Docosahexaenoic Acid, But Not Eicosapentaenoic Acid, Supplementation Reduces Vulnerability to Atrial Fibrillation

Running title: Ramadeen et al.; DHA, but not EPA, reduces vulnerability to AF

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Abstract:

**Background** - The potential health benefits of omega-3 polyunsaturated fatty acids (PUFAs) are usually studied using a combination of docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). This combination reduces vulnerability to experimentally induced atrial fibrillation (AF). It is unknown whether EPA and DHA have differential effects when taken alone. Using a model of pacing induced atrial hemodynamic overload we investigated the individual effects of EPA and DHA on vulnerability to AF and atrial remodeling.

**Methods and Results** - Thirty-four dogs were randomized into 3 groups all of which underwent simultaneous atrial and ventricular pacing (SAVP) at 220bpm for 14 days. One group received purified DHA (~1g/day) orally for 21 days beginning 7 days before pacing began. Similarly, one group received ~1g/day purified EPA. In a third (control) group, (No-PUFAs), 8 dogs received ~1g/day olive oil, 12 were unsupplemented. Electrophysiologic and echocardiographic measurements were taken at baseline and 21 days. Atrial tissue samples were collected at 21 days for histologic and molecular analyses.

Persistent AF inducibility was significantly reduced by DHA compared to No-PUFAs (0% [0-3%] median [25-75 percentiles] for DHA vs. 3.1% [2.2-11%] for No-PUFAs, P=0.007), but not by EPA (3.4% [1.9-8.9%]). DHA also reduced atrial fibrosis compared to No-PUFAs (11±6% vs. 20±4% respectively, P<0.05), whereas EPA did not (15±5%, P>0.05).

**Conclusions** - DHA is more effective than EPA in attenuating AF vulnerability and atrial remodeling instructural remodeling induced AF.

**Key words:** atrial fibrillation; collagen; fatty acids; remodeling
Background

Atrial fibrillation (AF) is the most common arrhythmia seen clinically and its prevalence is increasing. It is associated with significant morbidity and an elevated risk of death, primarily from stroke. Current treatments (cardioversion, antiarrhythmic drugs, ablation) are limited in their efficacy.

The marine derived n-3 polyunsaturated fatty acids (PUFAs) docosahexaenoic acid (DHA, 22:6n-3) and eicosapentaenoic acid (EPA, 20:5n-3) may have beneficial effects in the treatment of atrial fibrillation (AF). However, almost all studies in AF, and in other disease states, have tested the effects of EPA and DHA in combination. Although similar, DHA and EPA have a different length, shape, distribution and metabolic fate. Many studies have shown differential effects of DHA and EPA on cardiovascular endpoints such as hemodynamics, heart rate, endothelial function, and lipid status. However, EPA and DHA have been little studied in models of atrial fibrillation outside of post-cardiac surgery AF.

The simultaneous atrial and ventricular pacing model (SAVP) results in substantial structural remodeling of the atria and vulnerability to AF. We have previously shown that a fixed combination of DHA and EPA is effective in reducing atrial remodeling and inducible AF in this model. In the current study, we investigated the effect of purified DHA and purified EPA separately on vulnerability to AF in the SAVP model. We hypothesized that DHA would be more effective than EPA due to its higher concentration in heart tissue phospholipids.

Methods

The protocol was approved by the Animal Care Committee of St. Michael’s Hospital (Toronto, Ontario, Canada). The investigation conforms to the Guide for the Care and Use of Laboratory
Animals (NIH Publication No. 85-23, revised 1996). Detailed methods are available in the Online Supplement.

**Study groups**

This study was comprised of 3 groups totaling 34 dogs. All dogs were mongrels, 1-3 years old, 19-30kg, of either sex. Three groups were studied:

1. Paced 14 days, No-PUFAs (n=20)
2. Paced 14 days, DHA 21 days (n=8)
3. Paced 14 days, EPA 21 days (n=6)

All groups underwent a pacemaker implantation surgery and “end study” after 21 days, including echocardiographic and electrophysiologic measurements. Details of surgical procedures, sample collection, and preparation are available in the Online Supplement. Beginning 7 days prior to the start of pacing, eight dogs from group 1 were given 2.5mL/day olive oil (Filippo Berio Extra Virgin, San Migliarano, Italy) with no DHA and EPA, the rest were not supplemented. Dogs in group 2 were given 2.7mL/day purified DHA (yielding ~1000mg DHA [DHASCO, Martek Biosciences Corporation, Columbia, MD]). Dogs in group 3 were given 2.5mL/day purified EPA (yielding ~1078mg EPA [O3mega+ Joy, Genuine Health, Toronto, ON]).

