-controlled RF protocol at 30W (48°C, 17ml/min irrigation) and minimum
CF 10g resulted in pure R UE morphology at 7s and with mean lesion depth of 4.3mm, one would anticipate a high risk of inadvertent extra-cardiac thermal trauma from the practice of 20-40s / “a median of 45s” RF “per site” during recently conducted CF-guided PVI studies in humans.8,9

Here we provide the first-in-human report of pure R UE morphology change at sites of first RF application on the left atrial posterior wall (LAPW) during PVI, employing an objective and automated method for ablation catheter stability annotation fulfilling suitable criteria for a stable point of RF delivery in vivo, via custom software (VISITAG™ Module with CARTOREPLAY™, Biosense Webster Inc., Diamond Bar, CA). In addition we report concurrent, objectively annotated bipolar electrogram (BE) attenuation and impedance drop data (i.e. additional markers of RF effect in vivo10,11). Finally, through extended off-line catheter tip positional data analysis using the software R12, we report the relationship between measured catheter tip displacement during RF, time to pure R UE morphology and impedance drop at annotated LAPW ablation sites.

**Methods**

This ablation protocol and the technical approach to PVI was developed during an Institutional Review Board (IRB)-approved service evaluation, and was without influence from any recommendations for RF delivery derived from historical studies employing subjective methods of ablation catheter stability assessment; a complete description can be found online.13 Retrospective analysis of VISITAG™ Module and CARTOREPLAY™ data were performed following single-operator PVI, in consecutive unselected patients with symptomatic AF undergoing PVI according to current treatment indications.14 Briefly, all procedures were undertaken using general anesthesia (GA) with endotracheal intubation and
volume-controlled intermittent positive pressure ventilation: Tidal volume 6-8ml/kg; 14-16 breaths per minute as guided by end-tidal CO₂; positive end-expiratory pressure 5 cmH₂O; 50% inspired oxygen fraction. Single transseptal access was obtained with an SL1 (St Jude Medical Inc., Minneapolis, MN) sheath, following which either a NaviStar® THERMOCOOL® SMARTTOUCH™ (ST) F curve or NaviStar® EZSTEER® THERMOCOOL® SMARTTOUCH™ D/F catheter (Biosense Webster) via an Agilis™ NxT sheath (St Jude Medical Inc.) was placed in the left atrium (LA) via the first transseptal site. ACCURESP™ Module (Biosense Webster) respiratory training was checked with a duodecapolar LASSO® Nav catheter (Biosense Webster) in both the right superior pulmonary vein (PV) and the left inferior PV and applied prospectively as required to the CARTO®3 geometry (V.3, Biosense Webster).

CF-guided PVI was performed in temperature-controlled mode (17ml/min, 48°C) at 30W, with lesion placement guided by the VISITAG™ Module.¹³ The preferred sites of first RF application were at the LAPW opposite each superior PV ~1cm from the PV ostium, although in cases where constant catheter-tissue contact could only be achieved with maximal CF ≥70g, an adjacent LAPW site with lower peak CF was chosen; the target first-site ablation time was 15s. Circumferential PVI (entrance and exit block) was performed with continuous RF delivery, with additional point-by-point RF applied as required to achieve target parameters. Spontaneous recovery of PV conduction was assessed and eliminated during a minimum 20-minute wait. Dormant recovery was evaluated and targeted a minimum of 20 minutes after the last RF.¹⁵

VISITAG™ Module and CARTOREPLAY™ use

The VISITAG™ Module permits automated ablation site annotation through catheter “stability filters” utilising catheter tip CF and position data during RF application. The
Ablation catheter tip position is measured every 16/17 ms, from which the standard deviation (SD) is calculated over a minimum interval of 60 positions (i.e. 1 s). Consecutive ablation catheter tip positions within a user-defined maximum range for the SD (in mm) during ongoing RF are used to annotate a site meeting positional stability filter requirements. CF is measured every 50 ms, with CF filtering employing a user-defined minimum CF for a percentage of RF time at sites meeting positional stability filter requirements. Consecutive catheter tip positions satisfying both positional and CF stability filter requirements beyond the minimum user-defined duration (system minimum, 3 s) result in automated ablation annotation; a 3D and/or surface-projected tag may be used to denote the mean catheter position at each site. Post-ablation, annotated tags provide summative ablation data including RF duration, mean and range of CF, impedance and FTI (figure 1A and B; see box, left panels), while an export function permits extended data analysis. Following completion of a service evaluation (July – December 2013), the following VISITAG™ Module filter settings were used during this present report: Positional stability duration 3 s and range 2 mm; force-over-time 100% minimum 1 g (i.e. constant catheter-tissue contact). For extended ablation catheter positional stability analysis, R software code was written to analyse exported VISITAG™ Module data regarding catheter tip (position) SD and maximum displacement from the mean position annotated at each first site of LAPW ablation. Impedance and RF power data at 100 ms intervals during RF were obtained via the VISITAG™ Module export function.