Results for group 1 (paced control) have been previously reported and are presented here to place the DHA and EPA groups in their proper context.10

**Surgical procedures**

Thirty minutes prior to pacemaker implant procedure, or the 21 day “end study”, dogs were sedated with i.m. acepromazine (Atrovet, 0.05 mg/kg [Ayerst Veterinary Laboratories, Guelph, ON]), atropine (0.03 mg/kg [Alveda Pharmaceuticals Inc., Bellville, ON]), and buprenorphine (Temgesic, 0.02 mg/kg [Schering-Plough, Kirkland, QC]). Anesthesia was induced with i.v.
thiopental sodium (5% solution 0.25 mL/kg [Hospira Healthcare Corp., Saint-Laurent, QC]) for pacemaker implant, or i.v. propofol (Diprivan, 2.5-3.5 mg/kg [AstraZeneca, Mississauga, ON]) for the 21 day study. Anesthesia was maintained with isoflurane (1-2% [Halocarbon Products Corporation, River Edge, NJ]). Deep anesthesia was confirmed by noting the absence of spontaneous breathing and absence of jaw tonus. During 21 day study, while still under deep anesthesia, euthanasia was performed by rapid excision of the heart.

Electrophysiological study

Five bipolar, stainless steel, epicardial pacing/recording electrodes were sutured onto the heart at the RA appendage (RAA), LA appendage (LAA), high RA (SVC), low RA (IVC) and pulmonary veins (PVs). Atrial effective refractory period (AERP) was determined via the S1-S2 stimulus technique at 200 ms cycle length. Conduction time between the LAA and the SVC was calculated at 400 ms cycle length and presented as a measure of global atrial conduction time.

AF inducibility was measured via burst pacing at up to five electrodes (up to 10 bursts at each electrode). An AF “incident” was defined as any irregular atrial tachyarrhythmia that lasted more than 120 seconds with atrial rate >300bpm and was resistant to pace termination; “persistent” AF was defined as an AF incident that lasted more than 600 seconds (10 minutes).

PUFA isolation and quantification

EPA and DHA levels were measured separately in plasma and atrial tissue samples by gas-liquid chromatography as performed by Chen et al.12

Atrial electrophysiology

AF was defined as any irregular atrial tachyarrhythmia (atrial rate >300bpm) with duration ≥120 seconds, and resistant to pace termination. Global atrial conduction time was calculated as mean conduction time between the superior vena cava and the left atrial appendage (LAA). Atrial
effective refractory period (AERP) was measured via the S1, S2 stimulus technique and was defined as the longest S1-S2 interval not resulting in atrial capture.

Cardiac function (Echocardiography)

Trans-thoracic and trans-esophageal echocardiographic measurements were taken preoperatively in anesthetized dogs laying on their right side. During the 21 day study, the pacemaker was turned off at least 15 minutes prior to measurement.

Histology

All histological analyses were performed in a blinded fashion as described in. Atrial fibrosis was quantified by analysis of picrosirius red stained images of LAA tissue analyzed at 10X magnification (20-30 images per dog). Each image was analyzed with a thresholding function to calculate the fraction of pixels containing collagen. To ensure accuracy, each thresholded image was compared to the unedited original to make sure only collagen was included.

Mean atrial myocyte cross-sectional area was calculated from measurement of approximately 150 hematoxylin and eosin stained LAA cells at 40X as previously published.

Statistical analyses

In order to account for multiple attempts at inducing AF in each dog, AF inducibility was analyzed using a generalized estimating equation comparing each group to the DHA group (calculated by SAS v9.3, SAS Institute Inc., Cary, NC). All other analyses were performed as follows. Normality of data was tested with a Kolmogorov-Smirnov test (with a Dallal-Wilkinson-Lillefor P value). Separate comparisons were made of No-PUFAs to DHA and No-PUFAs to EPA with an unpaired t test for normally distributed groups (data shown as mean±SD in text), and a Mann-Whitney test for non-normally distributed groups (data shown as median
[25-75 percentiles] in text). Statistical results were calculated with GraphPad Prism 5 for Windows (v5.04, GraphPad Software Inc., La Jolla, CA). Data on bar graphs represent mean±SEM; data on box and whisker plots represents median with whiskers indicating minimum and maximum data. Comparisons are presented as No-PUFAs vs. DHA or No-PUFAs vs. EPA.

Results

PUFA integration

DHA supplementation resulted in significantly higher levels of DHA in plasma phospholipids compared to unsupplemented dogs (4.3±1.4% vs. 1.9±0.3%, P=0.004). DHA supplementation also increased DHA in atrial tissue phospholipids (see Figure 1). Similarly, EPA supplemented dogs had both greater plasma EPA levels than unsupplemented dogs (1.2±0.4% vs. 0.5±0.3%, P=0.01) and greater atrial tissue EPA levels (see Figure 1).

Atrial electrophysiology

Persistent AF could be induced with 3.1% [2.2-11%] (median [25-75 percentiles]) of bursts in the No-PUFAs group (see Figure 2). Significantly fewer episodes of persistent AF were inducible in the DHA group (0% [0-3%], P=0.007 compared to No-PUFAs group). In the EPA group, persistent AF was induced with 3.4% [1.9-8.9%] of bursts (P=0.04 compared to DHA group). When self-terminating episodes are included (≥2 mins, but <10 mins), AF could be induced with 7.3% [2.7-15%] of all bursts in the No-PUFAs group (P=0.11 compared to DHA group). In the EPA group, AF was induced with 5.6% [3.2-18%] of bursts (P=0.16 compared to DHA group). AF was induced in the DHA group with 1.3% [0-7.7%] of bursts. The median number of attempts at inducing AF was 37 [30-43], 40 [34-40] and 30 [21-43] for No-PUFA, DHA and EPA groups respectively (P>0.05 for comparisons). The median number of AF incidents induced per dog was 3 [1-4], 0.5 [0-2.5] and 2 [0.8-3.5] for No-PUFA, DHA and EPA.
groups respectively (P>0.05 for comparisons). The median number of persistent AF episodes per dog was 1 [1-3], 0 [0-1] and 1 [0.8-1.5] for No-PUFA, DHA and EPA groups respectively (P=0.02 No-PUFAs vs. DHA) (see Online Data Supplement Table 1). Neither DHA nor EPA affected atrial conduction time or AERP (see Table 1).

**Cardiac function**

The increase in atrial systolic and diastolic volumes caused by SAVP between baseline and 21 days was significantly smaller in the DHA group compared to the No-PUFAs group (see Figure 3, P=0.02 for LASV, P=0.04 for LADV). The change in atrial volume was not affected by EPA (P=0.7 for both LASV and LADV). Neither DHA nor EPA affected ventricular function (see Table 2, all P>0.05). Only a small number of interpolated values were used in the calculations (see Online Data Supplement Table 2).

**Atrial fibrosis**

Treatment with DHA significantly reduced atrial collagen area fraction compared to the No-PUFAs group (11±6% vs. 20±4% respectively, P=0.004, see Figure 4). Treatment with EPA did not affect fibrosis (15±5%, P=0.08).

**Atrial cellular hypertrophy**

Neither DHA nor EPA significantly affected the increase in atrial myocyte cross-sectional area induced by 14 days of SAVP (218±19μm², 171±15μm², 177±14μm² for No-PUFAs, DHA, EPA respectively; P=0.08 for No-PUFAs vs. DHA, P=0.1 for No-PUFAs vs. EPA).

**Discussion**

DHA, but not EPA, reduced vulnerability to persistent AF caused by 14 days of simultaneous atrial and ventricular pacing. DHA also reduced the atrial enlargement, and fibrosis that resulted from the pacing; EPA did not have a significant effect. Our previous work suggested that
PUFAs (DHA+EPA) may attenuate AF vulnerability by retarding the development of arrhythmogenic structural changes in the atria (inflammation, hypertrophy and fibrosis) brought on by pacing.\textsuperscript{10,13} The current study suggests that DHA is the more beneficial of the 2 components.

\textit{PUFA integration}

Compared to controls, 21 days of supplementation resulted in significantly higher levels of DHA in DHA supplemented dogs, and EPA in EPA supplemented dogs, in both plasma and tissue. This suggests that both DHA and EPA were available in circulating and cardiomyocyte-bound forms. A potential limitation of prior studies with PUFA supplementation is that not enough lead time was allowed for sufficiently high PUFA concentrations to be achieved to produce the desired effect. Greater levels of DHA were seen in cardiac tissue than EPA (regardless of supplementation), consistent with observations by others that DHA is accumulated in the heart, whereas EPA is thought to accumulate in plasma.\textsuperscript{14} The levels of integration observed in this study are comparable to other studies of PUFA supplementation in dogs.\textsuperscript{15} Had we supplemented for a longer duration it is likely that there would have been a small increase in PUFA levels.\textsuperscript{15} It is notable that DHA supplementation increased plasma EPA possibly indicative of retroconversion of DHA to EPA; this has also been observed previously.\textsuperscript{16}

\textit{Observations of the DHA effect in other studies}

Although no other studies have tested DHA and EPA separately in a model of structural remodeling induced AF, DHA is correlated with reduced incidence of AF in other models. In a rabbit study, Ninio et al. found that a DHA enriched diet led to lower AF inducibility and maintenance.\textsuperscript{17}

Kumar et al. showed that serum DHA, but not EPA, was associated with lower
inducibility of AF in a study of 61 patients without known AF and no structural heart disease.\textsuperscript{18} The same investigator showed similar results with AF recurrence after cardioversion in a study of 178 patients with persistent AF.\textsuperscript{19} A recent publication from the Cardiovascular Health Study observed that plasma DHA, but not EPA, was correlated with lower AF incidence in a cohort of over 3000 elderly Americans.\textsuperscript{20} Similarly, Kirkegaard et al. found that plasma DHA, but not EPA, was associated with a lower incidence of AF in 300 hemodialysis patients with cardiovascular disease.\textsuperscript{21} Data from 2174 middle aged men in the Kuopio ischemic heart disease risk factor study showed that serum DHA, but not EPA, was significantly inversely correlated with risk of AF.\textsuperscript{22}