From a theoretical perspective, a fundamental element of ablation catheter positional stability is constant (versus intermittent) catheter-tissue contact. However, respiration and cardiac cycle-induced motion may create displacement at the catheter-tissue interface even in the setting of constant contact. These may be accounted for using “annotation adjustment” algorithms; i.e. no displacement assumed if the catheter returns to consecutive end-expiratory
end-diastolic sites within a pre-defined distance range. However, this may introduce error since RF delivery can only be proven to be “single site” when measures of catheter-tissue interaction and tissue response to RF at sites without catheter displacement are defined, and shown to be present at sites demonstrating respiration or cardiac cycle-induced motion yet “adjusted” to a single site. Therefore, although a proprietary respiration adjustment algorithm was available (ACCURESP™, Biosense Webster), this was never applied to the VISITAG™ Module filter settings, since this tool has yet to undergo such validation in vivo.

CARTOREPLAY™ provides a means to retrospectively analyse electrogram data at all VISITAG™ Module annotated ablation sites, within 18 hours (data is deleted thereafter). During this present report, UE morphological analysis was performed immediately following case completion, employing histologically validated criteria for TM RF effect (i.e. pure R morphology change). UE filtering was 0.5-120Hz with analysis display speed 200mm/s and voltage scale 3.45 or 3.80mV. The time to pure R UE morphology was taken at the first of 3 consecutively occurring pure R complexes, no more than 1 of which could be an atrial ectopic. In order to facilitate summative analysis for all first-ablated LAPW sites and to minimise catheter instability due to cardiac cycle length changes, every RF application was performed during CS pacing at 600ms cycle length. Ablation catheter (distal pair) BE peak-to-peak amplitude was obtained using automated Wavefront Annotation (Biosense Webster). Pictorial evidence of the stated UE morphological changes was provided via a JPEG creation tool (e.g. figures 1A and B); all first-ablated LAPW site images are shown in the data supplement.

Statistical analyses were performed using GraphPad Prism version 4.03. Parametric data are expressed as mean [SD]; non-parametric data are presented as median (1st – 3rd quartile). Unpaired / paired t test or the Mann Whitney test was used to assess statistical significance for continuous data, as appropriate. P <0.05 indicated a statistically significant difference.
This work received IRB approval for publication as a retrospective service evaluation; all patients provided written, informed consent.

**Results**

Twenty-three patients underwent first-time PVI as described, between 1st December 2016 and 11th May 2017: 12 patients had persistent AF, 11 PAF; 17 were male (74%); mean age 58 [13] years and CHA₂DS₂-VASc score 1.4 [1.4]. Complete PVI was achieved in all without spontaneous / dormant recovery of PV conduction, following 16.3 [3.2] minutes of RF, with no procedural complications.

Pre-ablation, all first-ablated LAPW annotated sites demonstrated RS UE morphology (e.g. figure 1A and data supplement). For sites adjacent to the left PV, ablation-induced pure R UE morphology was identifiably achieved in 21/23 cases (e.g. figure 1B and data supplement). One case of inadvertent catheter displacement resulted in annotation termination at 4.43s when RS UE morphology remained present (supplementary figure S5A), while in another, gross artefact made UE morphology assessment impossible (supplementary figure S15A). For annotated sites adjacent to the right PV, ablation-induced pure R UE morphology was achieved in all. However, one case of inadvertent catheter displacement at RF onset resulted in 4.97s non-annotated RF delivery, so the time to pure R UE measurement was deemed invalid for analysis.