Possible mechanisms for the DHA effect

DHA and EPA are multipotent and putatively biologically active in a wide variety of areas from cardiac function to mental health to skin elasticity.\textsuperscript{23-24} Although they are similar, they differ in chain length and number of double bonds (DHA having 2 more carbons and 1 more double bond). As such they can have different interactions with receptors and enzymes and produce different metabolites.\textsuperscript{25} Given that DHA is more abundant in cardiac tissue, it is potentially more likely to have cardiac effects than EPA. Our group showed that AF inducibility in the SAVP model is correlated with increasing hypertrophy and fibrosis which may be triggered by early inflammation.\textsuperscript{10} In an in vitro model, DeCaterina et al. showed that DHA was more efficient than EPA in reducing inflammation.\textsuperscript{26} Additionally, certain products of DHA metabolism (D-series resolvins) have been shown to have anti-inflammatory properties.\textsuperscript{27} DHA mediated suppression of atrial inflammation could be a potential explanation for the results seen in the current study. Previous work has also shown that PUFAs alter the tissue response to injury at the genetic level.\textsuperscript{13} DHA, but not EPA, has been shown to be a ligand for nuclear receptors including FXR.
and RXR thus possibly affecting gene transcription.\textsuperscript{25,28} Other observed differences in DHA and EPA activity that may explain our observations include the ability of DHA, but not EPA, to block certain cardiac ion channels, especially repolarizing potassium channels, or membrane incorporated DHA mediated inhibition of cardiac adrenergic stimulation.\textsuperscript{11,29} Although these effects have been observed by others, we did not observe a PUFA effect on repolarization. A key to understanding the mechanism of the DHA effect may lie in the observation that DHA lowered the burden of persistent AF, but not AF induction. This may suggest that DHA is effective in reducing factors that perpetuate AF i.e. anatomical heterogeneities such as fibrosis, rather than factors leading to AF induction i.e. repolarization heterogeneities.\textsuperscript{30,31}

\textbf{Limitations}

This is an animal study with measurements taken only at baseline and after 14 days of SAVP. Structural remodeling is complex and evolves over time, thus the results presented here represent only a snapshot of the process. However, previous work has suggested that after 14 days there is established remodeling and AF inducibility, and that PUFA effects are visible at this time point. Additionally, we tested only one dose of DHA and EPA. This dose is however equivalent to clinically relevant doses in humans and significant incorporation into both plasma and tissue was observed.

\textbf{Conclusion}

Supplementation with DHA, but not EPA, reduces arrhythmogenic structural changes to the atria resulting from SAVP.

\textbf{Acknowledgments:} We gratefully acknowledge the generous donation of DHA from Martek Biosciences Corporation and pacemaker equipment from St. Jude Medical and Medtronic Inc. The authors would like to thank Zana Mariano and VladDragan for statistical assistance.
Funding Sources: This work was supported by the Canadian Institutes of Health Research [CGS-D to A.R.], the Ontario Government [OGS to A.R., OGSST to A.R.], the Heart and Stroke Foundation of Ontario [NA 6815 to P.D.], the Natural Sciences and Engineering Research Council of Canada [R.P.B.] and the Heart and Stroke Foundation of Canada [CS 6857 to K.A.C.]

Conflict of Interest Disclosures: None

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Table 1. Effect of DHA and EPA on atrial electrophysiologic parameters after 14 days of SAVP

<table>
<thead>
<tr>
<th></th>
<th>No-PUFAs</th>
<th>DHA</th>
<th>P</th>
<th>EPA</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Global atrial conduction time (GACT) (ms)</td>
<td>72±10</td>
<td>69±8</td>
<td>0.5</td>
<td>72±7</td>
<td>0.7</td>
</tr>
<tr>
<td>AERP (ms)</td>
<td>118±10</td>
<td>115±8</td>
<td>0.5</td>
<td>120±7</td>
<td>0.9</td>
</tr>
</tbody>
</table>

GACT measured between the superior vena cava and left atrial appendage at 400ms cycle length, AERP=atrial effective refractory period (measured at 200ms cycle length), DHA=docosahexaenoic acid, EPA=eicosapentaenoic acid. P values result from comparison of preceding column to No-PUFAs

Table 2. Effect of DHA and EPA on echocardiographic left ventricular parameters in 14 day SAVP dogs

<table>
<thead>
<tr>
<th></th>
<th>No-PUFAs</th>
<th>DHA</th>
<th>P</th>
<th>EPA</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>LVESV (Δ%)</td>
<td>99±26</td>
<td>87±40</td>
<td>0.5</td>
<td>123±43</td>
<td>0.3</td>
</tr>
<tr>
<td>LVEDV (Δ%)</td>
<td>52±29</td>
<td>29±9</td>
<td>0.1</td>
<td>50±23</td>
<td>0.9</td>
</tr>
<tr>
<td>LVEF (Δ%)</td>
<td>-47±6</td>
<td>-32±18</td>
<td>0.053</td>
<td>-46±18</td>
<td>0.9</td>
</tr>
</tbody>
</table>

LVESV=left ventricular end systolic volume, LVEDV=left ventricular end diastolic volume, LVEF=left ventricular ejection fraction, DHA=docosahexaenoic acid, EPA=eicosapentaenoic acid. P values result from comparison of preceding column to No-PUFAs

Figure Legends:

Figure 1. DHA and EPA integration into plasma and cardiac tissue. Panels A-B show relative % change in DHA [A] and EPA [B] in plasma phospholipid levels between baseline and 21 days. Panels C-D show absolute % of DHA [C] and EPA [D] in atrial tissue phospholipids at the 21 day study. *P<0.05, **P<0.01 compared to NP. NP=No-PUFAs, DHA=docosahexaenoic acid,
EPA=eicosapentaenoic acid.

**Figure 2.** Effect of DHA and EPA on AF inducibility in 14 day SAVP dogs. Effect of prophylactic supplementation with DHA and EPA on inducibility of AF episodes (AF incidence: atrial tachyarrhythmia≥2 mins) and persistent AF (AF persistence:≥10 mins) in dogs paced for 14 days. *P<0.05 compared to both No-PUFAs and EPA, SAVP=simultaneous atrioventricular pacing, NP=No-PUFAs, DHA=docosahexaenoic acid, EPA=eicosapentaenoic acid.

**Figure 3.** Effect of DHA and EPA on atrial volume in 14 day SAVP dogs. Effect of prophylactic supplementation with DHA and EPA on change in atrial volume between baseline and 21 days in dogs paced for 14 days. *P<0.05 compared to No-PUFAs, SAVP=simultaneous atrioventricular pacing, NP=No-PUFAs, DHA=docosahexaenoic acid, EPA=eicosapentaenoic acid.

**Figure 4.** Effect of DHA and EPA on atrial collagen area fraction in 14 day SAVP dogs. Effect of prophylactic supplementation with DHA and EPA on collagen area fraction in dogs paced for 14 days (panel A). Panels B-D show representative sections of left atrial appendage tissue stained with picrosirius red at 10X magnification (No-PUFAs [B], DHA [C], EPA [D]), scale bar represents 500μm. **P<0.01 compared to No-PUFAs, SAVP=simultaneous atrioventricular pacing, NP=No-PUFAs, DHA=docosahexaenoic acid, EPA=eicosapentaenoic acid.
Docosahexaenoic Acid, But Not Eicosapentaenoic Acid, Supplementation Reduces Vulnerability to Atrial Fibrillation

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_Circ Arrhythm Electrophysiol._ published online August 24, 2012;

_Circulation: Arrhythmia and Electrophysiology_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

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Print ISSN: 1941-3149. Online ISSN: 1941-3084

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SUPPLEMENTAL MATERIAL

DOCOSAHEXAENOIC ACID, BUT NOT EICOSAPENTAENOIC ACID, SUPPLEMENTATION REDUCES VULNERABILITY TO ATRIAL FIBRILLATION IN A CANINE MODEL

Methods:

Pacemaker implantation

Dogs were fasted overnight except for free access to water. Thirty minutes prior to the procedure dogs were sedated with i.m. acepromazine (Atrovet, 0.05 mg/kg [Ayerst Veterinary Laboratories, Guelph, ON]), atropine (0.03 mg/kg [Alveda Pharmaceuticals Inc., Bellville, ON]), and buprenorphine (Temgesic, 0.02 mg/kg [Schering-Plough, Kirkland, QC]). Blood samples were drawn from a vein in the left foreleg and stored on ice, in tubes containing 2 mg EDTA, until processed. Anesthesia was induced with i.v. thiopeptal sodium (5% solution 0.25 mL/kg [Hospira Healthcare Corp., Saint-Laurent, QC]). Dogs were then intubated with a 6.0-8.0 French E-T tube and ventilated with a mechanical ventilator (Harvard Apparatus, Inc., Holliston, MA, 16-18 breaths/min, 12-14 mL/kg tidal volume). Anesthesia was maintained with isoflurane (1-2% [Halocarbon Products Corporation, River Edge, NJ]). Deep anesthesia was confirmed by noting the absence of spontaneous breathing and absence of jaw tonus. Temperature was maintained at 37°C with a heating blanket. Under aseptic conditions, an incision was made in the lateral aspect of the neck to expose the left external jugular vein. Under fluoroscopic guidance, two steroid eluting, bipolar, IS-1 pacing leads (Pacesetter Tendril SDX, St. Jude Medical, Minneapolis, Minn) were inserted through the vein and fixed in the RA appendage and RV apex. Leads were connected to a “y-connector” (Lead Adaptor Kit, Medtronic Inc., Minneapolis, Minn) which connected the 2 leads to 1 bipolar IS-1 pacemaker (Model 5156 Verity ADx XL SR, St. Jude Medical, Minneapolis, Minn). Lead pacing thresholds were verified not to exceed 2.5 V and diaphragmatic stimulation was verified not to occur even with voltage as high as 10 V. Pacemaker, y-connector, and lead function were verified before recovering the dog. Dogs were placed on an anti-biotic regime (Baytril 5 mg/kg) for 1 week post implantation surgery.