Annotated first-ablated LAPW site data is shown in table 1. At left-sided sites there was a significantly shorter time to pure R UE morphology (4.9[2.1] s versus 6.7[2.5] s; p=0.02), with a significantly greater total impedance drop (13.5Ω versus 9.9Ω; p=0.003). This was not explained on the basis of a difference in the total RF duration, while both the mean CF and FTI were significantly lower for left-sided sites (11.2g versus 16.5g; p=0.002 and 167gs versus 244gs; p=0.004). Furthermore, while the maximum ablation catheter tip distance...
moved from the (annotated) mean point was not significantly different, the standard deviation of catheter tip motion was significantly greater at left-sided sites (1.4[0.3] mm versus 1.1[0.3] mm; p=0.001). There were no significant differences between the BE (distal ablation catheter pair) data, although there was a trend towards greater total percentage BE decrease at left-sided sites (61[19] % versus 51[24] %).

The first 15s of annotated impedance and percentage bipolar electrogram decrease data is shown in figure 2, with RF power below. At the times corresponding to the pure R UE morphology data in table 1, an impedance decrease of ~6-8Ω can be observed, accompanied by ~30% BE decrease for both left-sided and right-sided ablation sites. Notably, utilising this temperature-controlled RF protocol the delay to achieve 30W power was ~6s. Correlation between the time to pure R UE morphology and the following factors was assessed: Total impedance drop; mean CF; maximum catheter tip distance moved; ablation catheter tip position SD; and BE amplitude (annotation start). The only significant correlation was in right-sided sites and with impedance drop – Spearman r -0.43 (-0.72 to -0.005; p=0.048).

**Discussion**

The analyses described in this present report represent the first-in-human description of the response to RF application at LAPW first-ablated sites meeting a putative suitable definition for a stable point of catheter tip-tissue contact *in vivo*. As a result of adopting this novel RF annotation methodology, we have demonstrated that when an ablation catheter is maintained within a (SD) range of 2mm, acutely durable ablation effect was universally achieved following 15s temperature-controlled RF at 30W, with TM UE morphology change typically evident within 7s and associated with a BE attenuation of ~30% and impedance drop of ~6-8Ω. Furthermore, we have identified superior catheter tip-tissue energy coupling for left-
sided ablation sites, evidenced by significantly shorter time to pure R UE morphology and a significantly greater total impedance drop. Finally, the absence of a significant correlation between the time to pure R UE morphology change and either the maximum catheter tip distance moved / tip position SD provides supportive evidence for the appropriateness of the 2mm SD positional stability filter “working range”, without ACCURESP™ adjustment, annotation protocol.

That these data are out of keeping with current views regarding what constitutes a “conventional” approach to RF application during PVI should come as no surprise when considering the development of the current approach towards PVI and evidence available from other publications: (1) The original description of ~30s RF “per site”, aiming for 80% BE attenuation was without scientific validation, yet has remained unchallenged and perpetuated in methodology ever since; (2) the subjective methods used to derive current CF and FTI recommendations are flawed, compared to any investigations conducted utilising objective means of ablation catheter stability assessment. Specifically, in TOCCATA (the first study reporting a relationship between CF and clinical outcome following PVI), valid ablation records for analysis were those “with stable CF for at least 15 consecutive seconds”. However, for this methodology to be appropriate two assumptions must be satisfied, neither yet validated in vivo: (i) CF “stability” (however that is defined) must equate with a certain “fixed” degree of catheter tip-tissue positional stability; (ii) TM ablation during PVI must never be achieved within 15s. Therefore, these data should have been considered provisional, pending further investigations following appropriate methodological advances; (3) the LA wall is thin (e.g. computed tomography assessment pre-ablation mean thickness 1.9mm; range 0.5-3.5mm), yet laboratory preparations of irrigated RF using “conventional” RF duration result in far deeper lesions (e.g. in a bovine skeletal muscle model of intermittent catheter contact at 20W, an FTI of 200gs (CF range 0-22g).
resulted in 4mm depth lesions); similarly and as previously stated, in vivo investigations of CF-guided ablation in canines demonstrated pure R UE morphology change at 7s, with histologically confirmed TM ablation effect in 95% applications (power-controlled RF at 30W), and mean lesion depth 4.3mm; a report of real-time ovine lesion assessment utilising a novel combined ultrasound and RF ablation catheter in vivo, demonstrated rapid lesion development during right atrial epicardial ablation at 7W (i.e. 1.5mm depth at 10s, see figure 2A, Wright et al 2011); (6) esophageal temperature probe alerts during LAPW ablation at 25W (target CF 10-20g), occurred in 73% patients, with intra-luminal temperature 39°C illustrated as early as 7s following RF onset (see figure 2 from Halbfass et al 2017). The findings in this present report have numerous implications. Most importantly, we have demonstrated that the VISITAG™ Module and CARTOREPLAY™ may together be used as investigational tools during PVI, permitting an assessment of the TM nature of the tissue response to RF delivery. Clearly, this provides a foundation for further investigations in an effort to reduce the risk of esophageal thermal injury (e.g. evaluating the acute effectiveness of lower power delivery to the LAPW). Furthermore, in view of the objective and standardised nature of VISITAG™ Module annotation, any operator may similarly investigate lesion creation utilising their “usual approach” to ablation during CF-guided PVI. In view of the described evidence of rapid TM ablation effect in thin-walled atrial tissue, such investigations are likely to improve the safety profile of CF-guided PVI, through a movement away from the “conventional” view of a requirement for ~30s RF delivery “per site”, achieving 80% BE attenuation and FTI 400gs, towards shorter RF duration and possibly lower power delivery. This represents an important advance in RF ablation research methodology, since when the VISITAG™ Module is used as described in this present report, it comes as close as is presently possible to representing a “universal language” for the RF ablation “effector arm”. Consequently, and through the consistent application of CF and
positional stability filters described here, future research protocols may be optimised, practice harmonised and with published outcomes then replicated by any operator able to maintain the catheter position and CF stability in the manner described.