Pacing

Dogs were allowed to recover from surgery for at least 1 week before the start of the pacing protocol. The pacemaker was turned on to VVI mode at 220 bpm with a 5.0 V pulse amplitude and a 0.5-1.0 ms pulse width. Due to the Y-connector, both leads paced simultaneously resulting in simultaneous AV pacing at 220 bpm. Dual chamber pacing was verified by ECG analysis at the time of pacing commencement and termination.

End study (sacrifice study)

Dogs were fasted overnight except for free access to water. Thirty minutes prior to the procedure dogs were sedated with i.m. acepromazine (Atrovet, 0.05 mg/kg [Ayerst Veterinary Laboratories, Guelph, ON]), atropine (0.03 mg/kg [Alveda Pharmaceuticals Inc., Bellville, ON]), and buprenorphine (Temgesic, 0.02 mg/kg [Schering-Plough, Kirkland, QC]). Blood samples were drawn from a vein in the left foreleg and stored on ice, in tubes containing 2 mg EDTA, until processed. Anesthesia was induced with i.v. thiopeptal sodium (5% solution 0.25 mL/kg [Hospira Healthcare Corp., Saint-Laurent, QC]). Dogs were then intubated with a 6.0-8.0 French E-T tube and ventilated with a mechanical ventilator (Harvard Apparatus, Inc., Holliston, MA, 16-18 breaths/min, 12-14 mL/kg tidal volume). Anesthesia was maintained with isoflurane (1-2% [Halocarbon Products Corporation, River Edge, NJ]). Deep anesthesia was confirmed by noting the absence of spontaneous breathing and absence of jaw tonus. Temperature was maintained at 37°C with a heating blanket. Under aseptic conditions, an incision was made in the lateral aspect of the neck to expose the left external jugular vein. Under fluoroscopic guidance, two steroid eluting, bipolar, IS-1 pacing leads (Pacesetter Tendril SDX, St. Jude Medical, Minneapolis, Minn) were inserted through the vein and fixed in the RA appendage and RV apex. Leads were connected to a “y-connector” (Lead Adaptor Kit, Medtronic Inc., Minneapolis, Minn) which connected the 2 leads to 1 bipolar IS-1 pacemaker (Model 5156 Verity ADx XL SR, St. Jude Medical, Minneapolis, Minn). Lead pacing thresholds were verified not to exceed 2.5 V and diaphragmatic stimulation was verified not to occur even with voltage as high as 10 V. Pacemaker, y-connector, and lead function were verified before recovering the dog. Dogs were placed on an anti-biotic regime (Baytril 5 mg/kg) for 1 week post implantation surgery.
Inc., Bellville, ON), and buprenorphine (Temgesic, 0.02 mg/kg [Schering-Plough, Kirkland, QC]). Blood samples were drawn from a vein in the left foreleg and stored on ice, in tubes containing 2 mg EDTA, until processed. Anesthesia was induced with i.v. propofol (Diprivan, 2.5-3.5 mg/kg [AstraZeneca, Mississauga, ON]). Dogs were then intubated with a 6.0-8.0 French E-T tube and ventilated with a mechanical ventilator (Harvard Apparatus, Inc., Holliston, MA, 16-18 breaths/min, 12-14 mL/kg tidal volume). Anesthesia was maintained with isoflurane (1-2% [Halocarbon Products Corporation, River Edge, NJ]). Deep anesthesia was confirmed by noting the absence of spontaneous breathing and absence of jaw tonus. Temperature was maintained at 37°C with a heating blanket and ventilatory humidifier system. At the end of the study, with the dog still under deep anesthesia, the heart was quickly excised and tissue samples were taken. Correct placement of the pacing leads was verified by post mortem examination in most dogs.

**Electrophysiological study**

The pacemaker was turned off at least 30 minutes prior to starting the electrophysiological study. A median sternotomy was performed to expose the heart. Five bipolar, stainless steel, epicardial pacing/recording electrodes were sutured onto the heart at the RA appendage (RAA), LA appendage (LAA), high RA (SVC), low RA (IVC) and pulmonary veins (PVs).

Extrasystole pacing was performed at each pacing electrode (LAA, RAA, SVC, IVC, PVs) with an S1-S2 technique. Electrical stimulation was supplied by a Ventritex HVSO2 (St. Jude Medical, Minneapolis, Min) at twice stimulation threshold at 400 and 200 ms cycle length with a 2 ms pulse width for 30 seconds (to reach steady state). After 30 seconds of continuous pacing, 8 stimuli (S1) were applied followed by an extra stimulus (S2) beginning 100 ms after the last S1 stimulus. The S2 interval was increased in 20 ms intervals until capture was recorded. The S2 interval was then decreased by 10 ms until capture was lost. The S2 interval was then increased in 2 ms increments until capture was recorded again. The atrial effective refractory period (AERP) was determined to be the longest S1-S2 interval which did not result in capture. AERP was calculated as the mean of the AERPs measured at the LAA, RAA, SVC, IVC and PVs at 200 ms cycle length. Conduction time between the LAA and the SVC was calculated at 400 ms cycle length and presented as a measure of global atrial conduction time. Recordings taken just after steady state was reached at the LAA and SVC electrodes were analyzed. The time between the pacing stimulation on the LAA electrogram and the next activation (point of most negative dV/dt) on the SVC electrogram was taken to be the conduction time between the two electrodes (and vice versa SVC to LAA). The times from 3 consecutive stimulations at the LAA to the SVC, and 3 consecutive stimulations at the SVC to the LAA were averaged to produce a mean conduction time for each dog.

Burst pacing was performed at up to five pacing electrodes and recordings of the resulting activation patterns were made at all sites fitted with recording electrodes. Electrical stimulation was supplied by a Ventritex HVSO2 (St. Jude Medical, Minneapolis, Min) at 10 V, 10 Hz (100 ms cycle length), with a 2 ms pulse width for 10 seconds. Any irregular atrial tachyarrhythmia lasting more than 5 seconds was noted;
recordings were made if the arrhythmia lasted more than 60 seconds. A protocol to “break” the arrhythmia was employed just after the 60 second mark. A rapid burst of stimulations was applied through the same electrode that initiated the arrhythmia (10 V, 2 ms pulse width, 5-20 Hz, 2-5 sec duration). The protocol was repeated continuously until the 120 second mark, or until the arrhythmia stopped. If the arrhythmia did not stop, it was allowed to continue until self-termination or the 600 second mark at which point the dog was cardioverted with a 30-50 J shock from a Medtronic PhysioControl Lifepak 12 Defibrillator (Medtronic Inc. Minneapolis, Min) in synch mode. If no arrhythmia resulted from the burst, another burst was applied 10-12 seconds later, up to 10 bursts maximum per electrode. If an arrhythmia occurred, a rest period equal to at least half the duration of the arrhythmia was allowed before the next burst was applied. If more than 10 total minutes of arrhythmia were initiated by the same electrode, the protocol was stopped and started again on a new electrode.

An AF “incident” was defined as any irregular atrial tachyarrhythmia that lasted more than 120 seconds with atrial rate >300bpm and was resistant to pace termination; “persistent” AF was defined as an AF incident that lasted more than 600 seconds (10 minutes).

Echocardiography

Trans-thoracic and trans-esophageal echo was done preoperatively at the time of pacemaker implantation, and at the end study, with the dog intubated and anesthetized with 1-2% isoflurane, lying on the right side with a Sonos 5500 ultrasound system (Philips Ultrasound, Canada). An effort was made to ensure measurements were taken at the same location and angle in each dog to maximize comparability. At the end study the pacemaker was turned off at least 15 minutes prior to echocardiographic assessment.

**Trans-thoracic Echo**

A 1.8-3.6 MHz phased array transducer (S3, Philips Ultrasound) was used to obtain trans-thoracic recordings. From a parasternal approach, the LV short axis view was recorded at the mid-papillary muscle level for measurement of LV end systolic and diastolic area (LVESA, LVEDA). Apical 4 chamber and 2 chamber views were recorded for measurement of LV end-diastolic and end-systolic volumes, and ejection fraction (EF) according to the Simpson’s biplane method: \((\pi/4) \times \Sigma(ai)(bi)(L/n)\) (where \(n\) = the number of cylinders or discs of equal height taken from 2 chamber (aï) and 4 chamber (bï) recordings, \(L\) = length from apex to mitral valve annulus). Data from three consecutive beats were averaged in order to calculate each value.

**Trans-esophageal Echo**

A 5-7 MHz phased array multiplane trans-esophageal transducer (Philips Ultrasound) was used to obtain trans-esophageal recordings. LA area and length were measured from apical 4 chamber and 2 chamber views during the systolic and diastolic phases. LA systolic and diastolic volume (LASV, LADV) were calculated as follows: \((8/(3 \times \pi)) \times ((4 \text{ Ch LA area})(2 \text{ Ch LA area})/(\text{LA length}))\) (where LA length is the distance from LA
back wall to mitral valve annulus). Data from three consecutive beats were averaged in order to calculate each value.

**Calculations**

Changes in echo parameters were calculated as percentage changes between pacemaker implant and end study. If data from either timepoint were missing, the mean value from the group for that parameter at that timepoint was substituted so that percentage change could be calculated. Values “interpolated” in this manner were excluded from the final analysis if they fell outside the range of those values calculated from non-interpolated data.

**Sample collection and preparation**

As much as possible, care was taken to make sure corresponding samples were taken from approximately the same location in every dog.

**Plasma**

Whole blood was centrifuged at 2,200 rpm for 15-20 minutes at 5-10°C. Plasma was separated and frozen at -80°C. The remainder was discarded.

**Formalin**

Samples of LAA were preserved in 10% neutral buffered formalin (10% formaldehyde, 2% anhydrous sodium acetate, 88% water). Samples were fixed for at least 14 days at room temperature. Samples were prepared for histology by the pathology department at St. Michael’s Hospital (Toronto, ON) or The Centre for Phenogenomics (TCP, Toronto, ON).