The methodology presented here also represents a departure from studies utilising automated ablation site annotation yet failing to recognise the limitations of a CF filter permitting intermittent catheter-tissue contact (i.e. 30% minimum 5g) and the non-validated use of ACCURESP™ adjustment, when considering a suitable definition for a stable point of RF application in vivo. Furthermore, an inherent problem with any system of ablation lesion delivery founded upon assessments of CF and energy delivery alone is now clearly apparent when considering the differential ablative effect between left and right-sided sites demonstrated in this present report. However, this should also not be surprising, when recognising the recently reported impedance drop range of 0 to ~50Ω resulting from an ablation index (AI) value of 300; i.e. indicative of great variability in tissue RF effects (see figure 3 from Das et al).

Study limitations

All UE morphology data were collected retrospectively, so this report does not prove that modification of RF delivery based on real-time UE morphological assessment during VISITAG™ Module and CF-guided PVI is appropriate. However, this is an important area for further research, not least in view of the promising findings from a single-centre UE signal modification-based PVI study in humans, before the advent of CF-sensing and objective ablation site annotation. Furthermore, neither esophageal luminal temperature monitoring nor post-ablation endoscopic evaluation was employed; given the time to pure R UE morphology change of 3.1s demonstrated in figure 1 it is highly likely that some LAPW lesions were accompanied by inadvertent extra-cardiac thermal injury. Therefore, the
approach to ablation described in this report is not a direct recommendation for others to follow without careful consideration. Rather, through adopting the methodology described, any operator has the opportunity to identify the most appropriate means to achieve TM ablation during PVI, via insights into their “usual approach” using the VISITAG™ Module and CARTOREPLAY™.

The significantly greater time to pure R UE morphology at right-sided sites may in part have been due to increased LA wall thickness. Also, although the maximum distance of catheter tip motion did not differ between left and right-sided sites, there may have been a greater degree of LAPW motion beneath the catheter tip at right-sided sites, despite a significantly greater mean CF (i.e. out-of-phase motion). Nevertheless, the significantly greater total impedance drop at left-sided sites remains the first demonstration of superior regional catheter tip-tissue energy coupling *in vivo*. Although this finding cannot be fully explained at present, these data may indicate that the more oblique catheter orientation routinely obtained during left-sided ablation may result in a greater surface area of ablation electrode in contact with the tissue, with even a relatively small difference potentially resulting in significantly greater power delivery.\(^8\) While the mechanisms underlying these observations await identification, this represents an important unknown variable in any attempt to harmonise RF ablation practice based upon ablation lesion quality markers incorporating CF, RF duration and power alone.\(^8\)

Maintaining a stable catheter position within the chosen 2mm SD range may require some novel operator “adjustments” for respiration-induced motion, particularly when the use of ACCURESP™ adjustment use during PVI remains non-validated. There is presently no means to describe any compensatory sheath / catheter movements utilised during this report; jet ventilation may therefore improve the reproducibility of VISITAG™ Module and CF-guided PVI, but this requires further study. Moreover, even when adopting uniform weight-
adjusted ventilatory settings, variable lung capacity between patients may result in differing degrees of respiratory excursion, hindering reproducibility; the addition of a suitable scale to the ACCURESP™ respiratory indication window would theoretically help counter this problem, permitting patient-specific tidal volumes to be administered towards possibly greater uniformity of respiration-related catheter excursion.

An ex vivo study into the accuracy of ST catheter CF measurement demonstrated important CF error with parallel catheter orientation, of $6.6 \pm 5.9$ g. Although parallel orientation never occurred at these first sites of RF application in this present report, this issue represents a possible flaw to the use of 100% minimum 1g CF filter at all left atrial sites. A complete assessment of the effects of changing the CF filter minimum level was beyond the scope of this present report, but any operator seeking to perform entire PVI lesion sets during contact catheter-tissue contact should be aware of this important issue.

The availability of an imaging modality to help validate the TM nature of RF lesions in vivo would strengthen the findings of this present report; unfortunately there is no validated means to perform this at present. UE morphology change analyses are impossible during AF, since by definition, fibrillatory waves are random (data not shown, since all procedures were performed during CS pacing at 600ms in this present report). Finally, achieving durable PVI requires sufficient overlap of TM RF effect between adjacent lesions; these present data provide no information concerning the diameter of TM ablative effects. However, through conducting similar investigations to those described here, the diameter of RF-induced thermal injury may be identified during PVI; these data are presented in a pre-publication report online.
Conclusions

Through appropriate use of the VISITAG™ Module and CARTOREPLAY™ during PVI, the TM nature of the tissue response to RF may be investigated at objectively annotated first-ablated sites meeting a suitable definition for a stable point of catheter-tissue contact \textit{in vivo}.

These novel data presented indicate that the “conventional” approach to RF application during PVI probably inappropriately increases the risk of extra-cardiac thermal trauma, particularly at left-sided LAPW sites. When used appropriately, the VISITAG™ Module and CARTOREPLAY™ provide a novel research methodology with greater scientific rigour, potentially facilitating greater efficacy in RF ablation practice via suitably “tailored”

approaches to RF delivery.

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Conflicts of interest

None declared.

Author contributions

DRT devised the original ideas for this report, undertook all ablation procedures, performed post-procedural data collection, performed statistical analyses and wrote the manuscript. MM performed post-ablation data input and critically reviewed the manuscript. KS and AS
devised R code for position stability analyses, provided guidance on statistical methods and critically reviewed the manuscript.
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Table legends

Table 1: Left atrial posterior wall annotated ablation site data, according to proximity to the left or right pulmonary vein (left-sided / right-sided, respectively). For bipolar electrogram (BE) data, annotation “start” and “end” represent the first and last ablation catheter (distal pair) electrograms respectively, during automated VISITAG™ Module RF annotation. Data is shown as mean [SD] / median (25th – 75th centile), as appropriate. UE, unipolar electrogram; CF, contact force; FTI, force time integral; SD, standard deviation.

Figure legends

Figure 1: Left panel showing CARTO®3 LA geometry with VISITAG™ Module ablation site annotation displayed as 2mm radius spheres, with transition to red at 10s RF duration (transparent PA view) and right panel showing CARTOREPLAY™ screen with 24-hour clock timeline beneath. A: First RF application annotation onset at the LAPW during left PV isolation. “Location 1” is highlighted (greater tag diameter) with corresponding annotated summative ablation site data shown (box). CARTOREPLAY™ electrogram data at 200ms speed demonstrates pre-ablation RS UE morphology during CS pacing at 600ms cycle length (from top to bottom: ECG lead II; MAP 1-2 bipolar electrogram 1.33mV scale; MAP 1 UE 3.45mV scale; CS 1-2 and 5-6 bipolar electrograms). RF and VISITAG™ Module annotation onset is identified in the middle of the CARTOREPLAY™ screen as white and red/yellow horizontal bars at the bottom, respectively. B: CARTO®3 LA geometry and CARTOREPLAY™ screen showing time calliper at the onset of 3 consecutive pure R UE morphology complexes during CS pacing at “Location 1”, following 3.1s RF.
Figure 2: Impedance change and percentage bipolar electrogram decrease (ablation catheter distal pair) for the first 15s of annotated ablation site data according to proximity to the left or right PV (left-sided / right-sided, respectively). Error bars are shown in one direction for clarity. The RF power data represents a smoothed curve from all RF applications.
Figure 1A
Figure 1B
Figure 2