**PUFA phospholipid isolation and quantification**

Total lipids were extracted from frozen plasma or frozen atrial tissue according to the method of Folch, Lees, and Sloane Stanley using chloroform: methanol : 0.88% KCl (2:1 : 0.75 by vol.). Isolation of phospholipids was achieved by thin-layer chromatography (TLC). TLC G-plates (Cat. #5721-7; EMD Chemical, Gibbstown, NJ) were activated by heating at 100°C for 1 hour. Lipid classes were separated along with authentic standards in petroleum ether/diethyl ether/glacial acetic acid (80:20:1 by volume). Bands corresponding to total phospholipids, cholesterol, free fatty acids, triglycerides, and cholesterol esters were visualized under UV light after lightly spraying with 8-anilino-1-naphthalene sulfonic acid (0.1%w/v). Phospholipid bands were collected into test tubes with a known amount of heptadecanoic acid (17:0), then converted to fatty acid methyl esters with boron trifluoride in methanol.

Fatty acid methyl esters were analyzed using a Varian-430 gas chromatograph (Varian, Lake Forest, CA, USA) equipped with a Varian FactorFour capillary column (VF-23ms; 30 m • 0.25 mm i.d. • 0.25 lm film thickness) and a FID. Samples were injected in splitless mode. The injector and detector ports were set at 250°C. FAME were eluted using a temperature program set initially at 50°C for 2 min, increasing at 20°C/min, and
held at 170°C for 1 min, then at 3°C/min and held at 212°C for 5 min to complete the run at 28 min. The carrier gas was helium, set to a constant flow rate of 0.7 mL/min. Peaks were identified by retention times of authentic FAME standards (Nu-Chek Prep, Inc., Elysian, MN, USA). The concentration of each fatty acid was calculated by comparison with the internal standard (17:0) and converted to nmol% as performed by Chen et al.²

**Histology**

Histological analysis was performed via light microscopy using a Nikon Eclipse E800 microscope (Nikon, Inc.) with an attached Nikon DXM 1200 digital camera. Images were captured with Nikon ACT-1 software v2.70. Any image manipulation and analysis was performed with ImageJ software (Rasband, W.S., ImageJ, National Institutes of Health, Bethesda, MD, http://rsb.info.nih.gov/ij/).

**Atrial fibrosis**

Picrosirius red (PSR) staining was performed on sections of 14 day formalin fixed, paraffin embedded LAA tissue by the pathology department at The Hospital for Sick Children (Toronto, ON) or by The Centre for Phenogenomics (TCP, Toronto, ON).

PSR stained sections of 14 day formalin fixed, paraffin embedded LAA tissue were analyzed by light microscopy at 10X magnification. Approximately 7 images were taken per animal in black and white with a green interference filter to maximize contrast. Threshold was manually adjusted until only collagen containing pixels were highlighted (compared each picture to unedited original to ensure correctness). Area fraction was calculated based on largest area selected to exclude vessels and include mainly midmyocardium (avoiding edges). Analysis was done in a blinded fashion and measurements were repeated 3 times to ensure accuracy.

**Cellular hypertrophy**

Hematoxylin and eosin (H+E) staining was performed on sections of 14 day formalin fixed, paraffin embedded LAA tissue by the pathology department at St. Michael’s Hospital (Toronto, ON).

H+E stained sections of 14 day formalin fixed, paraffin embedded LAA tissue were analyzed by light microscopy at 40X magnification. Approximately 25 images were taken per animal. Images presenting large collections of myocytes cut in cross section were selected for further analysis. Myocytes were judged to be cut in cross section if they appeared circular in shape, with a well-defined membrane and large, circular, darkly staining nuclei appearing near the center of the cell. Myocyte outlines were traced and analyzed with the “circularity” function. A circularity of at least 0.8 (out of 1.0) was considered circular. Cross sectional area was measured by the “area” function. Approximately 150 cells were measured from each animal. All analysis was done in a blinded fashion and measurements were repeated 3 times to ensure accuracy.
Results:

**Atrial electrophysiology**

Table 1 – Detailed AF inducibility data

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Mean±SD 35.5±10.8  2.5±1.8  9.2±8.8  1.7±1.4  7.1±9.0

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Mean±SD 37.7±3.7  1.2±1.6  3.4±4.8  0.3±.5*  1.0±1.6*

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Mean±SD 31.2±12.9  2.2±1.7  10.8±14.0  1.2±1.0  6.1±8.5

BURSTS=number of attempts at inducing AF, #AFi=number of AF incidents, %AFi=percentage of bursts resulting in an AF incident, #PAF=number of persistent AF incidents, %PAF=percentage of bursts resulting in a persistent AF incident; *P<0.05 compared to No-PUFAs group
**Cardiac function**

Table 2 – Amount of interpolated echocardiographic data used

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LV=left ventricular, LA=left atrial, ESV=end systolic volume, EDV=end diastolic volume, EF=ejection fraction, SV=systolic volume, DV=diastolic volume

Reference List:
