INVESTIGATION OF THE EFFECTS OF N-3 POLYUNSATURATED FATTY ACIDS ON VULNERABILITY TO ATRIAL FIBRILLATION IN CARDIOMYOPATHY

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
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ABSTRACT

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Atrial fibrillation (AF) is a common and serious arrhythmia. Current treatments are of limited efficacy, and most do not treat the atrial structural remodeling (hypertrophy and fibrosis) that underlies most clinical AF. Our group has created an experimental dog model of atrial mechanical stretch called the simultaneous atrial and ventricular pacing (SAVP) model (which results in atrial fibrosis and susceptibility to AF) in order to study novel treatments for structural remodeling induced AF. Omega-3 polyunsaturated fatty acids (n3 PUFAs), particularly the marine derived forms eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), have been shown to be effective in treating arrhythmias (including AF) in some animal studies and clinical trials. The mechanism for this effect of n3 PUFAs is not well understood. In this study we sought to characterize the n3 PUFA effect on AF vulnerability, atrial electrophysiology, histology, and gene expression, and determine relevant mechanisms.

Dogs were paced for 0, 2, 7 or 14 days and given n3 PUFAs, olive oil or nothing. Prophylactic n3 PUFAs significantly reduced both AF vulnerability and conduction slowing in SAVP dogs (%AF inducibility: 9.2±8.8 vs. 4.7±6.3; global atrial conduction time: 75±11ms vs. 65±6ms [SAVP 14 days vs. SAVP 14 days with n3 PUFAs, P<0.05
for both comparisons). Prophylactic n3 PUFAs also reduced inflammation (mean CD18 grade: 2.1±0.8 vs. 1.3±0.6 [SAVP 2 days vs. SAVP 2 days with n3 PUFAs, P=0.055]),
hypertrophy (myocyte cross-sectional area: 498±64µm² vs. 322±111µm² [SAVP 14 days vs. SAVP 14 days with n3 PUFAs, P<0.05]),
and fibrosis (%collagen area vs. unpaced dogs: 178±58 vs. 127±37 [SAVP 14 days vs. SAVP 14 days with n3 PUFAs, P<0.05]). N3 PUFAs were also found to reduce the expression of structural remodeling related molecules such as TGF-β, EGF, ERK and Akt. N3 PUFAs given after some pacing had already occurred were found to be less effective at reducing AF vulnerability and structural remodeling.

The results of this study suggest that, in the SAVP model, n3 PUFAs reduce vulnerability to AF by attenuation of adverse structural remodeling at the genetic level.
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LIST OF ABBREVIATIONS

(ALA) α linolenic acid
(ADAM) a disintegrin and metalloprotease
(ARP) absolute refractory period
(APD) action potential duration
(Ang2R1) AngII receptor
(ACE) angiotensin converting enzyme
(ACEi) angiotensin converting enzyme inhibitors
(AngII) angiotensin II
(ARBs) angiotensin receptor blockers
(AADs) antiarrhythmic drugs
(AA) arachidonic acid
(AERP) atrial effective refractory period
(AF) atrial fibrillation
(ANP) atrial natriuretic peptide
(bpm) beats per minute
(BNP) brain natriuretic peptide
(CVD) cardiovascular disease
(JNK) c-Jun NH₂-terminal protein kinase
(CHF) congestive heart failure
(CABG) coronary artery bypass graft
(CAD) coronary artery disease
(CHD) coronary heart disease
(CRP) C-reactive protein
(COX) cyclooxygenase
(DAG) diacylglycerol
(DHA) docosahexaenoic acid
(ERP) effective refractory period
(EPA) eicosapentaenoic acid
(EF) ejection fraction
(ET-1) endothelin 1
(ELISA) enzyme-linked immunosorbent assay
(EGF) epidermal growth factor
(EETs) epoxeicosatrienoic acids
(ET1R) ET-1 receptor
(EST) expression sequence tag
(ECM) extracellular matrix
(ERK) extracellular signal related kinase
(FDR) false discovery rate
(FGF) fibroblast growth factor
(FAK) focal adhesion kinase
(FFA) free fatty acid
(GPCRs) G protein coupled receptors
(GEO) gene expression omnibus
(GSK3) glycogen synthase kinase 3
(GDP) guanine diphosphate
(GEF) guanine exchange factor
(GTP) guanine triphosphate
(H+E) hematoxylin and eosin
(HDL) high density lipoproteins
(hsCRP) high sensitivity C-reactive protein
(ICD) implantable cardioverter-defibrillator
(IVC) inferior vena cava (low RA)
(IP3) inositol 1,4,5-triphosphate
(IGF) insulin-like growth factor
(IL6) interleukin 6
(JAK) janus kinase
(LAA) left atrial appendage
(LADV) left atrial diastolic volume
(LASV) left atrial systolic volume
(LVEDA) left ventricular end diastolic area
(LVESV) left ventricular end systolic area
(LT) leukotrienes
(LA) linoleic acid
(LDL) low density lipoproteins
(mTOR) mammalian target of rapamycin
(MMP) matrix metalloproteinase
(MMPl) matrix metalloproteinase inhibitors
(MAPKs) mitogen activated protein kinases
(SMAD) mothers against decapentaplegic homolog
(MI) myocardial infarction
(MHC) myosin heavy chain
(NADPH) nicotinamide adenine dinucleotide phosphate
(NO) nitric oxide
(NFκB) nuclear factor kappa-light-chain-enhancer of activated B cells
(NFAT) nuclear factor of activated T cells
(n3 PUFAs) omega-3 polyunsaturated fatty acids
(OCT) optimal cutting temperature compound
(PPAR) peroxisome proliferator-activated receptors
(PIP2) phosphatidylinositol 4,5 bisphosphate
(PI3K) phosphoinositide 3-kinase
(PLA) phospholipase A
(PLC) phospholipase C
(PSR) picrosirius red
(PDGF) platelet derived growth factor
(POAF) post operative AF
(PCB) printed circuit board
(PG) prostaglandins
(Akt) protein kinase B
(PKC) protein kinase C
(PV) pulmonary vein
(QT-RT-PCR) quantitative real time PCR
(RCT) randomized controlled trial
(RVP) rapid ventricular pacing
(ROS) reactive oxygen species
(RTKs) receptor tyrosine kinases
(RGS) regulator of G protein signaling
(RR) relative risk
(RAS) renin angiotensin system
(RAA) right atrial appendage
(RMA) robust-multi array average
(SR) sarcoplasmic reticulum
(STAT) signal transducers and activators of transcription
(SAM) significance analysis of microarrays
(SAVP) simultaneous atrial and ventricular pacing
(SA) sinoatrial
(NCX) sodium calcium exchanger
(SACs) stretch activated ion channels
(SCD) sudden cardiac death
(SVC) superior vena cava (high RA)
(TUNEL) terminal deoxynucleotidyl transferase mediated dUTP-biotin nick end labeling
(TAK) TGF-β activated kinase
(TX) thromboxanes
(TIMP) tissue inhibitors of metalloproteinases
(TGF-β) transforming growth factor β
(TNF-α) tumor necrosis factor alpha
(VEGF) vascular endothelial growth factor
INTRODUCTION

Rationale:
Atrial fibrillation (AF) is the most common arrhythmia, and its prevalence is increasing. The presence of an arrhythmogenic substrate due to mechanical stretch induced structural remodeling (hypertrophy and fibrosis) of atrial tissue underlies most clinical AF. Current treatment paradigms are of limited efficacy, and most do not target the AF substrate. The Dorian lab has created an experimental dog model of atrial mechanical stretch called the simultaneous atrial and ventricular pacing (SAVP) model in order to study novel treatments for structural remodeling induced AF. The SAVP model induces atrial enlargement, fibrosis and susceptibility to AF.

Omega-3 polyunsaturated fatty acids (n3 PUFAs), particularly the marine derived forms eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), have shown promise as therapeutic agents in the treatment of AF. It is generally believed that n3 PUFAs have direct antiarrhythmic effects, however, recent studies have cast doubt on the idea that n3 PUFAs are antiarrhythmic in all patients. Animal studies have suggested that n3 PUFAs reduce AF, and may also have anti-structural remodeling properties. We designed a series of experiments to investigate the efficacy of n3 PUFAs in the treatment of AF, and to investigate the potential mechanisms of this beneficial effect.

Objective:
The primary objective of this study was to gain insight into the mechanisms by which n3 PUFAs may attenuate vulnerability to AF in the SAVP model by characterizing the effects of n3 PUFAs on atrial electrophysiology, structure, histology, and gene expression.
**Hypothesis:**
Our main hypothesis was that PUFAs would attenuate SAVP induced AF vulnerability by integrating into myocyte membranes, and interfering with the cell signaling processes that lead to structural remodeling.

**Experimental overview:**
This study was performed as 4 separate projects which are presented in chronological order in Section Four of the part of this thesis entitled “Experimental Work”. A brief summary of the study and the major experimental results from all projects combined can be found in Appendix One. This study used mongrel dogs, 1-3 years of age, ~20-30 kg for all projects. Dogs were implanted with a pacemaker, and both atria and ventricles were paced at 220 beats per minute for 0, 2, 7 or 14 days. Dogs received ~1 g/day oral n3 PUFAs (EPA only, DHA only, or EPA+DHA), ~1 g/day oral olive oil, or nothing.

**Project One - Effect of chronic, prophylactic PUFA administration on AF vulnerability and gene expression in a model of established atrial cardiomyopathy**

The major objective of this project was to identify mRNA expression profiles involved in AF vulnerability, and see whether they were altered by n3 PUFA therapy. The major hypothesis of this project was that AF vulnerability was related to structural remodeling, and that n3 PUFAs would reduce AF vulnerability and expression of structural remodeling related genes.

**Project Two - Effect of chronic, post-injury, PUFA administration on AF vulnerability in a model of established atrial cardiomyopathy**
Given that prophylactic n3 PUFAs in Project One were able to significantly attenuate AF vulnerability, this project was designed to test the ability of post-injury n3 PUFAs (n3 PUFAs started part way through the atrial injury process) to prevent AF vulnerability.

The major hypothesis of this project was that post-injury n3 PUFAs would not be as effective as prophylactic n3 PUFAs in reducing vulnerability to AF.

**Project Three – AF vulnerability, and the effect of prophylactic PUFA administration in a model of short term atrial stretch**

Given that increasing AF vulnerability appeared to be correlated with increasing structural remodeling in Projects One and Two, this project was designed to determine whether or not significant AF vulnerability existed before hypertrophy and fibrosis became established, but at a time when inflammation was estimated to be at peak levels. Additionally, this project tested the effects of n3 PUFAs on atrial electrophysiology, histology and gene expression early in the remodeling process.

The major hypotheses of this project were that significant AF vulnerability would not be present before structural remodeling had become established, and that n3 PUFAs would have no effect on any measured parameters in the absence of significant structural remodeling.

**Project Four - Effect of chronic, prophylactic, EPA or DHA alone on AF vulnerability in a model of established atrial cardiomyopathy**

Given the success of n3 PUFAs in reducing vulnerability to AF, this project was designed to test the efficacy of EPA and DHA alone (rather than in combination as was previously done) versus no PUFAs using the same experimental design set forth in Project One.

The major hypothesis for this project was that DHA would be more effective than EPA at reducing vulnerability to AF.
LITERATURE REVIEW

Section One: Atrial fibrillation

Background

Atrial fibrillation (AF) is the most common cardiac rhythm disturbance (arrhythmia). It is characterized by rapid, irregular atrial electrical activity resulting in uncoordinated atrial contraction; it can occur as a temporary condition (paroxysmal) or a persistent disease. AF is associated with significant morbidity and mortality; according to data from the Framingham study AF increases the risk of stroke up to 5 fold \(^{13}\) and doubles the risk of death \(^{14}\). AF is very common in the elderly \(^{15}\) and the number of patients with AF is growing rapidly. It is estimated that by the year 2050 the prevalence of AF will increase 2.5 times with anywhere from 5 to 10 million sufferers in the US alone \(^{16, 17}\). The aging population, increased detection and improved survival are all suggested to be fueling this increase. Hospitalization for AF cost at least 12.7 billion USD in 2005; this number may be close to 30 billion USD by 2050 if growth estimates are correct \(^{18}\).

Current treatments for AF are of limited efficacy; one recent study suggested that AF related mortality has remained unchanged for the last 20 years \(^{19}\). Treatment involves attempting to control atrial rhythm and/or rate of ventricular activation. Rhythm control can be accomplished with electrical cardioversion or surgery, both rate and rhythm control can be accomplished with antiarrhythmic drugs (AADs). Cardioversion and surgery (radiofrequency ablation \(^{20, 21}\) or Maze procedure \(^{22}\)) require hospitalization, are complex, and only moderately effective. In the case of cardioversion the procedure may have to be repeated quite often, and in the case of surgery only a subset of AF patients are eligible. For rhythm control the use of class Ic or class III AADs (according to the Vaughn Williams classification) is indicated \(^{23}\). For rate control beta blockers,
Ca${}^{2+}$ channel antagonists and cardiac glycosides can be added to the treatment regime. Several recent studies confirmed that rate control is at least equivalent to rhythm control in terms of mortality (AFFIRM 24, PIAF 25, HOT CAFE 26, STAF 27). However, adverse drug reactions were common in all trials ranging from 11% of patients to 28% depending on the drug used. Amiodarone, one of the most successful drugs for treating AF, had an incidence of adverse reactions of 11%-28% depending on the trial (AFFIRM 28, CTAF 29, PIAF 25). The CARAF study demonstrated that 33-57% of AF patients have contraindications or warnings against AADs 1. Together this indicates that AF is a serious problem, increasing in importance and in need of better treatment options.

**Epidemiology of AF**

Difficulties in the understanding and treatment of AF arise because it is a complex and multi-factorial disease. AF can be both a cause and consequence of multiple other diseases, and many patients have (sometimes several) comorbid conditions; AF also evolves over time becoming more persistent and less likely to terminate. Further difficulties arise due to the fact that most epidemiological studies of AF have involved almost exclusively white, Western cohorts and thus there is little information available from the global population. The prevalence of AF is known to increase with increasing age, and current estimates suggest anywhere from 9 to 20% of octogenarians currently have AF 16, 17, 30-32. Hypertension, diabetes, myocardial infarction (MI), congestive heart failure (CHF), valvular heart disease and stroke are all significant risk factors for AF 15,30,31. However, some AF is not correlated with aging or heart disease at all. This so called “lone AF” is typically diagnosed in younger patients who don’t exhibit the risk factors listed above. A genetic susceptibility to fibrillation may be the reason behind the AF seen in these patients. Indeed mutations in several genes
have been linked to AF including KCNQ1, KCNE2, KCNE3, Kir2.1, Kir3.4, GJA5, and SCN5A (reviewed by Benjamin et al. 33 and Tsai et al.34), although the importance of gene mutations in AF has been questioned by some 35. Hyperthyroidism and other endocrine disorders represent another important cause of AF 36,37. Transient cases of AF can be induced by many factors including alcohol consumption (“holiday heart syndrome”), pericarditis, myocarditis and cardiac surgery 38-40.

Nevertheless, in most patients, underlying heart disease is the most common cause of AF. Data from the CARAF study show that 56% of AF patients have some form of structural heart disease, 31% show ischemic heart disease, 22% have LV systolic dysfunction and 15% have heart failure 1. Hypertension is the most important condition leading to AF; studies suggest it is responsible for more AF than any other risk factor 31. The AFFIRM study showed that over 50% of AF patients had hypertension as the primary cardiac diagnosis 24; similar results have been reported in other studies 25-27. Hypertension leads to increased atrial pressures, atrial enlargement and scarring (fibrosis) 23. MI, valvular disease (especially mitral valve insufficiency leading to regurgitation), and heart failure have all been linked to hemodynamic changes resulting in atrial stretch, cell death and fibrosis 23,41,42.

The unifying pathology leading to AF in all these disease conditions is increased atrial mechanical stress (stretch) which results in atrial enlargement, hypertrophy, and fibrosis. This remodeling of atrial structure leads to conduction heterogeneity and abnormality.

**Electrophysiology of AF**

Regardless of its etiology AF is ultimately an electrical phenomenon. As such, its genesis and persistence are linked to changes in atrial action potential properties and
propagation. Under normal conditions, pacemaker cells in the sinoatrial (SA) node spontaneously generate an action potential; this triggers a spreading wave of action potentials through adjacent cardiomyocytes which eventually covers both atria.

**Action potentials**

The atrial action potential (which is different from the ventricular action potential) is characterized by the function and number of ion channels (which facilitate the entry and exit of ions from the cell) arranged on the membranes of atrial cardiomyocytes (see Figure 1). Inward currents occur when positive ions move into the cell causing it to “depolarize”, whereas outward currents occur when positive ions move out of the cell causing it to “repolarize”. Atrial cardiomyocytes have a resting membrane potential of about -80 mV. K⁺ ion channels generate an outward current (I\textsubscript{K1}) which maintains this negative potential. Movement of positively charged ions into the cell raises membrane potential. When the membrane potential reaches a level, termed the “threshold potential”, a massive inward current is generated by the opening of Na⁺ channels (I\textsubscript{Na}) triggering rapid depolarization; this is termed “phase 0” of the action potential. The membrane potential rises very rapidly to about +40 mV at which point the Na⁺ channels become inactive, and an outward current (I\textsubscript{to}) is generated by the opening of K⁺ channels causing a drop in membrane potential (phase 1). An inward current (I\textsubscript{Ca}) is created by the opening of Ca²⁺ channels; this depolarizing current is balanced by the repolarizing I\textsubscript{to} which creates a short plateau effect (phase 2). Very shortly thereafter, several more outward currents are generated by the opening of K⁺ channels; these are the ultra-rapid (I\textsubscript{Kur}), rapid (I\textsubscript{Kr}) and slow (I\textsubscript{Ks}) delayed rectifier currents (phase 3). These repolarizing currents, combined with the dwindling, then cessation of depolarizing currents, eventually return the membrane potential to the resting state (-80 mV), a level which is maintained once again by I\textsubscript{K1} (phase 4). There is a period of time during this
Figure 1 – Atrial action potential
Schematic representation of an atrial action potential. Height indicates membrane potential in mV, width indicates time. Numbers along the action potential tracing indicate the phase. Currents are listed underneath the tracing indicating the approximate time they are activated; arrows beside them indicate whether they are depolarizing (↑) or repolarizing (↓). APD = action potential duration, ARP = absolute refractory period, RRP = relative refractory period, ERP = effective refractory period.
cycle of depolarization/repolarization in which the cell is unable to generate another action potential, called the absolute refractory period (ARP), which is normally around 60-70% of the entire action potential duration (APD). The relative refractory period begins just after the ARP when it is possible to generate an action potential with a stronger than normal stimulus. The term effective refractory period (ERP, or AERP for “atrial ERP”) is used to describe the point during the RRP when it is possible to generate another action potential with double the normal minimum stimulus; this point normally occurs at around 80-90% of the APD. Generally, ion channels (once inactivated) cannot reopen again until a certain passage of time or a repolarization has occurred.

**Electrical propagation**

Cells are electrically coupled together via open channels which connect neighbouring myocytes called “gap junctions”; gap junctions are comprised of proteins called connexins. Diffusion of positive ions from adjacent depolarized cells through gap junctions raises the membrane potential to the threshold level in the secondary cells triggering an action potential. In order for this to occur, the adjacent cells must not be refractory. If all adjacent cells are refractory, the wave of electrical activity is extinguished and cells remain quiescent until their refractory period is over and they are excited by an adjacent cell, or they spontaneously generate an action potential.

**Mechanisms of AF**

The generation of action potentials and their normal propagation can be disrupted by several factors. Areas other than the SA node can sometimes generate spontaneous action potentials; these areas are called ectopic foci and they may “fire”
once, or at intermittent, or possibly regular intervals. The mechanism for this is usually either abnormal automaticity, or triggered activity (collectively called “ectopy”).

Changes to the electrical properties of the atria related to ion channel or connexin function are termed “electrical remodeling”, and can be important factors in the promotion of AF. However, “structural remodeling”, involving hypertrophy and especially fibrosis, is the most important factor leading to stretch related AF. The most common mechanism behind electrical and structural remodeling induced AF is called “reentry”.

Factors including genetics, drugs, and disease conditions can encourage both ectopy and reentry, thus leading to AF. Paradoxically, AF itself induces remodeling and thus makes future episodes more likely to occur and less likely to terminate. Wijffels et al. were the first to demonstrate this in a goat study and coined the phrase “AF begets AF”. This is consistent with data from human studies that show that rates of conversion and maintenance of sinus rhythm decrease in patients as AF duration increases.

The details of ectopy and reentry have been debated for over 100 years. One of the most comprehensive reviews of fibrillation studies done in the late 19th and early 20th centuries was written by Walter Garrey in 1924. An excellent review of the literature from as far back as 1896 was written by Nattel et al. in 2005. A very brief summary of some of the key details on ectopy and reentry follows below.

**Ectopy (automaticity and triggered activity)**

Abnormal automaticity can occur when K⁺ currents fail to maintain the resting membrane potential during phase 4 and allow the cell to reach threshold potential. A decrease in I_{K1} or the atrial specific acetylcholine sensitive I_{Kach} current can be responsible. Triggered activity is usually the result of abnormal Ca²⁺ cycling and takes
the form of an early afterdepolarization or a delayed afterdepolarization. Afterdepolarizations occur when Ca\(^{2+}\) inappropriately enters the cytosol either through \(I_{\text{Ca}}\) L-type channels or from the sarcoplasmic reticulum. The sodium calcium exchanger (NCX) extrudes the excess Ca\(^{2+}\) in exchange for Na\(^{+}\) in a 1:3 ratio. The resulting net inward current can raise the membrane potential to the threshold and trigger an action potential.

Pulmonary vein (PV) cardiomyocytes have been shown to depolarize spontaneously in animal and human studies \(^{49, 50}\). Excess ectopy from the PV region has been proposed as an important cause of AF (especially paroxysmal AF), and indeed isolation of the PVs by ablation has been shown to successfully abolish AF in humans \(^{21}\). Ectopic foci in areas other than the PVs are rare, but have been observed in humans; once again, ablation of these foci abolishes AF \(^{20}\). \(\beta\) adrenergic stimulation, or rapid atrial rates brought on by tachycardia or fibrillation causes increased Ca\(^{2+}\) uptake by myocytes potentially leading to diastolic Ca\(^{2+}\) release and afterdepolarizations \(^{51, 52}\).

**Reentry**

As an electrical wave propagates through the atria it may encounter non-excitatory tissues. These include refractory tissue, but also anatomical obstacles like vessels, valves, or fibrotic bundles composed of (non-conductive) collagen. Upon encountering these barriers propagation is blocked and the wave may be extinguished, or may “turn” towards excitable tissue. Thus a wave may circle around an obstacle and return to its starting point. The length of this circuit is called the pathlength, while wavelength refers to the distance traveled by the wave in one refractory period (the product of conduction velocity and refractory period). If the wavelength exceeds the pathlength, the wave will encounter refractory tissue upon returning to the starting point.
and, without adjacent excitable tissue, it will be extinguished. However, if the pathlength is larger, the wave can circle around indefinitely; this is the concept of reentry. The type of reentrant circuit just described is called a rotor. Rotors can behave like ectopic foci, constantly firing off waves of excitation to adjacent tissue as they rapidly circle. AF may be caused by a single rotor, or multiple rotors (provided the atria are large enough to support them) 47.

In the 1950s and 60s, Moe et al. proposed another form of reentry based on multiple wavelets 53, 54. They postulated that although fibrillation may be initiated by ectopic foci or rotors it could also degenerate into a pattern where “wavelets” were constantly being created by fractionation or annihilated by collision as they interacted with each other and anatomical obstacles.

In a series of experiments on rabbit atria in the 1970s, Allessie et al. proposed the “leading circle” mechanism to explain how single or multiple rotors could be maintained even in the absence of anatomical obstacles 55-57. They described the creation of a reentrant rotor with a wavelength the size of the shortest physiologically possible pathlength. This leading circle spawns centrifugal waves which activate the rest of the atria as well as centripetal waves that travel towards the center of the circle and annihilate each other (making the core of the circle constantly refractory and non-excitabile). Because the wavelength of the rotor is the size of the pathlength there is no excitable gap, in other words the leading edge of the rotor circles and excites its own tail as soon as the refractory period is over. This prevents disruption of the wave from a different source, and ensures the propagation of the rotor for another cycle. This model of reentry is termed “functional reentry” as opposed to “anatomical reentry”.

The leading circle model of reentry is consistent with much of the data observed concerning AF and its response to AADs and surgical treatment. Recently however, the
concept has been challenged by a much more complex model called “spiral wave reentry”. This model was developed from observations made in chemical reactions as well as mathematical projections. Although the model does not lend itself as quickly to physiological concepts as the leading circle model, it does deal with certain factors leading circle does not. These include wave shape and its relationship to conduction velocity, and the fact that excitable cores and excitable gaps have been observed in AF, both of which are not possible in the leading circle model. Leading circle theory also predicts that Na\(^+\) channel blockers should promote AF because they reduce conduction velocity; spiral wave theory suggests Na\(^+\) blockers may be antiarrhythmic, a prediction which is borne out by some experimental data. A good review of the differences between leading circle and spiral wave theories was written by Comtois et al. in 2005.

Although the precise mechanism by which fibrillatory activity is maintained is potentially complex, it is nevertheless important to understand as it will have implications for how the arrhythmia can be terminated. In general, factors which increase pathlength (like atrial enlargement), or reduce wavelength, may encourage AF by stabilizing reentry. Factors which promote heterogeneous changes in tissue conduction or create obstacles (like fibrosis) will also promote AF by promoting wave block, fractionation and reentry.

**Electrical remodeling as a cause of AF**

Rapid atrial rates brought on by atrial tachycardia, or AF itself, cause changes in tissue conduction properties. This electrical remodeling promotes further AF, and further electrical remodeling, and is an important reason why paroxysms of AF often lead to persistent AF. Maladaptive arrhythmogenic changes include decreased AERP
and decreased conduction velocity among others. Decreased AERP is arrhythmogenic since it results in decreased wavelength. Rapid atrial rates shorten AERP $^{64, 65}$ possibly due to downregulation of $I_{Ca}$ L-type channels occurring in response to elevated intracellular Ca$^{2+}$ levels $^{66}$. Other contributors to AERP shortening include acetylcholine, which promotes the function of the $I_{K(Ach)}$ current, and Ca$^{2+}$ channel blockers, which reduce $I_{Ca}$ $^{67}$. Similarly, decreased conduction velocity also decreases wavelength. Rapid atrial rates slow conduction $^{68}$ potentially because of downregulation of Na$^{+}$ channels $^{66}$ or gap junction proteins (specifically connexin 40) $^{69}$, data from human studies however are conflicting $^{70}$. Other factors such as loss of rate adaptation (potentially via inhibition/downregulation of $I_{to}$) $^{66, 71}$ and depression of SA node function $^{68}$ (both caused by rapid atrial rates) may also be important pro-fibrillatory mechanisms.

Although atrial stretch has been shown to cause AERP shortening and possibly produce afterdepolarizations $^{72, 73}$, electrical remodeling is not considered to be a direct result of stretch.

**Structural remodeling as a cause of AF**

Structural remodeling involves changes to atrial tissue structure, dimensions and contractility; the latter is sometimes separated into its own category called “mechanical remodeling”. As with electrical remodeling, structural remodeling can be a cause and/or consequence of AF. Most clinical data on AF pathology come from humans already in AF. As such, it is difficult to demonstrate conclusively that remodeling causes AF in humans, as opposed to simply being the result. Nevertheless, in combination with results from animal models, there are compelling data in support of structural remodeling as a cause of AF.
The process by which a mechanical insult, like stretch, results in structural remodeling is complex. Atrial tissue releases chemical messengers in response to stretch. These may include reactive oxygen species (ROS, which generate oxidative stress), cytokines (intercellular messenger proteins), chemokines (cytokines which can induce migration), and complement system proteins (members of the immune system) which activate the inflammatory response. Inflammation can be defined as the reaction of a living vascularized tissue to localized damage, involving increased local blood flow, and activation and migration of inflammatory cells (leukocytes) into the site of damage \(^{74}\). Damaged cells undergo apoptosis (programmed cell death) and leukocytes clear away dead or dying cells by phagocytosis (engulfment). Chemical messengers (cytokines, especially growth factors) promote the resolution of inflammation, hypertrophy of the remaining myocytes (muscle cells), and fibrosis. Hypertrophy describes cell growth and the production of new sarcomeres (intracellular contractile elements) \(^{75}\). Fibrosis involves the recruitment and maturation of fibroblasts into myofibroblasts which will deposit collagen either in extracellular spaces where there was no collagen before (reactive fibrosis), or in place of dead myocytes (reparative fibrosis) \(^{76}\). The result of this process is larger atria, with larger cells, with greater contractile strength, supported by a thicker collagen matrix than before. These changes are initially beneficial in that they enable the tissue to better withstand the force of stretch. However, as they progress, they become maladaptive and arrhythmogenic.

Each of the major steps in the process, whether the result of stretch or not, may promote AF. They are examined individually below:

**Oxidative stress, inflammation and cell death**

Oxidative stress has been linked with AF in several studies \(^{77-79}\). Mihm et al. showed an increase in protein oxidation (peroxynitrate formation) and hydroxyl radical
damage in patients with AF in 2001. Around the same time, Carnes et al. showed that the anti-oxidant vitamin C could reduce the incidence of AF post coronary artery bypass graft (CABG) surgery. It has been suggested that Ca$^{2+}$ overload leads to damage of mitochondrial DNA resulting in mitochondrial production of ROS. The resulting oxidative damage could affect structural or signaling proteins leading to AF vulnerability. However, the protective effects of anti-oxidant vitamins (C and E) on AF vulnerability (as well as AERP) could not be replicated in a dog model of AF.

Inflammation has long been associated with AF in both human and animal studies, and myocarditis has been detected in over 60% of AF patients in some instances. C-reactive protein (CRP) and high sensitivity CRP (hsCRP) are both markers of inflammation and elevated levels of both have been found to correlate with AF incidence and persistence in patients. CRP levels have been shown to have an inverse relationship with success of cardioversion; hsCRP levels have been shown to correlate with future incidence of AF in paroxysmal AF patients undergoing ablation. Interleukin 6 (IL6) is another marker of inflammation that has been shown to correlate with AF incidence in cardiac surgery patients. However, inflammation in the absence of any concomitant heart disease does not appear to be sufficient to induce AF in some studies.

Evidence for, and against, increased cell death in AF appears in the literature. Frustaci et al. observed increased atrial cell death near areas of inflammation in patients with lone AF. Similarly, Aime-Sempe et al. observed apoptosis in the atria of AF patients with valvular or coronary artery disease (CAD). Both Li et al. and Cardin et al. observed increased cell death in the same dog model of AF. However, several other groups have not observed cell death in patients or animal models with AF despite similar experimental methods being used. Although other signs of structural
remodeling were evident there was no sign of cell death in a clinical study involving 
CABG patients. Another clinical study involving persistent AF patients with mitral 
valve disease interestingly showed an increase in cell viability molecules. Results 
from two studies in a goat AF model also did not show any signs of atrial cell death. It 
was hypothesized by these investigators that AF may induce atrial myocyte 
differentiation, rather than death, as a means of survival. The reasons for the 
differences between these studies are not clear, but may revolve around differences in 
patient selection (animal model) or type of AF (persistent or paroxysmal or vulnerable 
substrate).

**Atrial enlargement and hypertrophy**

Larger atria can support more reentrant rotors or wavelets thus increasing AF 
persistence. Atrial enlargement has been linked to AF incidence and persistence in 
multiple human and animal studies. Indeed, specifically LA enlargement has 
been shown to be a significant risk factor in the development of AF, AF persistence, and 
risk of refibrillation.

Similarly, myocyte hypertrophy is linked to AF incidence and persistence. Increases in myocyte size approaching 200% have been observed in fibrillating atria. Hypertrophied cells show increased electrical uncoupling and conduction abnormalities which promote reentry.

**Fibrosis**

Increased levels of collagen or other extracellular matrix (ECM) proteins have 
been shown in humans with AF (both persistent and paroxysmal). Fibrosis has 
been observed in several animal models of AF and has been linked with conduction 
heterogeneity and a substrate vulnerable to reentrant arrhythmias. There has also been some suggestion that fibrosis could result in the production of
ectopic foci $^{99}$; however the mechanism behind this is unclear. Fibrosis is sufficient to maintain AF even in the absence of any other factors. Two groups have showed in a dog heart failure model, that even after heart failure was resolved, the residual fibrosis still caused conduction abnormalities and AF inducibility $^{94,100}$. Similarly, Verheule et al. showed in a transgenic mouse model that fibrosis alone could induce AF $^{101}$. Of all components of structural remodeling, fibrosis is usually the last to appear and is not usually reversible $^{87,94}$.

**Mechanotransduction**

Mechanotransduction is the process through which a mechanical stimulus, like stretch, results in a cellular response (like fibrosis for example). The exact molecular mechanisms behind this are convoluted and not well understood. Nevertheless, an effort will be made here to outline some of the key molecules and pathways that may be involved. First of all, it is important to note that multiple cell types are involved in this remodeling process; inflammatory cells, mast cells, fibroblasts and myocytes are examples. The interplay between autocrine, paracrine, and even endocrine signaling between these cells adds to the complexity of the process.

The force of stretch can be transduced by the plasma membrane, cytoskeleton, or ECM leading to changes in membrane permeability (and possibly electrical consequences) and activation or release of signaling molecules. Signaling molecules activate downstream effectors resulting in structural consequences via altered gene expression (mRNA production) and subsequent protein production. Major classes of molecule involved include: transcription factors (which promote transcription of DNA into mRNA), phospholipases (which cleave membrane phospholipids or activate other signaling molecules), protein kinases (which phosphorylate other molecules), and
mitogen activated protein kinases (MAPKs, which affect the function of transcription factors). Generally, stretch results in the release of pro-remodeling factors like angiotensin II (AngII), endothelin 1 (ET-1), growth factors like epidermal growth factor (EGF), insulin-like growth factor (IGF), transforming growth factor β (TGF-β), and hormones like atrial natriuretic peptide (ANP). A whole host of intracellular remodeling related proteins are also involved such as the Bcl-2 and caspase families (apoptosis regulating proteins), and the mothers against decapentaplegic homolog (SMAD), matrix metalloproteinase (MMP) and tissue inhibitors of metalloproteinases (TIMP) families (all fibrosis regulating proteins), among many others.

The precise way in which a myocyte senses and responds to stretch is not known, but some of the most common hypotheses are presented here (see Figure 2).

**Stretch activated ion channels**

Stretch activated ion channels (SACs) can be permeable to Na\(^+\), K\(^+\) or Ca\(^{2+}\). Their ability to sense stretch may come from integration with the cytoskeleton \(^{102}\). The resulting increase in intracellular Ca\(^{2+}\) (either through direct entrance of Ca\(^{2+}\) or extrusion of Na\(^+\) by NCX) leads to activation of protein kinase C (PKC) by diacylglycerol (DAG), and calcineurin by calmodulin \(^{103}\). Activation of PKC and calcineurin results in the activation of several MAPKs including extracellular signal related kinase (ERK) \(^{104}\) and the transcription factor, nuclear factor of activated T cells (NFAT) \(^{105}\). The first genes to be transcribed after the initiation of stretch are usually c-Fos, c-Myc, c-Jun, and Egr-1, the so called “immediate-early” genes \(^{102}\). These transcription factors promote transcription of other genes which help the cell respond to stress (usually by promoting hypertrophy). MAPKs have been shown to promote expression of immediate-early genes \(^{75,102,106,107}\).
Figure 2 – Mechanisms of mechanotransduction

Potential pathways activated by stretch ultimately resulting in structural remodeling (oxidative stress, inflammation, cell death, hypertrophy, fibrosis). Lines ending in a solid, black triangle indicate the former activates the latter. Lines ending in a solid black circle indicate the former inhibits the latter. Lines leading to an open, black diamond indicate the former have multiple effects. See text for details.
SAC related Ca\(^{2+}\) has also been shown to promote exocytosis of vesicles from myocytes \(^{108}\). AngII, ET-1, and various growth factors have been found to be secreted from myocytes under stress \(^{102}\). The release of these molecules can trigger remodeling responses in an autocrine and paracrine fashion.

**Stretch induced plasma membrane permeability**

Phospholipases can be activated as soon as 1 minute after initiating stretch; AngII release from myocytes has been detected within 5-10 minutes \(^{102}\). This rapid response suggests a very simple mechanism for activation or release in response to stretch. This could be related to SACs, but there is evidence for a non-Ca\(^{2+}\) related mechanism. Phospholipases and secretory vesicles are associated with the plasma membrane, and stretch increases the distance between phospholipids in the plasma membrane. Theoretically, this space could allow unknown factors access through the membrane and encourage phospholipase activation or vesicle exocytosis \(^{102}\). Phospholipases can have multiple actions; as an example, phospholipase A (PLA) can cleave fatty acids from the plasma membrane freeing them to serve as substrates in various reactions. Pro-inflammatory eicosanoids can be produced via fatty acid interaction with cyclooxygenase (COX) enzymes \(^{109}\). This is discussed in more detail later.

**G protein coupled receptor activation**

G protein coupled receptors (GPCRs) are transmembrane receptors which are associated with small proteins (G proteins) which dissociate upon ligand binding and activate downstream signaling pathways. GPCRs, like the AngII receptor (Ang2R1) and ET-1 receptor (ET1R), consist of the transmembrane receptor, a G\(_{\alpha}\), G\(_{\beta}\) and G\(_{\gamma}\) subunit. GPCR agonists (AngII, ET-1) are released in response to stretch and bind to their receptor causing the G\(_{\alpha}\) subunit to dissociate and activate phospholipase C (PLC). PLC
catalyzes the conversion of phosphatidylinositol 4,5 bisphosphate (PIP2) into inositol 1,4,5-triphosphate (IP3) and DAG \textsuperscript{103, 110}. IP3 stimulates release of Ca\textsuperscript{2+} from the sarcoplasmic reticulum (SR). As above, Ca\textsuperscript{2+} leads to activation of PKC (via DAG), and calcineurin (via calmodulin). PKC with calcineurin can activate the MAPKs ERK, c-Jun NH\textsubscript{2}-terminal protein kinase (JNK), and p38 by phosphorylating them \textsuperscript{104}. PKC can also stimulate the phosphorylation of ERK by activating Ras, an upstream activator of ERK \textsuperscript{107}. Activated MAPKs can translocate from the cytoplasm to the nucleus and promote the activity of transcription factors \textsuperscript{106, 111}. ERK can also stimulate PLA activity \textsuperscript{112} and activate mammalian target of rapamycin (mTOR). mTOR promotes both ribosomal production and protein synthesis; activation of mTOR may be a key step in the generation of hypertrophic proteins \textsuperscript{75, 103}. Stimulation of the Ang2R1 has also been shown to result in production of ROS via nicotinamide adenine dinucleotide phosphate (NADPH) oxidase \textsuperscript{113}. The precise sequence of events is unclear, however it appears that ROS may be an important contributor to downstream AngII mediated signaling (perhaps by activating PKC) \textsuperscript{114, 115}.

AngII is one of the best studied, and one of the most important, cardiac signaling molecules. It is produced extracellularly by conversion of angiotensinogen to angiotensin I by renin (in the liver), which is subsequently converted to AngII by angiotensin converting enzyme (ACE) (locally). This is the foundation of the renin angiotensin system (RAS). As mentioned above, stretch may stimulate release of intracellular AngII which may be produced in a non-renin, non-ACE dependant manner \textsuperscript{116}. Stretch has been shown to stimulate local ACE production as well \textsuperscript{117}, therefore it is difficult to tell whether circulating or intracellular AngII is more responsible for the activation of the Ang2R1 in stretch signaling \textsuperscript{116}. Nevertheless, AngII and the RAS are of great importance in stretch related remodeling.
**Receptor tyrosine kinase activation**

Receptor tyrosine kinases (RTKs), named for their ability to phosphorylate substrates at tyrosine residues, are another class of transmembrane receptor involved in stretch related signaling. Typically, ligands are growth factors like EGF, IGF, fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF) or platelet derived growth factor (PDGF). Upon ligand binding, RTKs undergo a conformational change that creates a binding site for so called “docking” or “adaptor” proteins. These proteins function to create binding sites or suitable conformations which allow further protein interactions. One such docking protein is Grb2 which is often coupled to a Sos protein. Sos is a guanine exchange factor (GEF) which aids other proteins in exchanging an associated molecule of guanine diphosphate (GDP) for a guanine triphosphate (GTP). When the Grb2+Sos unit binds to the RTK, it moves Sos into proximity with Ras. Sos facilitates the activation of Ras through its GEF functionality; Ras is ligated to GDP in its inactive state, but becomes active when ligated to GTP. Activated Ras leads to the activation of ERK via the Ras-Raf-MEK-ERK pathway. Activated Ras can also activate phosphoinositide 3-kinase (PI3K), and the janus kinase (JAK) signal transducers and activators of transcription (STAT) pathway. Transactivation of RTKs by GPCRs can also occur; GPCR activation of PLC leads to activation of a disintegrin and metalloprotease (ADAM) which frees membrane associated EGF, allowing it to activate the RTK receptor.

**TGF-β receptor activation**

TGF-β is a powerful remodeling factor and is common in its inactive form both in extra- and intracellular spaces. Activation of TGF-β can occur in response to a plethora of stimuli: ROS (H₂O₂, superoxide), MMPs, changes in pH, etc. Once in active form, binding of TGF-β to its receptor strongly promotes hypertrophy and fibrosis.
through activation of ERK (via Ras) and other MAPKs (via TGF-β activated kinase [TAK]), and the Smad transcription factors. 

TGF-β has a multitude of other effects and its precise action may depend on its concentration and environment. Generally, it is thought to aid the tissue in transitioning from an inflammatory state into a more hypertrophic/fibrotic state. It inhibits the activity of collagenases like MMPs, and promotes the activity of TIMPs (inhibitors of MMPs). TGF-β production is upregulated by AngII stimulation; this is considered to be one of the most important ways in which AngII stimulation results in structural remodeling.

**Integrin signaling**

Integrins are membrane proteins composed of 2 subunits, an α extracellular subunit and a β cytoplasmic subunit. Where the α subunit interacts with ECM proteins (collagen, fibronectin, vitronectin) is called a “focal adhesion”. The interaction generates signals which are transmitted to the β subunit, which can trigger multiple intracellular signaling pathways. Focal adhesions involve large, dense collections of proteins assembled around the integrin including kinases (FAK, paxillin), docking proteins (Fyn, Shc, p130), cytoskeletal binding proteins (talin, vinculin, α actinin), and others (Src, Grb2, Sos, Ras, PI3K, etc.) Signaling can be complex, reticulated, and at times redundant. Probably the most significant protein involved in integrin signaling is the tyrosine kinase, focal adhesion kinase (FAK). FAK has 4 or more locations at which it can be phosphorylated; phosphorylation at different sites may yield different results. FAK can be phosphorylated by the β subunit itself, Src, or even chemical signals (AngII, growth factors, etc.). Activation of FAK leads to the creation of a Grb2+Sos binding site. As above, the Grb2+Sos complex activates Ras which results in ERK
activation and PI3K activation. PI3K is an upstream activator of protein kinase B (Akt), a multipotent remodeling protein.

Akt phosphorylates Bad, a Bcl-2 family protein, trapping it in the cytoplasm and preventing it from performing its pro-apoptotic function. Similarly, Akt phosphorylates FOXO transcription factors trapping them in the cytoplasm and preventing them from transcribing pro-apoptotic genes. Nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) is a transcription factor that transcribes inflammatory and proliferative genes. IkB is usually bound to NFκB and traps it in the cytoplasm; Akt inhibits IkB thus allowing NFκB to translocate to the nucleus. Akt phosphorylates CREB, a transcription factor, allowing it to translocate to the nucleus. Akt also phosphorylates glycogen synthase kinase 3 (GSK3), inhibiting it. GSK3 inhibits multiple hypertrophic transcription factors, thus inhibition of GSK3 is strongly hypertrophic. Akt activates mTOR as well.

Overall, it can be seen that stretch leads to the release of signaling molecules like AngII and EGF which activate factors like ERK, Akt and TGF-β, leading to inflammation, hypertrophy and fibrosis (structural remodeling).

**Anti-structural remodeling agents as a treatment for AF**

Seeing that structural remodeling is an important contributor in the pathogenesis of AF, it is reasonable that agents which prevent structural remodeling may also prevent AF. Statins have been used successfully to treat AF in animals and humans. Statins are well known to have anti-inflammatory properties, but may also have anti-oxidant properties as well. The importance of the RAS system in structural remodeling has been outlined above. Agents which inhibit the RAS system, such as angiotensin converting enzyme inhibitors (ACEi), angiotensin receptor blockers (ARBs),
and matrix metalloproteinase inhibitors (MMPi), have been shown to prevent the development/maintenance of AF in both animal \textsuperscript{122-124} and human studies (TRACE \textsuperscript{125}, SOLVD \textsuperscript{126}, LIFE \textsuperscript{127}, CHARM \textsuperscript{128}). They have been shown to prevent, or even reverse, arrhythmogenic substrate development, specifically fibrosis \textsuperscript{122-124}. Given the prevalence of structural remodeling related AF, the ineffectiveness of AADs, and the complication of surgery, anti-structural remodeling agents represent an effective new tool in the treatment of AF. Discovery and development of new anti-structural remodeling therapies should be a priority in AF research.

\textbf{Modeling AF experimentally}

As seen from the above, AF is a complex disease with potentially multiple etiologies and multiple mechanisms of persistence. Studies have shown that AF patterns can be different depending on the underlying disease \textsuperscript{129}; as such, no single experimental model is perfectly suitable to study AF. For this reason, multiple experimental models have been devised. Many models use an \textit{in vitro} or Langendorff approach to study isolated cardiomyocytes or whole hearts. Although they are very useful in advancing our understanding of the mechanisms and signaling pathways involved in AF, these models suffer from the limitation of physiological relevance to intact organisms. There are however, a multitude of \textit{in vivo} animal models that have been developed, each with the intent of studying a particular clinical AF pathology (see Table 1).

Increased parasympathetic tone (cholinergic stimulation) can cause AF; the vagal stimulation model produces reliable increases in AF vulnerability \textsuperscript{130}. A sterile pericarditis model has been created to study cardiac surgery induced AF \textsuperscript{131, 132}. AF resulting from toxin exposure has been modeled; aconitine \textsuperscript{133} and cesium \textsuperscript{134} are
<table>
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<td>Congestive heart failure</td>
<td>Rapid ventricular pacing</td>
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**Transgenic models**

| Fibrosis                           | TGF-β overexpression           | Verheule 2004                  |
| Inflammation                       | TNF-α overexpression           | Saba 2005                      |
| RAS activation                     | ACE overexpression             | Xiao 2004                      |
| Conduction dysfunction             | Connexin 40 knockout           | Verheule 1999                  |
| Hypertension                       | Spontaneous hypertension       | Choisy 2007                    |
| Increased parasympathetic tone     | RGS knockout                   | Cifelli 2008                   |

LA = left atrium, TGF = transforming growth factor, TNF = tumor necrosis factor, RAS = renin angiotensin system ACE = angiotensin converting enzyme, RGS = regulators of G protein signaling
examples. Transgenic mouse models have also been developed to demonstrate the role of specific molecules in promoting AF and AF vulnerability: TGF-β overexpression\(^{101}\), tumor necrosis factor alpha (TNF-α) overexpression\(^{135}\), ACE overexpression\(^{136}\), connexin 40 knockout\(^{137}\), and regulators of G protein signaling (RGS) knockout\(^{138}\), among others.

AF induced by atrial tachycardia or AF related remodeling has been mimicked by several rapid atrial pacing models in sheep\(^{43, 89}\), dogs\(^{64, 66, 139, 140}\), and pigs\(^{97}\). Electrical changes develop quickly (30 minutes\(^{139}\)) and all models generally show AERP shortening and heterogeneity, contractile dysfunction, mitochondrial damage, and ion channel remodeling, along with increased AF inducibility. Faster rates (>400 bpm) and/or longer pacing durations (>6 weeks)\(^{43, 89, 97}\) tend to result in slight hypertrophy and fibrosis. These changes are all usually reversible upon cessation of pacing indicating an electrical remodeling based mechanism\(^{43}\).

**Models of structural remodeling induced AF**

Cornary artery ligation is a model of myocardial infarction\(^{141, 142}\) which produces AF inducibility via AERP prolongation, atrial conduction slowing, necrosis, and fibrosis. Mitral valve avulsion results in mitral regurgitation and exhibits increased AF vulnerability with AERP prolongation, LA enlargement, chronic inflammation, and fibrosis\(^{86}\). AF induced by cardiac shunt has been modeled by installation of an aorta-LA shunt\(^{143}\) or aorta-pulmonary artery shunt\(^{144}\). Shunting results in LA enlargement, cellular hypertrophy, and fibrosis. AV block has been shown to result in AF, due to atrial contraction against closed AV valves, and has been modeled by His bundle ablation\(^{145}\). Chronic AV block causes AF vulnerability, atrial enlargement, but no fibrosis. Hypertension has been modeled in sheep by *in utero* corticosteroid exposure (resulting in increasing hypertension with age)\(^{146}\). This model created atrial conduction
abnormalities, LA enlargement, and fibrosis, along with AF inducibility. Transgenic spontaneously hypertensive rats have also been studied; they show AF inducibility with increased fibrosis. Although these models provide valuable insights, ligation, avulsion, shunting, and ablation represent severe insults to the myocardium which may obfuscate AF pathology by creating non-physiological stresses.

Perhaps the best known structural remodeling based model of AF is the rapid ventricular pacing (RVP) model created to mimic CHF. It has been implemented in sheep and dogs. Results differ somewhat between the models; AERP increased in sheep, but not in dogs, atrial conduction slowed in dogs, but not in sheep. Nevertheless, all studies showed increases in AF inducibility, atrial enlargement and fibrosis. As a model of LV damage and CHF, the animals develop severe pulmonary edema, ascites, and sometimes die before significant AF is inducible. AF and CHF have an important relationship in that as many as 50% of patients with severe heart failure will develop AF, however perhaps as few as 15% of all AF patients have CHF. Thus a model involving structural remodeling without overt heart failure may apply to more clinical situations, and may suffer less from complicating factors like ventricular failure, pulmonary distress and premature mortality.

**Simultaneous atrial and ventricular pacing (a model of atrial stretch)**

The SAVP model was developed in dogs by the Dorian lab as a model of stretch induced structural remodeling leading to AF. The design of SAVP was based on the observation that atrioventricular nodal reentry tachycardias produce profound increases in atrial pressure (leading to “neck pounding” and “shirt flapping” in humans) due to the simultaneous contraction of the atria and ventricle. Thus it was hypothesized that SAVP would produce more severe LA remodeling without severe LV damage.
In a recent study, SAVP at 220 bpm for 2 weeks was shown to result in severe LA remodeling, only moderate LV remodeling, with no mortality, and only mild ascites in less than 50% of the animals. Sustained AF was induced in 83% of dogs via burst pacing, and single extra-stimulus pacing induced AF in 50% of dogs. There was conduction slowing, heterogeneity, and increased fibrosis. Plasma ANP doubled (22 to 46 pg/mL), and brain natriuretic peptide (BNP) tripled (7 to 24 pg/mL) indicating some degree of ventricular dysfunction.

In a head-to-head comparison of SAVP and RVP, SAVP was shown to cause an immediate, significant increase in peak pulmonary capillary wedge pressure (surrogate of LA end diastolic pressure) compared to RVP (19 vs. 15 mmHg). SAVP generated significantly more systolic PV flow reversal and reverse flow velocity than RVP (>800 vs. <300 cm/sec). After 2 weeks, LA systolic area increased significantly more with SAVP than RVP (88% vs. 77%). Additionally, LA fractional area shortening (a measure of LA function) decreased significantly more with SAVP than RVP (>45% vs. 26%), whereas LV fractional area shortening decreased significantly less with SAVP than RVP (~26% vs. 55%). This indicates a greater degree of LA dysfunction, but a lesser degree of LV dysfunction with SAVP as compared to RVP. There was also greater inducibility, maintenance, and persistence of AF with SAVP as compared to RVP. All RVP dogs developed pulmonary edema, half developed ascites, and one third died before completing the study. No SAVP dogs developed pulmonary edema, less than half developed ascites, and none died during the study.

Overall, this suggests that SAVP is an effective model of primarily atrial stretch related cardiomyopathy and AF, without overt ventricular failure, arguably making it relevant to more clinically seen AF than any other model.
Section Two: Omega-3 polyunsaturated fatty acids

Background

Omega 3 polyunsaturated fatty acids (n3 PUFAs) have recently emerged as natural, multipotent treatments for a wide variety of disease conditions. Interest in using PUFAs, specifically the marine derived n3 PUFAs eicosapentaenoic acid (EPA), 20:5(n3) and docosahexaenoic acid (DHA), 22:6(n3), to treat heart disease has grown exponentially since it was first proposed in the 1970s.

Fatty acids are essential biological molecules with a carboxylic acid head and an unbranched carbon chain tail that can be saturated (no double bonds), monounsaturated (having a single double bond), or polyunsaturated (having multiple double bonds). PUFAs are classified based on the position of the first double bond relative to the methyl group on the end (called the “omega” or “n” carbon); n3s have the first double bond on the third carbon, n6s have the first double bond on the sixth carbon, and so on (see Figure 3). Fatty acids are ubiquitous in the body being key components of cell membranes, hormones, energy stores, and helping to regulate blood pressure, inflammation, platelet aggregation, and even mood.

Major sources of EPA and DHA are marine algae, fatty cold water fish like salmon, mackerel, sardine, tuna, and herring. Plants, including flax and nuts, are sources of another n3 PUFA, α linolenic acid (ALA), 18:3(n3). Sources of n6 PUFAs are plants like corn, safflower, and sunflower. Levels of fats are concentrated in animals that consume these food sources, thus animal meats can have different compositions depending on whether they are corn fed, fish fed, farmed, wild, etc. Western diets have changed over the last century and consumption of n6 PUFAs has increased dramatically, while consumption of n3 PUFAs has declined. Thus the ratio of n3s/n6s in the diet has fallen precipitously from an estimated 1:5 in ancient times, to
Figure 3 – Fatty acid structures
as low as 1:50 today, even as total fat consumption has increased significantly\textsuperscript{152, 155}. The widespread use of cooking oils like corn oil, sunflower oil, and the cultivation of corn fed cattle has contributed to this change.

**PUFA biochemistry**

Humans have no ability to manufacture ALA or the n6 PUFA linoleic acid (LA), 18:2(n6). As such, they are termed “essential” fatty acids, meaning they must be supplied in the diet\textsuperscript{156}. Humans have limited ability to convert ALA into EPA and DHA, or LA into arachidonic acid (AA), 20:4(n6), thus they are not essential in the strictest sense\textsuperscript{157}. Both ALA and LA require elongase, $\Delta5$, and $\Delta6$ desaturase enzymes in order to be converted into their long chain derivatives (see Figure 4)\textsuperscript{155}. Levels of conversion vary greatly between studies, but on average <10% of ALA is converted to EPA with an efficiency around 0.2%; similarly, <5% of ALA is converted to DHA with an efficiency around 0.05%\textsuperscript{158}. Conversion of LA to AA is generally more efficient, and AA levels vastly exceed EPA and DHA levels in most human tissue\textsuperscript{159, 160}. This could be the result of high intakes of LA and the monopolization of available enzymatic machinery by LA, leading to low rates of successful conversion of ALA to EPA and DHA\textsuperscript{157}. Whatever the reason, therapeutic levels of EPA and DHA for treatment of any disease condition can rarely be reached without dietary intake or artificial supplementation.

Fatty acids are absorbed in the intestines and shuttled around the body in protein containing structures called lipoproteins (chylomicrons, high density lipoproteins [HDL], low density lipoproteins [LDL], etc.). Fats usually exist in the body esterified to glycerol in triglycerides (containing 3 fatty acids), in phospholipids (containing 2 fatty acids and a phosphate group), or cholesterol esters (containing 1 fatty acid and a cholesterol molecule). Fat stored in adipose tissue is typically in triglyceride form; cell membranes
N3 and n6 fatty acids compete for the same elongase and desaturase enzymes in order to produce longer chain products. N6 fatty acid conversion is depicted on the left, n3 fatty acid conversion is depicted on the right. AA = arachidonic acid, EPA = eicosapentaenoic acid, DHA = docosahexaenoic acid.
are composed of phospholipids. Unesterified fats can also exist individually bound to serum albumin and are part of the “free fatty acid” (FFA) pool. The FFA pool also includes fatty acids from triglyceride breakdown as well as fatty acids cleaved from phospholipids by phospholipases (PLA for example) which can be metabolically active.

**PUFA metabolism**

N3 or n6 PUFAs cleaved from the plasma membrane by PLA can be metabolized into biologically active products called eicosanoids (see Figure 5). Using the same set of enzymes, different PUFAs produce metabolites with different properties. AA can be metabolized by COX enzymes to produce prostaglandins (PG) of the 2-series and thromboxanes (TX) of the 2-series; EPA is metabolized by COX to produce 3-series PGs and 3-series TXs. In inflammatory cells, AA can be metabolized by lipoxygenase enzymes to produce leukotrienes (LT) of the 4-series; EPA can be metabolized by lipoxygenases to produce 5-series LTs. In general, AA metabolism proceeds with much greater efficiency than EPA metabolism. An increase in the proportion of n3 PUFAs in the plasma membrane usually means a decrease in the proportion of n6 PUFAs, and an increase in the likelihood of EPA being cleaved for metabolism, therefore reducing overall eicosanoid synthesis.

AA derived TXs like TXA$_2$ are pro-thrombotic vasoconstrictors; EPA derived TXs like TXA$_3$ are also pro-thrombotic vasoconstrictors, but are far weaker. Similarly, AA derived LTs like LTB$_4$ have powerful pro-inflammatory, vasoconstricting properties; EPA derived LTs like LTB$_5$ have similar actions, but are less potent. In contrast, both AA and EPA derived PGs (PGI$_2$, PGI$_3$ respectively) are similarly anti-thrombotic and vasodilatory. Nevertheless, in general, AA derived eicosanoids tend to promote thrombosis, inflammation, and vasoconstriction. The result of EPA metabolism tends to be less thrombotic, less inflammatory, and vasodilatory. This is due both to the reduced
Figure 5 – Fatty acid metabolism
Fatty acids can be metabolized by the same enzymes, but generate products with varying properties.
Fatty acids are: AA = arachidonic acid, EPA = eicosapentaenoic acid, DHA = docosahexaenoic acid
Enzymes are: EPOX = cytochrome p450 epoxygenase, COX = cyclooxygenase, LOX = lipoxygenase
Products are: EET = epoxyeicosatrienoic acid, PG = prostaglandin, TX = thromboxane, LT = leukotriene
potency of EPA derived eicosanoids and reduced efficiency of EPA metabolism. Both EPA and DHA can be metabolized by COX and then lipoxygenase enzymes into products termed “resolvins” and “protectins”. These compounds have only recently been discovered, but reportedly have powerful anti-inflammatory and pro-resolution properties. AA can also be metabolized into epoxyeicosatrienoic acids (EETs) by cytochrome p450 epoxygenase enzymes; EETs have various anti-inflammatory and vasorelaxation properties.

N3 and n6 PUFAs (as well as their eicosanoids) are also substrates for the peroxisome proliferator-activated receptors (PPAR). Upon ligand binding, these nuclear receptors activate transcription factors which transcribe genes related to fatty acid metabolism: triglyceride production, β-oxidation (metabolizing fats for energy), etc. N3 PUFAs reduce triglyceride synthesis by downregulating triglyceride assembly proteins and promote β-oxidation by upregulating acyl-coenzyme A oxidase, a key enzyme in the β-oxidation pathway.

**N3 PUFAs as treatment for disease**

In recent years, n3 PUFAs have been tested in numerous disease conditions and have been reported to have multiple mechanisms of action, many of which are related to structural remodeling. A review of approximately 1,000 publications released between 2004 and 2008 involving n3 PUFAs and either oxidative stress, inflammation, apoptosis, hypertrophy, or fibrosis revealed research into fields as diverse as cancer, cardiovascular disease (CVD), and mental health (see Figure 6). A broad range of, sometimes contradictory, effects have been reported including pro- and anti-inflammatory actions, pro- and anti-apoptotic actions, and pro- and anti-oxidant actions (see Figure 7). Work has been divided relatively equally between clinical studies, small
Figure 6 – Areas of n3 PUFA research
A review of ~1,000 recent publications found via PubMed database search using a search term encompassing n3 PUFAs and structural remodeling revealed research in the indicated areas and proportions. The “OTHER” category includes research into skin inflammation, periodontitis, lupus, spinal cord injury, traumatic brain injury, sepsis, fetal development, and maximum lifespan, among others.

Figure 7 – Published n3 PUFA effects in various disease conditions
A review of ~1,000 recent publications found via PubMed database search using a search term encompassing n3 PUFAs and structural remodeling revealed that n3 PUFAs have the indicated actions in various models and disease conditions.
Figure 8 – Models used in n3 PUFA research
A review of ~1,000 recent publications found via PubMed database search using a search term encompassing n3 PUFAs and structural remodeling revealed that research involving n3s has occurred in the indicated models and proportions. Large animal models include dog, pig, horse, monkey, rabbit, hamster, cow and gerbil.
animal studies and cell culture models; however, far less work has been done in large animal models (which may be more physiologically relevant than small animal or cell culture models) (see Figure 8). Although it should be noted that there may be some publication bias (negative results are published less often), these data do provide some insight as to the most commonly reported actions of n3 PUFAs.

N3 PUFAs have been shown to suppress tumour growth\textsuperscript{163}, prevent the onset of dementia\textsuperscript{164}, have anti-aging effects on skin\textsuperscript{165}, even reduce incidence of violent behaviour\textsuperscript{166,167}, among a whole host of other reported effects.

**N3 PUFAs and CVD**

Data from numerous clinical studies suggest that dietary fat intake is linked to the development of CVD\textsuperscript{168}. However, in the 1970s, Dyerberg and Bang published a series of studies showing that Greenland “Eskimos”, despite subsisting on a diet containing a great deal of whale, seal, and fish fat, had virtually no incidence of CVD\textsuperscript{169}. This was in contrast to observations in neighbouring Danish populations which had much higher incidence of CVD, but similar fat intakes (albeit from non-marine sources)\textsuperscript{169,170}. This led to the suggestion that the low CVD rates among Eskimos were the result of predominantly marine derived n3 PUFA intake and their putative anti-atherogenic/anti-thrombotic effect\textsuperscript{171}.

Since then, interest in using n3 PUFAs to treat CVD has exploded. A search on http://clinicaltrials.gov/ shows 109 active trials (as of June 2010) involving PUFAs and CVD. Searching PubMed (http://www.ncbi.nlm.nih.gov/sites/entrez) for publications in the last 10 years with a search term encompassing n3 PUFAs and cardiovascular research returned over 3,500 papers. These studies investigate manifold areas of CVD in diverse populations using multiple methodologies; understandably the results are not
always clear, and occasionally are conflicting. Tables 2 and 3 divide trials into observational and randomized controlled trial (RCT) categories. Below, the same trials are presented according to primary endpoint.

**Sudden cardiac death**

There have been large observational trials which have looked at fish intake and risk of sudden cardiac death (SCD). The Physicians’ Health Study followed over 20,000 men for 11 years and found that increasing fish intake was associated with reduced risk of SCD, but did not have any effect on MI or total CVD mortality\(^\text{172, 173}\). In contrast, a Japanese cohort study looked at over 40,000 middle aged subjects for 9 years and found that increasing fish intake was associated with an increased risk of SCD in people with the highest level of fish consumption despite the reductions in other conditions\(^\text{174}\).

The Gruppo Italiano per lo Studio della Sopravvivenza nell’Infarto Miocardico Prevenzione (GISSI) study was an RCT that randomized over 11,000 post-MI patients to either 1.0g/day n3 PUFAs, vitamin E, both, or none. After only 4 of the scheduled 12 months of follow up, the relative risk of SCD was reduced by n3 PUFAs (RR 0.47), as was total mortality\(^\text{175}\). Contrary to this is the DART-2 RCT which took over 3,000 men with angina and randomized them to receive dietary advice to increase fish consumption, increase fruit consumption, both, or neither. After between 3 and 9 years of follow up, significant increases were seen in the relative risk of SCD (RR 1.54) and CVD death (RR 1.26) in the group counseled to eat fish\(^\text{176}\).

**Coronary heart disease and MI**

Several large observational trials have looked at fish intake and risk of coronary heart disease (CHD) and MI with positive results. The Nurses’ Health Study followed approximately 85,000 women for 16 years and found that increasing fish intake was associated with reduced risk of CHD and non-fatal MI\(^\text{177}\). The same Japanese cohort
<table>
<thead>
<tr>
<th>Study</th>
<th>Subjects</th>
<th>Follow-up</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nurses' Health Study Hsu 2002</td>
<td>8468 healthy women</td>
<td>18 years</td>
<td>fish intake on RR of CHD: 1-3 months = 0.79, 1 week = 0.71, 2-4 week = 0.69, 5+ week = 0.65, fish intake 5+ week on RR of disease: CHD death = 0.65, non-fatal MI = 0.73</td>
</tr>
<tr>
<td>Health Professionals Follow-up Study Ascherio 1985</td>
<td>44985 healthy men</td>
<td>6 years</td>
<td>fish intake on RR of CHD: highest quintile vs. lowest quintile = 1.12, any fish consumption vs. no fish consumption = 0.74, increasing fish intake not associated with further decrease in CHD</td>
</tr>
<tr>
<td>The Japan Public Health Center-Based Cohort I Iso 2006</td>
<td>41570 healthy</td>
<td>9 years</td>
<td>fish intake on RR (between highest and lowest quintile) of disease: CHD = 0.63, MI = 0.44, SCD = 1.14</td>
</tr>
<tr>
<td>Physicians' Health Study Albert 1998</td>
<td>20551 healthy men</td>
<td>11 years</td>
<td>fish intake on RR of SCD: 1 week = 0.48, fish intake not associated with MI, non SCD, total CV mortality</td>
</tr>
<tr>
<td>Yuan 2001</td>
<td>16244 men</td>
<td>9 years</td>
<td>fish intake on RR (between highest and lowest quintile) of disease: MI = 0.41, total mortality = 0.79, fish intake not associated with stroke, other IHD</td>
</tr>
<tr>
<td>Physicians' Health Study (sub-analysis) Guallar 1996</td>
<td>14916 healthy men</td>
<td>5 years</td>
<td>fish intake not associated with MI incidence</td>
</tr>
<tr>
<td>Seven Countries Study (sub-analysis) Cornen 2000</td>
<td>2700 healthy men</td>
<td>20 years</td>
<td>fatty fish intake associated with ↓ CHD mortality (RR 0.56)</td>
</tr>
<tr>
<td>Chicago Western Electric Study (sub-analysis) Daviglus 1997</td>
<td>1822 healthy men</td>
<td>30 years</td>
<td>fish intake (36g/day) on RR of disease: CHD = 0.62, sudden MI = 0.4, non-sudden MI = 0.95</td>
</tr>
<tr>
<td>Campos 2008</td>
<td>1619 post MI</td>
<td>none</td>
<td>↑ adipose/serum ALA associated with ↓ MI (RR 0.41-0.61)</td>
</tr>
<tr>
<td>EURAMIC Guallar 1996</td>
<td>639 post MI</td>
<td>none</td>
<td>adipose DHA levels not associated with MI incidence</td>
</tr>
</tbody>
</table>
Table 2 – continued

<table>
<thead>
<tr>
<th>Study</th>
<th>Subjects</th>
<th>Follow up</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Multiple Risk Factor Intervention</td>
<td>94 men with CHD</td>
<td>none</td>
<td>↑ plasma DHA associated with ↓ CHD</td>
</tr>
<tr>
<td>Trial (sub-analysis) Simon 1995</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>RR = relative risk, CHD = coronary heart disease, MI = myocardial infarction, SCD = sudden cardiac death, CV = cardiovascular, IHD = ischemic heart disease, ALA = α-linolenic acid, DHA = docosahexaenoic acid</td>
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</tr>
</tbody>
</table>

Table 3 – Randomized controlled trials studying the effect of n3 PUFAs on cardiovascular disease

<table>
<thead>
<tr>
<th>Study</th>
<th>Subjects</th>
<th>Intervention</th>
<th>Follow up</th>
<th>Effect of n3 PUFAs</th>
</tr>
</thead>
<tbody>
<tr>
<td>JELIS Yskoyama 2007</td>
<td>10645 hypercholesteremic</td>
<td>1.0g/day EPA</td>
<td>6 years</td>
<td>18% ↓ major coronary events, ↓ unstable angina, non-fatal coronary events</td>
</tr>
<tr>
<td>GISSI Marchioli 2002</td>
<td>11323 post MI</td>
<td>1.0g/day n3</td>
<td>12 months</td>
<td>↓ total mortality (RR 0.69), SCD (RR 0.47) → total cholesterol</td>
</tr>
<tr>
<td>GISSI-HF Tavezzu 2008</td>
<td>7000 NYH II-IV</td>
<td>1.0g/day n3</td>
<td>4 years</td>
<td>↓ time to death or hospital admission for CV reason (RR 0.92), total mortality (RR 0.91)</td>
</tr>
<tr>
<td>DART-2 Bur 2003</td>
<td>3114 men with angina</td>
<td>fish diet</td>
<td>3-9 years</td>
<td>↑ CV death (RR 1.26), SCD (RR 1.54)</td>
</tr>
<tr>
<td>DART Bur 1989</td>
<td>2093 post MI men</td>
<td>fish diet</td>
<td>2 years</td>
<td>↓ total mortality (29%) → infarction, IHD death</td>
</tr>
<tr>
<td>Lyon Diet Heart Study de Lorenzo 1994</td>
<td>500 post MI</td>
<td>ALA diet</td>
<td>5 years</td>
<td>↓ coronary events, total mortality</td>
</tr>
</tbody>
</table>

R = randomized, PI = placebo controlled, BI = blinded, EPA = eicosapentaenoic acid, MI = myocardial infarction, RR = relative risk, SCD = sudden cardiac death, NYH = New York Heart Association, CV = cardiovascular, IHD = ischemic heart disease, ALA = α-linolenic acid, DHA = docosahexaenoic acid
study mentioned above noted a reduction in CHD and MI \(^ {174}\). A Chinese cohort study followed over 18,000 men for 9 years and noted a reduction in acute MI and total mortality with increasing fish intake, but no effect on stroke or other ischemic heart disease \(^ {178}\). A sub-analysis of the Seven Countries Study looked at the diets of 2,700 men and showed that fish intake was associated with a decreased risk of CHD mortality \(^ {179}\). A sub-analysis of the Chicago Western Electric Study followed just under 2,000 men for 30 years and found that fatty fish intake was associated with a reduced risk of CHD and sudden MI \(^ {180}\). A Costa Rican study looked at 1,819 post MI patients and 1,819 case matched controls, and determined that adipose or dietary ALA levels were associated with a reduced risk of MI \(^ {181}\). Finally, a small sub-analysis of the Multiple Risk Factor Intervention Trial analyzed data from 94 men with CHD and 94 controls, and found an inverse relationship between plasma DHA and CHD \(^ {182}\).

Some observational trials however have reported negative results. The EURAMIC study looked at DHA levels in the adipose tissue of 639 post MI patients and 700 case matched controls. The authors concluded that there was no association between DHA levels and risk of MI \(^ {183}\). The Health Professionals Follow-up Study included almost 45,000 healthy men and followed them for 6 years. Although they noticed that the risk of CHD decreased in men that consumed any amount of fish versus those that consumed no fish, they observed an increase in CHD when fish intake increased from 1 serving per month to 6 servings per week \(^ {184}\).

Several RCTs have looked at the effect of n3 PUFAs on CHD and MI. The Japan EPA Lipid Intervention Study (JELIS) looked at over 18,000 hypercholesterolemic patients given 1.8g/day EPA plus a statin, or a statin alone. After 5 years of follow up, the study demonstrated EPA was associated with a 19% reduction in major coronary events, a decrease in unstable angina, and a decrease in non-fatal coronary events \(^ {185}\).
The Lyon Heart Diet Study randomized post MI patients to receive a “prudent Western” diet or a “Mediterranean” diet (rich in ALA). Those on the Mediterranean diet saw a reduction in coronary events and total mortality compared to those on the prudent Western diet\textsuperscript{186}.

**Total mortality**

A follow up study to the GISSI trial mentioned above, called the GISSI heart failure (GISSI-HF) study, took 7,000 New York Heart Association Class II-IV patients and randomized them to either 1.0g/day n3 PUFAs or placebo. After 4 years, relative risk was reduced for both total mortality (RR 0.91) and CVD death/hospitalization (RR 0.92). The Diet and Reinfarction Trial (DART) randomized over 2,000 post-MI men to receive different dietary advice, including increasing consumption of fatty fish. After 2 years, total mortality was reduced 29% in the group counseled to eat fish compared to those getting no fish advice\textsuperscript{187}.

In summary, there is evidence from the above trials that n3 PUFAs may indeed have a beneficial effect in preventing CVD despite the existence of some negative results.

**Mechanisms of n3 PUFA benefit in CVD**

Putative mechanisms for n3 PUFA prevention of CVD typically revolve around an antiarrhythmic hypothesis or an anti-ischemic hypothesis; these 2 are not mutually exclusive. The antiarrhythmic actions of n3 PUFAs were first suggested by McLennan et al. studying reperfusion arrhythmias in rats\textsuperscript{188}; dietary n3 PUFAs led to complete suppression of arrhythmias. This led to studies in a dog MI model using i.v. n3 PUFAs\textsuperscript{189}; n3 PUFAs similarly suppressed exercise induced arrhythmias. These studies resulted in the discovery that n3 PUFAs have the ability to inhibit Na\textsuperscript{+} channels by promoting their entry into, and impeding their recovery from, the inactive state after an
action potential. This suppresses $I_{Na}$, which slows conduction and lowers heart rate $^7,^8$. N3 PUFAs may also lower heart rate by reducing the activity of SA node cells $^8,^{190}$. There is also evidence that n3 PUFAs interact with L-type Ca$^{2+}$ channels to inhibit Ca$^{2+}$ entry and thus reduce Ca$^{2+}$ induced arrhythmias $^8,^{191}$. These actions of n3 PUFAs are reversible (when PUFAs are washed out with delipidated albumin), indicating that PUFAs are not covalently bound in triglycerides or phospholipids when exerting these effects $^8$.

Anti-ischemic actions of n3 PUFAs involve their potential anti-atherogenic, plaque stabilizing, and vasodilatory properties. LDL is a key component in the creation of atherosclerotic plaques; n3 PUFAs can reduce LDL release by lowering synthesis of triglycerides $^{109}$. N3 PUFA eicosanoid formation (or lack of n6 PUFA eicosanoid formation) may result in suppression of inflammation and thrombosis via only weakly inflammatory and platelet aggregatory product formation (TXA$_3$, LTB$_5$). Lowered inflammation and thrombosis suppress plaque growth and encourage stability. Vasodilatation can be encouraged by n3 PUFA eicosanoid formation (PGI$_3$, TXA$_3$, LTB$_5$). There is also evidence that EPA can increase nitric oxide (NO) release, thus encouraging vasodilatation $^{192}$. Dilated vessels and absent or stable plaques will reduce the incidence of an ischemic event.

**N3 PUFAs and markers of CVD**

Given the mainly positive results of trials looking at n3 PUFAs and CVD, and the suggestion that the mechanism of n3 PUFA benefit is direct prevention of arrhythmic or ischemic events, a plethora of smaller trials have been performed to test various aspects of that theory. Results from observational trials are presented in Table 4; results from clinical studies are divided into categories below.
**Heart rate and arrhythmias**

N3 PUFAAs generally lower resting heart rate and promote heart rate variability (which should prevent arrhythmias)\(^ {193-206}\), although this is not seen in every case\(^ {194, 195, 199, 207-209}\). Some trials show reductions in premature atrial and ventricular complexes, and promotion of sinus rhythm\(^ {210-214}\). However, 3 RCTs failed to show any benefit of n3 PUFAAs on implantable cardioverter defibrillator (ICD) intervention in patients with ventricular tachycardia or fibrillation\(^ {215-217}\) (see Table 5).

**Lipid profile**

Trials looking at the effect of n3 PUFAAs on lipid profile generally, but not always, show a reduction in triglycerides and an increase in HDL\(^ {193, 214, 218-237}\). A few trials show reductions in LDL\(^ {214, 226, 234, 237}\), although some show an increase\(^ {238}\). Similarly, total cholesterol is reduced in some trials\(^ {214, 232-234}\), and elevated in others\(^ {238}\) (see Table 6).

**Endothelial function and inflammation**

Results from trials looking at the effect of n3 PUFAAs on vascular/endothelial function, blood pressure, thrombosis, and inflammation are more varied. Vascular and endothelial function generally improve with n3 PUFA administration\(^ {195, 197, 206, 208, 239-242}\). Blood pressure is generally reduced\(^ {193, 198, 200, 204, 205, 207, 231}\), though not always\(^ {194, 219}\). Some trials show reductions in platelet aggregation\(^ {243}\), although there is no consensus on the n3 PUFA effect on thrombotic factors (fibrinogen, von Willebrand factor, FV, FX, etc.)\(^ {205, 218, 224, 229, 244-247}\). Markers of oxidative stress and inflammation (superoxide, interleukins, CRP, cellular adhesion molecules, isoprostanes, etc.) are often reduced\(^ {211, 214, 223, 233, 246, 248-251}\), though in some cases they are found to be increased or unaffected\(^ {224, 237, 249, 251-255}\) (see Table 7).
Table 4 – Observational studies of the effect of n3 PUFAs on markers of cardiovascular disease

<table>
<thead>
<tr>
<th>Study</th>
<th>Subjects</th>
<th>Follow up</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Galleneggie 2003</td>
<td>9756 healthy men</td>
<td>none</td>
<td>↑ fish intake associated with ↓ TG, HDL, HR, diastolic BP</td>
</tr>
<tr>
<td>CARDIA, Anchor 1996</td>
<td>1872 healthy young</td>
<td>none</td>
<td>fish intake not associated with changes in fibrinogen, FVII, FVIII, VWF</td>
</tr>
<tr>
<td>ATTICA, Chrysostou 2007</td>
<td>1614 men</td>
<td>none</td>
<td>↑ fish intake associated with ↓ QTc interval</td>
</tr>
<tr>
<td>CARDIO, Papagianni 2006</td>
<td>848 ACS</td>
<td>none</td>
<td>↑ fish intake associated with ↓ chance of developing ACS</td>
</tr>
<tr>
<td>Nishizawa 2006</td>
<td>161 healthy men</td>
<td>none</td>
<td>↑ RBC n3 levels associated with ↓ brachial-to-ankle pulse wave velocity</td>
</tr>
</tbody>
</table>

TG = triglycerides, HDL = high density lipoprotein, HR = heart rate, BP = blood pressure, VWF = von Willebrand factor, ACS = acute coronary syndrome, RBC = red blood cell

Table 5 – Randomized controlled trials studying the effect of n3 PUFAs on heart rate and arrhythmias

<table>
<thead>
<tr>
<th>Study</th>
<th>Subjects</th>
<th>Intervention</th>
<th>Follow up</th>
<th>Effect of n3 PUFAs</th>
<th>Rs</th>
<th>Pr</th>
<th>Bi</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOFA, Blannard 2006</td>
<td>548 ICD</td>
<td>2.00g/day FO</td>
<td>12 months</td>
<td>↓ ICD intervention for VF/VT, all cause mortality</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Leaf 2005</td>
<td>402 ICD</td>
<td>2.50g/day EPA/DHA</td>
<td>12 months</td>
<td>↓ ICD intervention for VF/VT</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Grimsgaard 1998</td>
<td>224 healthy men</td>
<td>4.00g/day EPA or 4.00g/day DHA</td>
<td></td>
<td>DHA ↓ HR, EPA ↓ HR</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Raitt 2006</td>
<td>200 ICD</td>
<td>1.30g/day n3</td>
<td>0-2 years</td>
<td>↓ ICD intervention for VF/VT</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Kanomaki 2007</td>
<td>187 persistent AF, IHD</td>
<td>1.0g/day n3</td>
<td>12 months</td>
<td>↑ maintenance of sinus rhythm</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dyerberg 2006</td>
<td>87 healthy men</td>
<td>4.00g/day n3</td>
<td>8 weeks</td>
<td>↓ resting HR, ↓ HR</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Dyerberg 2004</td>
<td>87 healthy men</td>
<td>4.00g/day n3</td>
<td>8 weeks</td>
<td>↓ HR</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Geelen 2005</td>
<td>84 healthy with PVCs</td>
<td>1.5g/day n3</td>
<td>14 weeks</td>
<td>↓ resting HR, ↓ PVCs</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Geelen 2003</td>
<td>84 healthy elderly</td>
<td>3.5g/day FO</td>
<td>12 weeks</td>
<td>↓ HR</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Geelen 2002</td>
<td>84 healthy elderly</td>
<td>1.5g/day n3</td>
<td>12 weeks</td>
<td>↓ QT interval, QRS-T-angle, QRS duration, T peak-to-end duration</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Geelen 2005</td>
<td>74 healthy with PVCs</td>
<td>1.5g/day n3</td>
<td>14 weeks</td>
<td>↓ QT interval, QRS-T-angle, U wave amplitude</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sjoberg 2010</td>
<td>67 obese</td>
<td>2.0-6.0g/day n3</td>
<td>12 weeks</td>
<td>↑ HR</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Study</td>
<td>Subjects</td>
<td>Intervention</td>
<td>Follow up</td>
<td>Effect of n3 PUFAs</td>
<td>Rn</td>
<td>Pl</td>
<td>B1</td>
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<td>--------------------------------------------------------</td>
<td>----</td>
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<td>----</td>
</tr>
<tr>
<td>Singer 2004</td>
<td>65 healthy with arrhythmias</td>
<td>1.0g/day n3</td>
<td>6 months</td>
<td>↓ PVCs, PACs</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Eksi 1996</td>
<td>63 hypertensive</td>
<td>3.7g/day n3</td>
<td>16 weeks</td>
<td>↓ HR</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Christensen 1999</td>
<td>60 healthy</td>
<td>6.6g/day n3 or 2.6g/day n3</td>
<td>12 weeks</td>
<td>↑ HRV in men, ↓ HRV in women</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Mori 1999</td>
<td>69 hyperlipidemic</td>
<td>4.0g/day EPA or 4.0g/day DHA</td>
<td>6 weeks</td>
<td>DHA ↓ HR</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Holguin 2006</td>
<td>69 healthy elderly</td>
<td>2.0g/day FO</td>
<td>6 months</td>
<td>↑ HRV</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Christensen 1997</td>
<td>55 post MI</td>
<td>6.2g/day n3</td>
<td>12 weeks</td>
<td>↑ HRV, ↓ PVCs</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Rameau 2005</td>
<td>60 healthy elderly</td>
<td>2.0g/day FO</td>
<td>6 months</td>
<td>↑ HRV</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Park 2002</td>
<td>40 healthy</td>
<td>4.0g/day EPA or 4.0g/day DHA</td>
<td>4 weeks</td>
<td>EPA ↓ resting HR</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Theobald 2007</td>
<td>38 healthy</td>
<td>0.7g/day DHA</td>
<td>12 weeks</td>
<td>↓ resting HR</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Hamad 2006</td>
<td>38 post MI</td>
<td>1.0g/day n3</td>
<td>12 weeks</td>
<td>↓ HRV</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Shah 2007</td>
<td>26 healthy</td>
<td>1.0g/day FO</td>
<td>2 weeks</td>
<td>↓ resting HR</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
</tbody>
</table>

Rn = randomized, Pl = placebo controlled, B1 = blinded, ICD = implantable cardioverter defibrillator, FO = fish oil, VT = ventricular fibrillation, VF = ventricular tachycardia, EPA = eicosapentaenoic acid, DHA = docosahexaenoic acid, HR = heart rate, AF = atrial fibrillation, IHD = ischemic heart disease, HRV = heart rate variability, PVC = premature ventricular complex, PAC = premature atrial complex, MI = myocardial infarction
<table>
<thead>
<tr>
<th>Study</th>
<th>Subjects</th>
<th>Intervention</th>
<th>Follow up</th>
<th>Effect of n3 PUFAs</th>
</tr>
</thead>
<tbody>
<tr>
<td>OPTILIP Sanders 2006</td>
<td>256 healthy</td>
<td>n3 enriched diet</td>
<td>6 months</td>
<td>↓ TG, ↓ LDL</td>
</tr>
<tr>
<td>Harrison 2004</td>
<td>213 hyper-cholesterolemic/hypertensive</td>
<td>2.0g/day DHA</td>
<td>5 weeks</td>
<td>↑ HDL, ✈ LDL</td>
</tr>
<tr>
<td>Park 2009</td>
<td>136 hyper-triglyceremic</td>
<td>0.5-2.0g/day n3</td>
<td>20 weeks</td>
<td>↑ total cholesterol, LDL, ✈ TG, ✈ VLDL, ✈ HDL</td>
</tr>
<tr>
<td>Bains 1992</td>
<td>125 CAD</td>
<td>4.5g/day EPA</td>
<td>6 months</td>
<td>↓ TG, ✈ total cholesterol</td>
</tr>
<tr>
<td>Schwellenbach 2006</td>
<td>116 CAD and hyper-triglyceremic</td>
<td>1.0g/day DHA or 1.2g/day EPA+DHA</td>
<td>8 weeks</td>
<td>✈ TG</td>
</tr>
<tr>
<td>Castro 2007</td>
<td>96 healthy</td>
<td>0.5g/day n3</td>
<td>6 weeks</td>
<td>✈ TG</td>
</tr>
<tr>
<td>de Roos 2001</td>
<td>81 healthy</td>
<td>3.5g/day EPA</td>
<td>6 weeks</td>
<td>✈ HDL</td>
</tr>
<tr>
<td>Singer 2004</td>
<td>96 healthy with arrhythmias</td>
<td>1.0g/day n3</td>
<td>6 months</td>
<td>✈ TG, ✈ total cholesterol, LDL, plasma lipids, ✈ HDL</td>
</tr>
<tr>
<td>Damsgaard 2008</td>
<td>64 healthy men</td>
<td>3.1g/day n3</td>
<td>6 weeks</td>
<td>✈ TG, ✈ total cholesterol, glucose, insulin, LDL</td>
</tr>
<tr>
<td>Sacks 1995</td>
<td>60 CHD</td>
<td>8.0g/day n3</td>
<td>36 months</td>
<td>✈ TG, ✈ coronary artery diameter</td>
</tr>
<tr>
<td>Durrington 2001</td>
<td>58 CHD and hyper-triglyceremic</td>
<td>2.0g/day n3</td>
<td>12 months</td>
<td>✈ TG, ✈ VLDL, ✈ HDL, LDL</td>
</tr>
<tr>
<td>Mori 2000</td>
<td>56 hyperlipidemic men</td>
<td>4.0g/day EPA or 4.0g/day DHA</td>
<td>6 weeks</td>
<td>✈ TG, ✈ total cholesterol</td>
</tr>
<tr>
<td>Dunstan 1997</td>
<td>55 NIDDM, dyslipidemic men</td>
<td>3.8g/day n3</td>
<td>8 weeks</td>
<td>✈ TG, ✈ HDL</td>
</tr>
<tr>
<td>Markkamiin 1997</td>
<td>47 healthy men</td>
<td>0.9g/day n3</td>
<td>4 weeks</td>
<td>✈ plasma lipids, apolipoproteins</td>
</tr>
<tr>
<td>Petersen 2002</td>
<td>42 diabetic</td>
<td>4.0g/day FO</td>
<td>8 weeks</td>
<td>✈ TG, ✈ HDL, ✈ LDL</td>
</tr>
<tr>
<td>Norden 2001</td>
<td>42 hyperlipidemic</td>
<td>1.7g/day n3</td>
<td>5 weeks</td>
<td>✈ HDL</td>
</tr>
<tr>
<td>Norden 1998</td>
<td>41 hyperlipidemic</td>
<td>4.0g/day n3</td>
<td>6 weeks</td>
<td>✈ TG, ✈ total cholesterol, apolipoproteins</td>
</tr>
<tr>
<td>Hong 2004</td>
<td>40 CHD and dyslipidemic</td>
<td>3.0g/day n3</td>
<td>8 weeks</td>
<td>✈ TG, ✈ total cholesterol</td>
</tr>
<tr>
<td>Study</td>
<td>Subjects</td>
<td>Intervention</td>
<td>Follow up</td>
<td>Effect of n3 PUFAs</td>
</tr>
<tr>
<td>---------------</td>
<td>-------------------</td>
<td>---------------------------------------</td>
<td>-----------</td>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>Morcas 1997</td>
<td>40 dyslipidemic</td>
<td>1.8g/day EPA or 1.2g/day DHA</td>
<td>4 weeks</td>
<td>↓ total cholesterol, TG, LDL</td>
</tr>
<tr>
<td>Stark 2000</td>
<td>36 healthy woman on HRT</td>
<td>2.4g/day EPA+1.6g/day DHA</td>
<td>4 weeks</td>
<td>↓ TG</td>
</tr>
<tr>
<td>Rajaram 2009</td>
<td>25 hyperlipidemic</td>
<td>fish diet</td>
<td>4 weeks</td>
<td>↓ TG, ↑ HDL</td>
</tr>
<tr>
<td>Chavan 2005</td>
<td>22 CABG</td>
<td>8.0g/day</td>
<td>6 weeks</td>
<td>↓ vLDL, TG, ↑ HDL</td>
</tr>
</tbody>
</table>

Rn = randomized, PI = placebo controlled, BI = blinded, LDL = low density lipoprotein, DHA = docosahexaenoic acid, HDL = high density lipoprotein, TG = triglycerides, PO = fish oil, vLDL = very low density lipoprotein, CAD = coronary artery disease, EPA = eicosapentaenoic acid, CHD = coronary heart disease, NIDDM = non insulin dependent diabetes mellitus, HRT = hormone replacement therapy, CABG = coronary artery bypass graft.
### Table 7 – Randomized controlled trials studying the effect of n3 PUFAs on vascular/endothelial function, blood pressure, thrombosis and markers of inflammation

<table>
<thead>
<tr>
<th>Study</th>
<th>Subjects</th>
<th>Intervention</th>
<th>Follow up</th>
<th>Effect of n3 PUFAs</th>
<th>Rn</th>
<th>PI</th>
<th>El</th>
</tr>
</thead>
<tbody>
<tr>
<td>Traseid 2009</td>
<td>963 high risk, elderly men</td>
<td>2.4g/day n3</td>
<td>3 years</td>
<td>↓ IL-1β</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OPTILIP Sanders 2006</td>
<td>258 healthy elderly</td>
<td>n3 enriched diet</td>
<td>6 months</td>
<td>↓ fibrinogen, FXIIIa, FVIIIc, FXIIa</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Grimsaard 1996</td>
<td>224 healthy men</td>
<td>4.0g/day EPA or 4.0g/day DHA</td>
<td>5 months</td>
<td>↑ BP</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Harrison 2004</td>
<td>213 hypercholesterolemic/ hypertensive</td>
<td>2.0g/day DHA</td>
<td>5 weeks</td>
<td>↓ BP</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Kanisli 2007</td>
<td>187 persistent AF or IHD</td>
<td>1.0g/day n3</td>
<td>12 months</td>
<td>↓ CRP</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Knutstad 2003</td>
<td>171 high risk, elderly men</td>
<td>2.4g/day EPA+DHA</td>
<td>16 months</td>
<td>↑ VCAM</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Finnegan 2003</td>
<td>160 hyperlipidemic</td>
<td>1.7g/day EPA+DHA</td>
<td>6 months</td>
<td>↑ FVIIa, FVIIIc, FVIIIa, FXIIIa, FXIIa, fibrinogen, PA-1</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Mori 1997</td>
<td>120 hypertensive and hyperlipidemic</td>
<td>3.7-7.2g/day n3</td>
<td>12 weeks</td>
<td>↓ platelet aggregation</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Dyerberg 2005</td>
<td>87 healthy men</td>
<td>4.0g/day n3</td>
<td>8 weeks</td>
<td>↓ arterial compliance, flow mediated vasodilation</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Dyerberg 2004</td>
<td>87 healthy men</td>
<td>4.0g/day n3</td>
<td>8 weeks</td>
<td>↓ arterial pressure, arterial compliance</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Geelen 2004</td>
<td>84 healthy elderly</td>
<td>1.6g/day n3</td>
<td>12 weeks</td>
<td>↓ CRP</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Geelen 2003</td>
<td>64 healthy elderly</td>
<td>3.6g/day FO</td>
<td>12 weeks</td>
<td>↓ baroreflex sensitivity</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>de Roos 2003</td>
<td>81 healthy</td>
<td>3.6g/day FO</td>
<td>8 weeks</td>
<td>↓ inflammation</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Sjögren 2010</td>
<td>67 obese</td>
<td>2.0-6.0g/day n3</td>
<td>12 weeks</td>
<td>↑ large artery compliance, ↑ BP, small artery compliance</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Singar 2004</td>
<td>86 healthy with arrhythmias</td>
<td>1.0g/day n3</td>
<td>8 months</td>
<td>↓ thromboxane B2</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Damsgaard 2000</td>
<td>84 healthy men</td>
<td>3.1g/day n3</td>
<td>8 weeks</td>
<td>↓ fibrinogen, CRP, IL-6, VCAM, adiponectin</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Esa 1996</td>
<td>83 hypertensive</td>
<td>3.7g/day n3</td>
<td>16 weeks</td>
<td>↓ BP</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mori 2003</td>
<td>59 diabetic, hypertensive</td>
<td>4.0g/day EPA+DHA</td>
<td>6 weeks</td>
<td>↓ F2 isoformases, ↑ CRP, IL-6, TNFα</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Mori 2000</td>
<td>59 hyperlipidemic men</td>
<td>4.0g/day EPA or 4.0g/day DHA</td>
<td>6 weeks</td>
<td>DHA ↓ forearm blood flow, EPA ↑ forearm blood flow</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Study</td>
<td>Subjects</td>
<td>Intervention</td>
<td>Follow up</td>
<td>Effect of n3 PUFAs</td>
<td>Rn</td>
<td>PI</td>
<td>BI</td>
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<td>------------------</td>
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<td>--------------------------------------------------------</td>
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<td>----</td>
</tr>
<tr>
<td>Mori 1999</td>
<td>59 hyperlipidemic</td>
<td>4.9g/day EPA or 4.9g/day DHA</td>
<td>6 weeks</td>
<td>DHA ↓ BP</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Mori 1999</td>
<td>55 NIDDM, dyslipidemic</td>
<td>3.8g/day n3</td>
<td>8 weeks</td>
<td>↓ P2-isoprostanes</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Dunstan 1999</td>
<td>56 diabetic</td>
<td>3.6g/day n3</td>
<td>8 weeks</td>
<td>↓ tissue plasminogen activator ↑ FVIIc ↓ PAI-1, fibrinogen</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Johansen 1999</td>
<td>54 CHD</td>
<td>5.1g/day n3</td>
<td>8 weeks</td>
<td>↓ PAI-1, thrombomodulin ↑ VCAM</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hill 2007</td>
<td>50 healthy</td>
<td>1.8g/day DHA</td>
<td>12 weeks</td>
<td>↓ superoxide, inflammation ↔ cytokine production, chemotaxis, neutrophil adhesion</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Marcum 1997</td>
<td>47 healthy young men</td>
<td>0.9g/day n3</td>
<td>4 weeks</td>
<td>↔ fibrinogen, VWF</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Nørh 2003</td>
<td>42 hyperlipidemic</td>
<td>1.7g/day n3</td>
<td>5 weeks</td>
<td>↓ FVIIIa, FVIIIc, FVII-Ag</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Nørh 2001</td>
<td>42 hyperlipidemic</td>
<td>1.7g/day n3</td>
<td>5 weeks</td>
<td>↓ systolic BP</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Hong 2004</td>
<td>40 CHD, dyslipidemic</td>
<td>3.0g/day n3</td>
<td>9 weeks</td>
<td>↓ CRP</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Park 2002</td>
<td>40 healthy</td>
<td>4.9g/day EPA or 4.9g/day DHA</td>
<td>4 weeks</td>
<td>EPA ↓ diastolic BP ↔ platelet activation</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Thebald 2007</td>
<td>38 healthy</td>
<td>0.7g/day DHA</td>
<td>12 weeks</td>
<td>↓ diastolic BP ↔ arterial compliance, CRP, a-selectin, VWF, isoprostanes</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Cao 2009</td>
<td>26 healthy elderly</td>
<td>0.2g/day DHA</td>
<td>12 weeks</td>
<td>↑ coronary flow velocity during hyperemia ↔ coronary flow velocity at rest</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>de Mante 2009</td>
<td>27 healthy</td>
<td>Fish diet</td>
<td>8 weeks</td>
<td>↔ IL-1β, ICAM</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Shah 2007</td>
<td>26 healthy</td>
<td>1.0g/day FO</td>
<td>2 weeks</td>
<td>↑ vasodilation</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Lhama 2005</td>
<td>22 CABG</td>
<td>8.0g/day</td>
<td>5 weeks</td>
<td>↔ apoptosis, inflammation</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
</tbody>
</table>

Rn = randomized, PI = placebo controlled, BI = blinded, IL = interleukin, EPA = eicosapentaenoic acid, DHA = docosahexaenoic acid, BP = blood pressure, AF = atrial fibrillation, IHD = ischemic heart disease, CRP = C-reactive protein, VCAM = vascular cellular adhesion molecule, PAI = plasminogen activator inhibitor, FO = fish oil, TNF = tumor necrosis factor, NIDDM = non-insulin dependent diabetes mellitus, CHD = coronary heart disease, VWF = von Willebrand factor, ICAM = intercellular adhesion molecule, CABG = coronary artery bypass graft.
The often conflicting results of trials looking at the effect of n3 PUFAs on CVD, or CVD markers, highlight our incomplete understanding of the mechanisms by which n3 PUFAs may be beneficial\(^9,256\). Results of the 3 failed ICD trials mentioned above are a strong indicator that simple ion channel blockade is an unsatisfactory hypothesis to explain how n3 PUFAs may prevent arrhythmic events. Evidence is accumulating that n3 PUFAs are actually proarrhythmic in some patients while antiarrhythmic in others\(^257\).

Although it does seem that n3 PUFAs reduce triglycerides, data supporting LDL reduction are not as convincing. Lack of consensus on n3 PUFA effects on thrombosis and inflammation also contribute to doubts that improving plaque stability is the mechanism by which n3 PUFAs prevent ischemic events.

It is probably not incorrect to say that n3 PUFAs inhibit ion channels, improve endothelial function, lower triglycerides, blood pressure, thrombosis, and inflammation, however it is evident that they do not do these things in all patients, all the time. It is difficult to deny that n3 PUFAs show benefit in the treatment of CVD, however it is difficult to conclude that we have discovered how n3 PUFAs achieve their effect. This raises the exciting prospect that there are as yet unexplored avenues of therapeutic n3 PUFA activity that need to be investigated.

**N3 PUFAs and AF**

**Clinical studies**

There are relatively few trials of n3 PUFAs dealing specifically with AF (see Tables 8 and 9). The Danish Diet, Cancer and Health Study was an observational study of almost 48,000 people over 6 years. The authors concluded that plasma n3 PUFA levels were not associated with incidence of AF or atrial flutter\(^258\). This is in contrast to the Cardiovascular Health Study which was an observational study of almost
### Table 8 – Observational studies of the effect of n3 PUFAs on AF

<table>
<thead>
<tr>
<th>Study</th>
<th>Subjects</th>
<th>Follow up</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Danish Diet, Cancer, and Health Study</td>
<td>47949 healthy</td>
<td>6 years</td>
<td>Plasma n3 levels not associated with incidence of atrial flutter, AF</td>
</tr>
<tr>
<td>Frost 2006</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cardiovascular Health Study</td>
<td>4315 healthy</td>
<td>12 years</td>
<td>Fish intake on RR of AF: 1-4/week = 0.72</td>
</tr>
<tr>
<td>Mozaffarian 2004</td>
<td>elderly</td>
<td></td>
<td>5+4/week = 0.69</td>
</tr>
<tr>
<td>Kuopio Ischemic Heart Disease Risk Factor</td>
<td>2174 healthy</td>
<td>17.7 years</td>
<td>↑ serum DHA associated with ↓ AF</td>
</tr>
<tr>
<td>Study Vitanen 2003</td>
<td>men</td>
<td></td>
<td>RR = 0.62 highest vs. lowest quartile</td>
</tr>
</tbody>
</table>

RR = relative risk, DHA = docosahexaenoic acid

### Table 9 – Randomized controlled trials studying the effect of n3 PUFAs on AF

<table>
<thead>
<tr>
<th>Study</th>
<th>Subjects</th>
<th>Intervention</th>
<th>Follow up</th>
<th>Results</th>
<th>Rn</th>
<th>Pi</th>
<th>Bi</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heid ravott 2010</td>
<td>168 CABG/valve repair</td>
<td>1.2g/day EPA+1.0g/day DHA</td>
<td>to discharge</td>
<td>↑ POAF</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Cali 2005</td>
<td>160 CABG</td>
<td>2.0g/day n3</td>
<td>to discharge</td>
<td>↓ POAF (15% vs. 33% for controls)</td>
<td>X</td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>Sarawanan 2010</td>
<td>106 CABG</td>
<td>2.0g/day n3</td>
<td>to discharge</td>
<td>↑ hospital stay</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Heidt 2009</td>
<td>102 CABG</td>
<td>1.0mg/kg FO</td>
<td>to discharge</td>
<td>↓ POAF (17% vs. 31% for controls)</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
</tbody>
</table>

Rn = randomized, Pi = placebo controlled, Bi = blinded, CABG = coronary artery bypass graft, EPA = eicosapentaenoic acid, DHA = docosahexaenoic acid, POAF = post operative atrial fibrillation, FO = fish oil
5,000 healthy elderly people over 12 years. The authors concluded that increased fish intake was associated with decreased risk of AF; relative risk of AF was 0.72 with fish intake 1-4 times per week. The Kuopio Ischemic Heart Disease Risk Factor Study was another observational study involving over 2,000 healthy men over 17 years. The authors showed that serum DHA levels were associated with a reduced risk of AF (RR 0.62 highest vs. lowest quartile).

The only RCTs on the effect of n3 PUFAs on AF involve post cardiac surgery AF, presumably arising from post surgical inflammation. Two of the studies (both involving over 100 patients) gave in excess of 2.0 g/day of n3 PUFAs beginning at least 5 days before surgery and continued to discharge; neither of these studies saw a reduction in post operative AF (POAF). Two additional studies however, concluded that n3 PUFAs significantly reduce the incidence of POAF (from 33% down to 15% \(^4\), or 31% down to 17% \(^2\)). Both these trials included over 100 patients each and followed a similar protocol to the two negative trials.

**Animal studies**

There are several animal studies that suggest n3 PUFAs may be beneficial in the treatment of AF. In 2007, Sarrazin et al. reported that dietary n3 PUFAs reduce AF incidence in a dog model of vagal stimulation induced AF. The authors hypothesized that downregulation of connexin 40 by n3 PUFAs was a possible mechanism. N3 PUFAs were found to attenuate acute electrical remodeling in dogs subjected to rapid atrial pacing for 6 hours; dogs receiving a concurrent infusion of n3 PUFAs were protected from AERP shortening. AERP was not prolonged during sinus rhythm by n3 PUFAs leading the authors to suggest that acute administration of n3 PUFAs may not directly alter atrial electrophysiology the way they have been suggested to in the ventricle. In a rabbit Langendorff model increased atrial pressures led to reduced
AERP, increased inducibility and maintenance of AF. Hearts from rabbits fed fish oil showed an attenuation of pressure mediated AERP shortening, and required greater atrial pressures to induce and maintain AF.

Probably the 2 studies most relevant to the effect of n3 PUFAs on in vivo structural remodeling induced AF are by Sakabe et al. in 2007 and the Dorian lab in 2008. Sakabe et al. observed the effect of n3 PUFAs in dog models of RAP and RVP induced AF. The authors showed that n3 PUFAs did not prevent electrical remodeling in chronic RAP and did not reduce the maintenance of AF. In the RVP model however, they showed a significant reduction in the maintenance of AF as well as attenuation of CHF induced LA and LV mechanical function, LA fibrosis, and LA levels of one activated form of ERK and p38. The Dorian lab studied the effect of n3 PUFA supplementation in the dog SAVP model. The data showed that n3 PUFAs significantly reduced both inducibility and maintenance of AF, as well as reducing conduction slowing and heterogeneity. Additionally, n3 PUFAs were found to reduce fibrosis, collagen mRNA levels, and MMP activity.

These studies certainly provide evidence that n3 PUFAs may be effective in the treatment of AF. The lack of both clinical studies, and animal studies in clinically relevant models, investigating the effect of n3 PUFAs on AF indicates a gap in our efforts to explore an area where n3 PUFAs have definitely shown some promise as therapeutic agents.
EXPERIMENTAL WORK

Section One: Background

Atrial fibrillation (AF) is the most common arrhythmia and its prevalence is increasing. It is associated with substantial morbidity and mortality, especially increased risk of stroke. The presence of an arrhythmogenic substrate due to mechanical stretch induced structural remodeling of atrial tissue underlies most clinical AF. Current treatment paradigms revolve around the use of antiarrhythmic drugs (AADs), cardioversion, and surgery; these are of limited efficacy and may be associated with adverse effects and significant cost. Additionally, most treatment options are not targeted at the substrate which produces AF vulnerability. The Dorian lab has created an experimental dog model of atrial mechanical stretch, called the simultaneous atrial and ventricular pacing (SAVP) model, in order to study novel treatments for structural remodeling induced AF. This model produces chronic atrial pressure overload leading to atrial enlargement, fibrosis, mechanical dysfunction, conduction heterogeneity, and AF vulnerability.

Omega-3 polyunsaturated fatty acids (n3 PUFAs), particularly the marine derived forms eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), have been shown to be effective in treating cardiovascular disease (CVD) in some clinical trials. N3 PUFAs have been linked with reductions in the development of coronary heart disease (CHD) and myocardial infarction (MI); in post MI patients n3 PUFAs may reduce all-cause and sudden cardiac death (SCD). Hypotheses concerning the n3 PUFA mechanism of benefit include antiarrhythmic, anti-inflammatory, lipid normalizing, or vascular/endothelial protective functions. However, inconsistent results in several clinical trials have raised doubts that these are the mechanisms through which n3 PUFAs mediate their beneficial effects. Little investigation has
been done looking at the effect of n3 PUFAs on AF. Current clinical studies are almost evenly divided over whether or not n3 PUFAs are beneficial to patients with AF \(^4\), \(^5\), \(^258\), \(^261\).

We have previously shown that n3 PUFAs reduce the incidence of AF in our SAVP model, possibly via an anti-fibrotic mechanism \(^12\). In the following projects I have sought to characterize more clearly the development of atrial cardiomyopathy and AF vulnerability as a result of SAVP, and to investigate the mechanism of n3 PUFA benefit on AF vulnerability. This was done through an analysis of atrial electrophysiology, echocardiography, mRNA levels, protein levels, and histology.
Section Two: General methods
The following is a discussion of the general approaches used in the projects which comprise this study (described in Section Four), and their potential limitations.

Model considerations

In vivo vs. in vitro

Our use of a large animal (dog) in vivo approach in this study has advantages and disadvantages. Alternatives might have included the use of in vitro cell cultures, Langendorff perfused hearts or in vivo small animal models. Large animal in vivo studies have the advantage of physiological relevance to human clinical situations. Atrial disease conditions can be modeled reasonably accurately in animals like pigs and dogs, more so than in smaller animals which may exhibit marked differences in cardiac physiology. Differences in ion channel activity, action potential shape, and myocardial reactions to stress have been noted in rodents compared to humans\textsuperscript{266}. Reliable and relevant models of atrial (as opposed to ventricular) fibrillation are also difficult to achieve in rodents due to small atrial size and rapid sinus rates. In vivo disease models also include relative contributions from other organs and systems which may be absent in cell culture or isolated organ set-ups; endocrine signaling plays a significant role in the pathogenesis of heart disease. This “whole organism” approach to studying disease may result in insights that are more immediately applicable to humans.

On the other hand, it is practically impossible to completely control a large animal in vivo study the way it is possible in a cell culture study. The multi-factorial nature of disease progression in in vivo studies yields results that can only demonstrate correlations between experimental conditions and observed outcomes, rather than causation. Preservation of tissues for molecular or histological analysis can also be
problematic; tissues may take several minutes to be harvested during which time they may be damaged or degraded. The contribution of a particular gene or gene product to disease pathology cannot easily be tested due to the lack of large animal transgenic models. Additionally, large animals are expensive and require complex care facilities, and therefore, investigators are usually compelled to use fewer animals than would be used in a small animal project. As such, fewer timepoints and experimental conditions can be studied. Nevertheless, dog pacing models, such as the SAVP model, have been widely used by multiple groups and have yielded valuable treatment insights 12, 64, 81, 86.

**SAVP**

The SAVP model induces atrial cardiomyopathy by causing the atria to contract against closed tricuspid and mitral valves. This produces an immediate and consistent increase in atrial pressure (pressure overload) resulting in atrial enlargement, leading to mitral regurgitation and subsequent volume overload 2, 3, 150. We feel this is the most relevant experimental model of atrial stretch induced cardiomyopathy currently known. However, the rapid pacing also induces a moderate amount of ventricular failure 2, 3 and thus it is not a completely specific model of atrial dysfunction. Failing ventricles activate the renin angiotensin (RAS) system, cause the release of natriuretic peptides, etc.; all of these factors contribute to the development of AF 122-128. Thus the AF in this model cannot be considered to be solely the result of atrial stretch.

SAVP at 220 bpm (the dogs’ sinus rate is usually between 80 and 100 bpm) for 14 days reliably induces AF 2, 3; theoretically, pacing at lower rates may generate less ventricular dysfunction while still maintaining AF inducibility. We have performed some pilot studies using SAVP at 200 and 180 bpm; while AF inducibility remains, ventricular dysfunction does not decrease (unpublished observations). It remains to be determined
whether or not there is a combination of SAV pacing rate and duration that will produce maximal atrial cardiomyopathy with little or no ventricular damage.

A limitation of this study is that hemodynamic data were not collected. Heart rate was monitored, but only in anesthetized dogs. The effect of SAVP on resting heart rate and blood pressure in conscious dogs in this study is not known.

Dogs

Our implementation of the SAVP model involves the use of mongrel dogs (mostly hound crosses) 1-3 years of age (~7-21 human years of age), 20-30 kg, of either sex. An attempt was made to distribute dogs evenly across all groups according to age, weight and sex (although in some cases we were limited by the selection of dogs that were available from the breeder, see Appendix Three for dog information). Originally, our group used beagle dogs for SAVP, however it was noted that these dogs died of heart failure before developing AF (unpublished observations). Using mongrel dogs reduces the chance that other such breed specific abnormalities will be introduced into the study.

These dogs are quite young, but structural remodeling induced AF occurs mainly in the elderly. It may have been possible to perform SAVP in elderly dogs, however we decided to use young dogs in order to avoid the risk of severe heart failure.

Due to the relatively small amount of molecular work that is done in dogs (as compared to rodents), there is a scarcity of antibodies and primers for protein level and gene expression assays. Therefore, detailed mechanistic results are often difficult to achieve in a dog model. However, an effort has been made to obtain or create antibodies and primers, where feasible, in order to produce as detailed a study of mechanism as possible.
**Diet and supplementation considerations**

We chose to supplement our dogs with dosages of PUFAs around 1 g/day. Our consultations with lipid experts (Dr. Bruce Holub, University of Guelph [Guelph, ON]; Dr. Richard Bazinet, University of Toronto [Toronto, ON]) led us to believe that dogs were similar to humans in their ability to metabolize PUFAs, and that our dog dosages were comparable to a human dosage on a weight/weight basis. Thus a 1 g/day dose in our 20-30 kg dogs can be considered equivalent to a 2.5-3 g/day dose in a 60-80 kg human. Such a dose is easily achievable through the use of over-the-counter supplements from grocery or drug stores. Many groups use large doses of PUFAs in their studies (>5 g/day)\(^{11,225,237}\), however Mozaffarian and Rimm found that the benefits of n3 PUFA supplementation level off after dosages exceed 0.5-1.0 g/day\(^{153}\). Thus our dog dosage of approximately 1 g/day was considered realistic and likely to produce an effect.

The dogs were all fed the same dog chow, however we did not attempt to control the volume of food intake; thus the diets were not isocaloric. As in humans, dog appetites range widely based on size, activity level, mood and individual preference. Moderate heart failure induced by pacing sometimes caused a loss of appetite. In these cases, in accordance with ethics regulations, regular chow was supplemented with special “treats” in order to encourage dogs to eat. An effort was made to provide treats equally to all dogs, however it is possible that highly symptomatic dogs had a different dietary composition than asymptomatic dogs. It may also be possible that n3 PUFAs affect hunger and feeding behaviour. There is no evidence for this in the literature as far as I have been able to ascertain, neither was this observed during the study, however this possibility was not directly tested. It is therefore a limitation of this study that no record was kept of the exact food intake for each dog. Available information on dog chow used in this study can be found in Appendix Two.
Dogs were weighed at the time they were entered into the study, however they were not weighed at the end. Our previous experience with this model led us to believe that dog weights did not fluctuate significantly during the course of the study. Dogs with heart failure symptoms may have lost appetite, but this typically did not happen until close to the sacrifice day. Changes in weight, adiposity, muscularity, etc. throughout the duration of the study may have affected how supplemented PUFAs were metabolized. A limitation of this study is that dog weight and body composition was not assessed after baseline. Available information on the dogs used in this study can be found in Appendix Three.

In early projects of the study, placebo fats were not used to control for the addition of PUFAs to the diet. At the time, we felt that the addition of a relatively small (~1 g) dosage of PUFAs to the regular diet would not affect the study given the other uncontrolled differences in diet discussed above. However, in later projects, several control groups were added using olive oil (primarily containing monounsaturated fat and very little n3 PUFA) as a placebo to control for dietary composition and daily handling stress. Olive oil is a commonly used placebo in PUFA studies and is considered a neutral fat without significant cardiac effects\textsuperscript{260, 261}.

**Analysis considerations**

In the absence of the ability to perform a great deal of reliable gene expression and protein level analysis (due to antibody and primer limitations), much reliance was placed on histological analysis to give indications as to the pathology of SAVP induced disease and the effect of PUFA supplementation. Histological methods were well designed and executed, however they are qualitative or semi-quantitative at best. Care was taken to excise tissue from the same location in every dog, to process and stain the
tissue in an identical manner, and to analyze the tissue with the same algorithm. Nevertheless, differences in terms of tissue orientation, fixation, stain quality, etc. can affect the results. With these limitations in mind, the strength of our results is in the concordance of many lines of evidence, including what molecular results were obtainable, histology, echocardiography and electrophysiology.
Section Three: Detailed methods

The following is a detailed description of methods used in the projects presented in Section Four. A discussion of each method, including benefits and potential limitations, is presented in a section entitled “commentary” underneath each method where appropriate.

Pacemaker implantation

Dogs were fasted overnight except for free access to water. 30 minutes prior to beginning the procedure, dogs were sedated with i.m. acepromazine (Atrovet, 0.05 mg/kg), atropine (0.03 mg/kg), and buprenorphine (Temgesic, 0.02 mg/kg). 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. thiopental sodium (5% solution 0.25 mL/kg). 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%). 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, Min) 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, Min) which connected the 2 leads to 1 bipolar IS-1 pacemaker (Model 5156 Verity ADx XL SR, St. Jude Medical, Minneapolis, Min). 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.

Commentary: Acepromazine is a sedative and anti-nausea drug often used in combination with atropine and buprenorphine in dogs. Our selected dose is in the accepted range of approximately 0.01-0.05 mg/kg i.m. Atropine is an anti-cholinergic drug (blocks AcH receptors) thus preventing parasympathetic stimulation. It is used primarily to prevent bradycardia induced by other drugs in the sedation/anesthesia protocol. Buprenorphine is an µ-opioid receptor partial agonist with analgesic properties 20-40X greater than morphine. It is commonly given to dogs i.m. (due to large first pass effects) in dosages around 0.01-0.03 mg/kg for analgesia before induction of anesthesia. Thiopental sodium (sodium pentothal) is a barbiturate, acting on the GABA receptors in the brain and spinal cord. It is a commonly used anesthetic with a rapid elimination rate (15-20 minutes) and is used mainly to allow intubation so that anesthesia can be maintained with an inhalant. It is known to cause cardiac depression. Thiopental sodium is cheap and is commonly used in healthy dogs with no side effects. Isoflurane is an inhaled anesthetic known to cause cardiac depression, but is very commonly used as a maintenance anesthetic for dogs (1-2%). The mechanism of isoflurane mediated anesthesia and pain reduction are not clear.

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 beginning the procedure, dogs were sedated with i.m. acepromazine (Atrovet, 0.05 mg/kg), atropine (0.03 mg/kg), and buprenorphine (Temgesic, 0.02 mg/kg). 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 (2.5-3.5 mg/kg as Diprivan [10 mg propofol/mL or 1% soln]). 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%). 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.

Commentary: These sedatives and anesthetics have potential cardiovascular effects. Acepromazine has been known to cause decreases in heart rate and cardiac output, and is often left out of the premedication when dealing with debilitated animals; we have not observed this. Buprenorphine is commonly used in combination with propofol and isoflurane; it remains in the body for many hours (highly variable), but there are no commonly reported cardiac effects of buprenorphine relevant to our study. Propofol is a sedative commonly used for induction of anesthesia in dogs. It is a vasodilator and causes hypotension, but dogs in heart failure have been observed to die
when induced with thiopental sodium (unpublished observation). All dogs were induced with propofol for their end study, thus any cardiac effects were equally distributed among all 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. Four bipolar, stainless steel, epicardial pacing/recording electrodes were sutured onto the heart at the RA appendage (RAA), LA appendage (LAA), high RA (SVC) and low RA (IVC). A “clock-face” electrode (16 unipolar, equidistant recording electrodes with a central bipolar pacing electrode, radius 7.5 mm) was sutured onto the posterior wall of the LA (straddling the AV groove and inferior pulmonary vein ostia) (PVs). Intra-cardiac electrograms were recorded in bipolar mode at a filter setting of 30–300 Hz and in unipolar mode at a filter setting of 0.05–300 Hz by a custom acquisition system (AQUI 2 Electrophysiological Recording System v4.0, Cartesian Labs, Toronto, ON) recording at 5 KHz. After the recordings had been made, the clock-face electrode was removed and replaced by a “plaque” electrode at the same location (7X8 grid of unipolar recording electrodes with ~2.6-2.8 mm inter-electrode distance). Plaque electrode intra-cardiac electrograms were recorded in unipolar mode with a filter setting of 1–1000 Hz with the same acquisition system as above. Alternatively, a flexible Printed Circuit Board (PCB) electrode array (7X9 grid of unipolar recording electrodes with ~2.0 mm inter-electrode distance with a central bipolar pacing electrode) was used in place of both the “clockface” and “plaque”. Flexible PCB intra-cardiac electrograms were recorded in unipolar mode with a filter setting of 1–1000 Hz with the same acquisition system as above. Electrophysiological recordings were analyzed with custom software (AQUI 2 v4.0, Cartesian Labs, Toronto, ON) (see Figure 9).
Panel A shows the “clock-face” electrode (16 unipolar, equidistant recording electrodes with a central bipolar pacing electrode, radius 7.5 mm). Panel B shows the “plaque” electrode (7X8 grid of unipolar recording electrodes with ~2.6-2.8 mm inter-electrode distance). Only the first 7 columns of the plaque were active. Panel C shows the PCB array (7X9 grid of unipolar recording electrodes with ~2.0 mm inter-electrode distance with a central bipolar pacing electrode).
Commentary: In the first project, the clock-face electrode was used exclusively. The plaque was designed after the completion of the first project to address concerns about the large inter-electrode distance with the clock-face electrode (particularly between central pacing electrodes and the recording electrodes at the periphery). The plaque allows more exact detection of activation patterns with less possibility of spurious, confounding data. However, it does not have the capability to pace the heart, thus the clock-face electrode was still necessary for applications involving pacing. Later, the PCB array had both the desired recording resolution and the ability to pace the heart, thus it replaced both the clock-face and plaque electrodes.

**Extrasystole pacing, effective refractory period and global conduction time**

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.

Commentary: The S1-S2 technique for measuring AERP is well established in similar dog models. Global atrial conduction time was used as a surrogate of conduction speed. Because the size of the atria differs between dogs, and we did not have an exact measurement of distance between the LAA and SVC, we did not have a method of calculating conduction velocity. Equipment failures occasionally prevented us from obtaining electrophysiological data from all electrodes. We used the LAA and SVC because they had the most complete data; they also represent the far right side and far left side of heart. Conduction between the right and left atrium occurs via Bachmann’s bundle (conductive tissue running from the SA node to the LA). An increase in the transit time for the electrical signal to reach the LAA can result from an increase in atrial size, electrical uncoupling and/or the presence of obstacles to conduction (fibrotic patches).

**Burst pacing and AF**

Burst pacing was performed at each pacing electrode 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; “persistent” AF was defined as an AF incident that lasted more than 600 seconds (10 minutes).

Commentary: Burst pacing is a well established method for testing AF inducibility in similar dog models. At the beginning of the study, we defined an AF incident as any irregular atrial tachyarrhythmia that lasted more than 60 seconds; later we redefined AF to be any irregular atrial tachyarrhythmia that lasted more than 120 seconds. This constrained the labelling of an AF incident to only those arrhythmias that were resistant to pace termination. AF is difficult to define in an experimental setting. Parameters such as atrial rate/cycle length/dominant frequency can be used to define AF; episode length, repeatability/organization index can also be used. However, these are all continuous variables which require the setting of arbitrary cut offs in order to define AF. The use of pace termination to define AF is both biologically sound (true AF should not typically be pace terminable) and non-continuous (it is binary).
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, Bothell, WA). An effort was made to ensure measurements were taken at the same location and angle in each dog to maximize comparability.

Trans thoracic

A 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 ejection fraction (EF) according to the Simpson method: VTI x π (d/2)^2 (where d = LV outflow tract diameter (LVOT), VTI = velocity time integral of LVOT flow). A modified Simpson’s rule was used to calculate LV volumes from the summation of LV cross sectional areas as follows: (π/4) x ∑(ai)(bi)(L/n) (where n = the number of cylinders or discs of equal height taken from 2 chamber (ai) and 4 chamber (bi) recordings, L = length from apex to mitral valve annulus). Data from three consecutive beats were averaged in order to calculate each value.

Trans esophageal

A 5 to 7 MHz phased array multiplane trans esophageal transducer 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 x π)) x ((4 Ch LA area)(2 Ch LA area)/(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.

Commentary: Echocardiographic images and calculations are highly dependant on the position and angle of the probe used to make the recordings. Care was taken to place all dogs in the same position for each echo, and to use the same machine, probes and technician to make all the recordings and measurements. The measurements were then rechecked by a highly experienced cardiologist.

Anesthesia can affect cardiac function. In a previous study, our group attempted to perform echocardiographic measurements on conscious dogs, however it was found that dog movement made the data less reliable than desired. Thus, we elected to perform echocardiography on all dogs under anesthesia.

**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.
**Liquid N$_2$**

Samples of LAA were snap frozen in liquid N$_2$ and stored at -80$^0$C.

Commentary: At -80$^0$C enzymatic activity slows down considerably; therefore proteases and DNA/RNAases that would normally degrade proteins and nucleic acid chains would not be able to function. This allows preservation of tissue for later molecular analysis.

**Formalin**

Samples of LAA were preserved in 10% neutral buffered formalin (10% formaldehyde, 2% anhydrous sodium acetate, 88% water). One sample was fixed for at least 14 days at room temperature (14 day formalin). A second sample was put on a shaker at room temperature for 24-36 hours, then put into 70% ethanol (70% ethanol, 30% saline) (1 day formalin). Samples were prepared for histology by the pathology department at St. Michael’s Hospital (Toronto, ON) or The Centre for Phenogenomics (TCP, Toronto, ON).

Commentary: Formalin “fixes” tissue by cross-linking proteins and pushing out water, thus making tissues harder. It prevents enzymatic activity which would degrade tissue. It is also anti-septic/anti-bacterial. Longer fixation times (weeks) can lead to denaturation of protein structure and loss of antigens. Thus, antibodies used for immunohistological staining which are based on antigen recognition may not work even with “antigen retrieval” methods. Therefore, a trial and error based approach was used to determine the method for 1 day formalin fixation which best suited the immunohistological applications used.

**Optimal cutting temperature**

A sample of LAA was embedded in Tissue-Tek Optimal Cutting Temperature Compound (OCT) (Sakura Finetek, Torrance, CA) and frozen in a bath of isopentane
cooled over dry ice, or over a bath of liquid N\textsubscript{2}, then stored at -80\textdegree C. The section was then prepared for histology by The Centre for Phenogenomics (TCP, Toronto, ON).

Commentary: OCT is composed of a resin-polyvinyl alcohol, benzalkonium chloride (an anti-fungal agent), and polyethylene glycol (to lower freezing temperature)\textsuperscript{267}. Its purpose is to provide a medium into which tissues can be embedded rapidly, and which is hard enough to be sliced with a microtome (as opposed to formalin-paraffin fixation that takes days). Placing the sample in cold isopentane is a commonly used technique to rapidly freeze the OCT embedded sample. Isopentane freezes at -160\textdegree C, thus it can be cooled over dry ice or liquid N\textsubscript{2} to -80\textdegree C and still remain in liquid form\textsuperscript{268}.

**RNA extraction**

RNA was extracted from snap frozen LAA tissue samples as follows. Approximately 50 mg of tissue were crushed with a mortar and pestle filled with liquid N\textsubscript{2}. Powder was homogenized with 1 mL of TRIzol reagent (Invitrogen, Carlsbad, CA), and then incubated for 2-3 minutes at 25\textdegree C. 0.2 mL chloroform were added, the solution was shaken, incubated 2-3 minutes at 25\textdegree C, and then centrifuged (12,000 g, 15 min, 4\textdegree C). RNA remains in the upper (non-chloroform) phase. The supernatant was recovered and mixed with 0.5 mL of isopropyl alcohol to precipitate RNA. The mixture was centrifuged (12,000 g, 10 min) and the pellet was recovered; RNA remains in the pellet. The pellet was washed in 1 mL of 75% ethanol, vortexed and centrifuged (7,500 g, 5 min, 4\textdegree C). The supernatant was discarded and the pellet was air-dried for 5 min, and then dissolved in 50 \mu L of RNAase free water. Purity was verified by spectrophotometry (NanoDrop ND-100, NanoDrop Technologies, Rockland, DE). An absorbance ratio 260 nm/230 nm of 1.8 - 2.1 was considered acceptable.

Commentary: Care was taken not to allow the tissue or powder to melt so as to preserve the RNA until it could be extracted. However, despite the best efforts,
homogenization may have resulted in slight melting of the powder. RNAases destroy RNA, hence the need to use RNAase free water.

RNA absorbs light at 260 nm, but organic contaminants absorb at 230 nm, therefore the 260/230 ratio gives an idea of the level of organic contamination present in the sample.

**Protein extraction**

Protein was extracted from snap frozen LAA tissue samples as follows. Approximately 50 mg of tissue were crushed with a mortar and pestle filled with liquid N₂. Lysis buffer and protease inhibitors were added to the powder and the mixture was sonicated. The mixture was then centrifuged at 1,400 rpm for 15 minutes. The supernatant (protein) was recovered, and the pellet was discarded. Protein concentration was determined with BioRad DC Protein Assay (Bio-Rad, Hercules, CA) according to manufacturer’s instructions.

Commentary: Lysis buffer is essentially a detergent that destroys cell membranes and releases the contents of cells. Protease inhibitors are necessary to prevent destruction of the proteins by endemic protease enzymes which will become active as the sample thaws.

**CD45 stain**

CD45 staining was performed on OCT embedded, frozen sections of LAA tissue by The Centre for Phenogenomics (TCP, Toronto, ON) with the LS-C43970 antibody (Lifespan Biosciences, Seattle, WA).

Commentary: CD45, also known as protein tyrosine phosphatase receptor type C, is an antigen on B and T cells. In discussion with the pathologists who performed this stain, it was suggested that this antibody did not produce ideal staining in canine
tissues. There appears to have been a greater degree of background and non-specific staining than desired (especially “edging”).

**CD18 stain**

CD18 staining was performed on 1 day formalin fixed, paraffin embedded sections of LAA tissue, or OCT embedded, frozen sections of LAA tissue, by The Centre for Phenogenomics (TCP, Toronto, ON) with the CA16.3C10 antibody (University of California, Davis, CA).

Commentary: CD18 is an antigen found on leukocytes and thus this stain can be used to help identify inflammation.

**TUNEL stain**

Terminal deoxynucleotidyl transferase mediated dUTP-biotin nick end labeling (TUNEL) staining was performed on sections of 1 day formalin fixed, paraffin embedded LAA tissue, or OCT embedded, frozen sections of LAA tissue, by The Centre for Phenogenomics (TCP, Toronto, ON).

Commentary: Terminal deoxynucleotidyl transferase (TdT) binds to 3’ OH ends of DNA. The rest of the staining process involves the TdT mediated incorporation of biotinylated deoxyuridine at 3’ OH ends of DNA which can be detected by light. Because apoptosis is characterized by DNA fractionation, there are an elevated number of 3’ OH ends. These ends are marked by the TUNEL staining procedure and made visible under the light microscope.

In discussions with collaborating apoptosis experts (Dr. Kim Connelly, University of Toronto [Toronto, ON]), it has been suggested that TUNEL staining is not always successful in cardiomyocytes and a negative result does not always mean no apoptosis is present. It has also been published that TUNEL staining does not only indicate apoptosis, but possibly also necrosis. Thus, TUNEL staining alone is not sufficient to
determine the presence or absence of apoptosis and we have combined it with measurement of caspase-3 protein activity (see below).

**Hemotoxylin and eosin stain**

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).

Commentary: H+E is a dual stain where the positively charged hematoxylin stains anionic components (generally nucleic acids), and the negatively charged eosin stains cationic components (generally proteins).

**Picrosirius red stain**

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).

Commentary: PSR is comprised of Sirius red and picric acid. It stains collagen red and gives all other cellular components a pale yellow colour. Sirius red is the coloured component, but when picric acid is omitted, red staining is pervasive; the mechanism by which picric acid prevents indiscriminate staining is not known.

**PUFA phospholipid integration**

Total lipid extracts were prepared from frozen plasma or frozen atrial tissue samples according to the method of Bligh and Dyer. Briefly, approximately 1 mL of plasma, or 200 mg of tissue, was washed repeatedly with a mixture of chloroform and methanol (1:2 by volume) in order to separate phospholipids from the rest of the mixture. A known amount of internal standard as diheptadecanoyl phosphatidylcholine was added to the mixture. After vortexing repeatedly and centrifuging, the chloroform (lipid) phase
was recovered, concentrated, and applied to thin-layer chromatographic plates for the isolation of the phospholipid fraction followed by transmethylation and fatty acid analyses by gas-liquid chromatography on a Varian 3800 gas-liquid chromatograph (Palo Alto, CA) with a 60-m DB-23 capillary column (0.32 mm internal diameter). See Stark et al. and Madden et al. for details of gas-liquid chromatography.

Commentary: Phospholipids in plasma are components of lipoproteins (HDL, LDL, chylomicrons, etc.). They form the surface monolayer, sequestering triglycerides and cholesterol inside. In tissue, phospholipids are found in cell membranes. Based on a discussion with a collaborating lipid expert (Dr. Richard Bazinet), we feel that the results of this method accurately reflect the concentrations of PUFAs in phospholipids and are not biased by contributions from adipose cells (which contain PUFAs in triglyceride form), cholesterol esters, etc. In both plasma and tissue, phospholipids are separated out in the chloroform layer, leaving the rest of the components in the methanol.

Results for this method are reported as percentages of each fatty acid as a proportion of the entire fatty acid pool. We chose not to present the data as absolute concentration of fatty acids due to the fact that a change in the concentration of a particular fatty acid could reflect a change in the level of that particular fatty acid alone, or a change in the size of the lipid pool as a whole. For the purposes of our study, a representation of how fatty acid levels were changing in relation to one another was judged to be more important than presenting absolute levels of fatty acids.

RNA microarray

RNA microarray was performed by the Microarray Facility at The Center for Applied Genomics (TCAG, Hospital for Sick Children, Toronto, ON). Microarray was performed with a GeneChip Canine Genome 2.0 Array (Affymetrix, Santa Clara, CA); 1 array was
used per dog. The array is a 64 format, 11 micron design and contains 11 probe pairs per probe set. It contains 42,860 canine probe sets, >18,000 expression sequence tag (EST)/mRNA-based transcripts and >20,000 non-redundant canine gene predictions. Oligonucleotide probe lengths are 25 mer. Hybridization controls are bioB, bioC, bioD from E. coli and cre from P1 bacteriophage. Poly-A controls are dap, lys, phe, thr, trp from B. subtilis. Housekeeping genes are beta-actin, elongation factor 1 and GAPDH. Control genes are actin, beta-3-adrenergic receptor, glucose-6-phosphatase, GAPDH and cytochrome p450. Detection sensitivity is 1:100,000. Technical information concerning this array can be found on the Affymetrix website (http://www.affymetrix.com/support/technical/byproduct.affx?product=canine-20).

Commentary: RNA microarrays are tools for measuring gene expression. They allow for the identification and quantification of mRNA in a given sample. Microarrays are designed with a given organism in mind and usually contain the capacity to measure whole (known) genome expression, often comprising tens of thousands of genes. Arrays are comprised of wells organized in a grid pattern on a plate or “chip”. One well corresponds to one EST. One or several ESTs correspond to a single gene. Inside each well are 11 cDNA primers or “oligos” which contain a complimentary sequence to an EST of interest. Subject mRNA is bonded to biotin (biotinylation) and dropped into each well where hybridization takes place with the primer. The array is washed to remove unbound mRNA, and then stained with streptavidin-phycoerythrin. Streptavidin binds strongly to the biotin on any remaining subject mRNA. Phycoerythrin has fluorescent properties and fluorescent intensity is recorded from each well. Intensity of fluorescence is proportional to mRNA concentration.

**Data analyses**
Fluorescent intensity values recorded from each chip were normalized with the Robust-Multi Array Average (RMA) package in Bioconductor software (Bioconductor v2.1, http://www.bioconductor.org). Quality filters were applied to log2 data based on normalized raw intensity values (excluded ESTs with raw intensity values ≤50 and absent in over half the experimental conditions). Summarization was performed by median polish. Remaining ESTs were analyzed with the Significance Analysis of Microarrays (SAM) algorithm. This non-parametric algorithm performs t-tests on repeated permutations of the log2 data for each gene to determine genes with significant differences in fluorescent intensity. A multiclass SAM analysis (100 permutations, K nearest neighbours imputation) was run using the SAM add-in for Microsoft Excel (SAM v3.02, http://www-stat.stanford.edu/~tibs/SAM/). A false discovery rate (FDR) of 4.6% (delta 0.51) was considered acceptable. We allowed the FDR to rise so that we could later compare AF related gene expression pattern changes in our dogs with those documented in humans. The resulting significant ESTs were hierarchically clustered by an unsupervised algorithm using JMP statistical software (JMP v7.01 SAS Institute Inc). The significant ESTs were then sorted according to fold change in intensity value between treatment groups. ESTs with a fold change ≥1.5 between 2 treatment groups were considered to show a significant treatment effect. Note that q values (output from SAM) were not used to determine which ESTs had significant differences in expression between 2 treatment groups because SAM output was based on a comparison of all 3 groups. ESTs were translated into their respective genes and analyzed by Ingenuity Pathway Analysis software (Ingenuity Systems, Redwood City, CA, http://www.ingenuity.com/) to determine significant functional associations. P-values less than 0.05 were considered significant. Gene lists were then manually curated, to determine function, by searching public databases on
PubMed (http://www.ncbi.nlm.nih.gov/sites/entrez) and SOURCE (http://smd.stanford.edu/cgi-bin/source/sourceSearch) as well as the Ingenuity private database (Ingenuity Systems, Redwood City, CA). Genes found to have specific functional associations were hierarchically clustered using the same method as above. All significant ESTs were then reanalyzed by Ingenuity Pathway Analysis and broken into functional groups. Separate hierarchical clusters for each functional group were then assembled with JMP.

Commentary: Intensity levels are normalized by the RMA software algorithm, both within an array itself, and across all arrays where intensity values will be compared, and reported in log2 form. A further normalization must be performed in order to remove background noise and eliminate intensity values generated by non-specific hybridization. This involves removing from consideration any ESTs with intensity values below an arbitrarily defined cut off point across all samples. The higher the cut off is set, the more easily the analysis algorithms will be able to detect significant patterns in gene expression due in part to the fewer number of ESTs that will be left to analyze, and in part to the fact that at higher intensity values, the differences in intensity between samples tend to be proportionately smaller. The cut off value can be raised even further if confirmation of microarray results with RT-PCR is desired. An intensity value of around 60 (in base 10) is usually required in order to successfully confirm with RT-PCR, which is typically well above the level of background noise.

Remaining EST intensity values were compared with the SAM algorithm which is designed specifically to deal with large data sets and highlight significant differences between a priori designated groups. The algorithm assigns a score to each EST on the basis of change in gene expression relative to the standard deviation of repeated measurements. A point for each EST is plotted on a graph of observed intensity vs.
expected intensity; comparing gene sets with identical intensity values will yield the line of identity. The adjustable threshold defines a gap on either side of the line of identity within which points are not considered significantly different. Points falling outside the gap are considered significantly differently expressed than expected. The size of the gap implicitly determines the FDR (large gaps yield smaller FDRs, but fewer ESTs will be considered significant and vice versa).

Ingenuity software does not determine the “significance” level of individual genes; it assumes that any gene input into the program has already been calculated to be significant. P-values and scores generated by Ingenuity are a measure of the likelihood that the input list contains functionally related genes by chance. For example, the P-value assigned to the “renal hypertrophy” function represents the certainty with which one could say the genes on the input list disproportionately relate to renal hypertrophy. The calculation basically involves looking at the ratio between all known renal hypertrophy genes and all known genes in the genome, then comparing that to the ratio of renal hypertrophy genes in the input list compared to the number of genes in the input list in total. Ingenuity cannot determine the “direction” of the effect (whether pro- or anti-hypertrophic).

**Comparison with human microarray data**

A search for publicly available microarray datasets of humans with AF in NCBI Gene Expression Omnibus (GEO) DataSets (http://www.ncbi.nlm.nih.gov/geo/) yielded 1 dataset published by Barth et al. 276. Briefly, RAA tissue samples had been taken from 30 patients undergoing open heart surgery for valve repair or coronary artery bypass grafting (CABG). Ten of the patients had persistent AF defined as duration of AF longer than 3 months, while 20 patients had no history of AF and were in sinus rhythm. An RNA microarray was performed with an Affymetrix U133A+B Gene Chip
(Affymetrix, Santa Clara, CA). The normalized data were downloaded from the PubMed site and a 2 class SAM analysis (100 permutations, K nearest neighbours imputation) was performed; a FDR of 4.62% was considered acceptable. Human ESTs with significant differences in expression between AF patients and sinus patients were compared to dog ESTs with significant differences in expression between SAVP dogs and unpaced dogs according to the following method. Dog ESTs exhibiting a fold change ≥1.5 induced by SAVP alone were converted to their corresponding human ESTs. To standardize the annotation data between species and microarray platforms, probes from each chip used were matched to the human Affymetrix U133 Plus 2.0 microarray chip. Probe sets were matched based on probe sequences identity as available from the annotation and probe sequence files downloaded from Affymetrix (http://www.affymetrix.com/index.affx) or using Resourcerer (http://compbio.dfci.harvard.edu/cgi-bin/magic/r1.pl). In Resourcerer ESTs are identified using the Eukaryotic Gene Ortholog (EGO) database (http://compbio.dfci.harvard.edu/tgi/ego/). Sequence similarities are identified by stringent pair-wise comparison between tentative consensus sequences available from The Institute for Genomic Research (TIGR) Gene Indices (http://compbio.dfci.harvard.edu/tgi/). Significantly altered human ESTs were then compared to converted dog ESTs to determine instances of overlap.

Quantitative real time PCR

Quantitative real time PCR (QT-RT-PCR) was performed as follows. cDNA was created from 10 µl of RNA with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) according to manufacturer’s instructions. Briefly, 10 µL of sample RNA were combined with kit contents and placed in a thermal cycler
(MJ Mini, Bio-Rad, Hercules, CA). A single cycle (10 min @ 25°C, 120 min @ 37°C, 5 min @ 85°C, end @ 4°C) was run to create cDNA. Real time PCR reaction was performed with the Universal Master Mix kit (Applied Biosystems, Foster City, CA) according to manufacturer’s instructions. Briefly, primers were prepared as 1X dilutions and loaded with kit contents and cDNA onto a 384 well plate. All reactions were done in triplicate. The plate was loaded into a 7900 HT Fast Real-Time PCR System machine with SDS v2.3 software for the real time reaction. The reaction consisted of 1 cycle (50°C, 2 min) then 1 cycle (95°C, 10 min) followed by 40 cycles (95°C, 15 sec, 60°C, 1 min) completed in approximately 90-100 minutes.

Commentary: QT-RT-PCR allows a quantitative measurement of mRNA levels. It is based on the PCR reaction, which duplicates existing copies of cDNA molecules. Multiple cycles of duplication result in exponential increases of cDNA quantity. The rate of increase in cDNA quantity is proportional to the amount of cDNA in the original sample. The rate of increase can be measured by fluorescence. With Taqman technology, the primer is engineered with a fluorophore on the 5’ end and a quencher on the 3’ end. Polymerase activity releases the fluorophore from the primer. With the quencher no longer in proximity to the fluorophore, fluorescence can be detected. With SYBR Green technology, a dye is added to the reaction that binds to double stranded DNA and fluoresces when excited. cDNA is synthesized from mRNA with the cDNA reverse transcription kit. A buffer (MgCl, CaCl₂, etc.), free nucleotides (dNTPs), random hexamer primers (small primers which allow reaction to start), distilled water, and 10 µL of sample RNA are combined and placed in the thermal cycler. At low temperatures the polymerization reaction occurs and the single stranded mRNA is fitted with nucleotides to make a double stranded molecule. The high temperature phase anneals the molecule so that the mRNA and new (complementary) cDNA strands separate. There
is now a cDNA molecule for every mRNA molecule present in the sample. The primer for the gene of interest is put in with the cDNA strands and will bind only to the cDNA corresponding to the gene of interest. The master mix kit contains polymerases and free nucleotides which will handle the polymerization reaction on all strands with a primer bound. A similar cycle of polymerization and annealing repeats multiple times, each time essentially doubling the quantity of cDNA corresponding to the gene of interest (limited by availability of free nucleotides, enzyme stability, etc.). Constant recording of fluorescence emitted during the reaction will yield a curve showing the rate of rise in fluorescent activity.

Primers were obtained from Applied Biosystems (Applied Biosystems, Foster City, CA) or designed according to the following method. A search was performed on the NCBI nucleotide database (http://www.ncbi.nlm.nih.gov/sites/entrez?db=nucleotide) for a dog sequence for the gene of interest. Where multiple sequences were found, those with NCBI reference sequence numbers beginning with NM_ were preferred (indicates complete sequence). The sequence number was input into the Primer-BLAST tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi) which proposed a series of primers. Primers producing products less than 200 base pairs in length and spanning exon-intron-exon boundaries were preferentially selected. Forward and reverse primer sequences were sent to ACGT Corp where they were manufactured (ACGT Corp, Toronto, ON).

Commentary: It is important to design primers such that they span exon-intron-exon boundaries. DNA sequences contain both exons and introns, however mature mRNA molecules have introns excised. Thus, it is possible to differentiate DNA and mRNA for the same gene in this manner. Primers which span exon-intron-exon boundaries will not be able to bind to DNA (since the intron is missing from the primer).
Therefore, one can be certain that only mRNA is being amplified by the PCR reaction. The NCBI nucleotide database indicates gene sequences that have been verified and analyzed for intron and exon boundaries by giving them the NM_ prefix; XM_ sequences have not been so carefully analyzed.

**Protein measurement**

*Western blot*

Western blots were performed as follows. Approximately 50 µg of protein were loaded onto a 4-20% Tris-Glycine polyacrylamide gel (Invitrogen, Carlsbad, CA) immersed in sodium dodecyl sulfate (SDS) and electrophoresis was performed at 100 V for 2 hours. The gel was then soaked in transfer buffer (Tris-Glycine, 20% methanol), covered with nitrocellulose paper and placed in an electrophoresis device for 2 hours at 80 V. Protein on the nitrocellulose paper was stained with Ponceau S to verify successful transfer. Paper was incubated with primary antibody overnight at 4°C. Incubation with secondary antibody occurred 14 hours later. Fluorescence was allowed to develop on film for 1-10 minutes.

Commentary: Western blot is a method of protein identification and quantification. It requires that proteins be isolated from a sample, separated based on weight, then tagged with a fluorescent antibody that recognizes a certain antigen known to be on the protein of interest. Positive identification of the protein of interest is based on observing fluorescent activity. The first electrophoresis phase is referred to as the SDS-PAGE. In this phase, the electric current pulls proteins vertically down through increasingly thick Tris-Glycine gel (4-20%). Small proteins move more quickly and easily, and therefore make it further down the gel in the allotted time. Thus, proteins are separated by weight (size). The second phase is called transferring; the electrophoresis pulls the proteins
horizontally off the gel and onto the nitrocellulose paper while maintaining their vertical arrangement. The third phase is called blocking; the nitrocellulose paper itself must be completely coated in protein that will not bind to the antibody thus ensuring no spurious fluorescence (the proteins in skim milk are used as the blocking agents). The fourth phase is incubation with the primary antibody. This antibody is engineered to bind to a specific antigen on the protein of interest. It is possible that the antigen could appear on more than one protein. In that case, positive identification would be based on fluorescence and protein weight, i.e. the fluorescent band would have to appear in the correct position on the film. The secondary antibody binds to the primary antibody and is engineered with a portion that is vulnerable to cleavage by an enzyme like horseradish peroxidase. The cleavage product is strongly fluorescent and can expose film like a photograph.

**TNF-α level**

TNF-α levels were measured via enzyme-linked immunosorbent assay (ELISA) using a canine TNF-α immunoassay kit (CATA00, R&D Systems, Minneapolis, Min) according to manufacturer’s instructions. Briefly, canine heart tissues were homogenized in phosphate buffered saline, and total protein concentrations were determined by Lowry’s Assay. Tissue homogenates containing 200 µg of protein were added to a microplate coated with a mouse monoclonal antibody specific for canine TNF-α and incubated for 2 hours at room temperature. After the supernatant was removed and the microplate was washed with a washing buffer, a polyclonal antibody against canine TNF-α conjugated to horseradish peroxidase was added to each well and incubated for 2 hours at room temperature. Following aspiration and washing, a colour reagent mixture was added to each well and incubated for 30 minutes; the reaction was stopped by adding a stop solution. Recombinant canine TNF-α was run in
parallel with the tissue samples to create a standard curve. Optical density was determined using a SpectraMax M5 microplate reader (Molecular Devices, Sunnyvale, CA) at 450 nm with a correction wavelength at 540 nm.

Commentary: TNF-α is a cytokine that plays an important role in inflammation and apoptosis signaling. It promotes inflammation by upregulating leukocyte adhesion molecules and by acting as a chemotactic agent.

ELISA is a commonly used method for detecting protein levels. This ELISA kit employs a sandwich ELISA methodology whereby the sample is “sandwiched” between the primary antibody on the microplate underneath, and the secondary antibody which is applied over top of the sample.

**Caspase-3 activity**

Caspase-3 activity assay was performed with an EnzChek Caspase-3 Assay Kit #1 (Molecular Probes, Eugene, OR) according to manufacturer’s instructions with the following modification. Fluorescence (excitation/emission) was performed at 320 nm/405 nm rather than 342 nm/441 nm recommended by the manufacturer. Our experience with this kit leads us to believe that 320 nm/405 nm yields more reliable results. Briefly, approximately 15 mg of tissue were homogenized (manual crushing followed by sonnication) in a mixture of lysis buffer and distilled water (19:1 ratio). The resulting mixture was centrifuged and 200 µL of supernatant were removed. 50 µL of supernatant were combined with 50 µL of Z-DEVD-AMC substrate from the kit in a well on a plate. All samples were run in triplicate. Samples were allowed to incubate for 30 minutes with light agitation. Excitation/emission fluorescence was measured at 320 nm/405 nm.

Commentary: Apoptosis is characterized by chromatin compaction, cell shrinkage and formation of membrane “blebs”. The caspase family of proteases, particularly
caspase-3, plays an important role in apoptosis. It cleaves specifically the amino acid sequence Asp-Glu-Val-Asp (DEVD). The EnzChek Caspase-3 Assay Kit #1 allows the detection of DEVD protease activity. The kit contains a substrate, Z-DEVD-AMC, which is weakly fluorescent in the UV range (330/390 nm excitation/emission), but strongly fluorescent upon proteolytic cleavage (320/405 nm excitation/emission).

**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/).

**Positive CD45 cell count**

CD45 stained sections of OCT embedded, frozen tissue were analyzed by light microscopy at 40X magnification with an exposure time of 200 ms. 15-20 images were taken per animal. Images were taken of primarily midmyocardial areas with high cellularity due to non-specific staining around the edges and vessels. Cells were considered positively stained if they had heavily stained (dark brown) membranes. Areas with diffuse, non-specific staining were avoided. Positively stained cells were manually counted in each image. All analysis was done in a blinded fashion.

Commentary: This analysis can be considered only qualitative or semi-quantitative. It is difficult to accurately count positively stained cells in areas where there is a high degree of stain. In discussion with a collaborating pathologist, it was decided that this antibody could not produce the high quality staining necessary for confident analysis of
dog tissues. Therefore, this antibody was abandoned after one analysis in favour of the CD18 antibody.

**Positive CD18 stain count**

CD18 stained sections of 1 day formalin fixed, paraffin embedded LAA tissue were analyzed by light microscopy at 10X magnification with an exposure time of 250 ms. The entire specimen was photographed. Cells were considered positively stained if they had heavily stained (dark brown) membranes. Each picture was assigned a grade between 0 and 4 indicating the volume of positive stain present in the image (0 no visible staining, 4 pervasive positive staining). Figure 10 shows representative images for each grade on the scale.

Commentary: There are several sets of criteria that can be used to assess myocarditis such as the Dallas Criteria, the World Health Organization’s Marburg Criteria, etc. The Dallas Criteria are the most commonly used in North America, however, several recent publications have questioned whether the Dallas Criteria are adequate for assessing myocarditis. Points such as differing investigator opinions, sampling bias and an excessive number of false negatives have been raised as criticisms. In order to avoid criteria based methods for assessing inflammation, a simple “positive cell count” system was used here. This eliminates investigator bias, however still suffers from sampling bias. This is potentially mitigated by the fact that tissue samples were taken from approximately the same location in all dogs.

**Positive TUNEL stain analysis**

See Project Two and Project Three descriptions for details (in Section Four).

**Myocyte cross sectional area**

H+E stained sections of 14 day formalin fixed, paraffin embedded LAA tissue were analyzed by light microscopy at 40X magnification with an exposure time of 200
Figure 10 – CD18 grading scale
Numbered panels show example CD18 stained images at 10X for each grade in the scale (0 no positive staining, 1-4 increasing levels of positive staining).
ms. 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. At least 20 myocytes matching the criteria were required in order for the animal to be included in the analysis. All analysis was done in a blinded fashion and measurements were repeated three times to ensure accuracy.

Commentary: This analysis can be considered only qualitative or semi-quantitative, however it is a well established and still commonly used method for analyzing myocyte area.\textsuperscript{279,280}

\textbf{Collagen area fraction}

PSR stained sections of 14 day formalin fixed, paraffin embedded LAA tissue were analyzed by light microscopy at 40X magnification with an exposure time of 500 ms. 3-4 images were taken per animal. Images were converted to 32 bit tiffs, the “subtract background” (light background, rolling ball radius defaulted to 50) algorithm was applied followed by the “enhance contrast” (default options) algorithm. The images were then converted to 8 bit tiffs for analysis. 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 three times to ensure accuracy.
Commentary: This method can be considered only qualitative or semi-quantitative, however it is a well established and still commonly used method for analyzing collagen content in heart tissue\textsuperscript{279, 280}.

**Fibrillar vs. total collagen**

PSR stained sections of 14 day formalin fixed, paraffin embedded LAA tissue were analyzed by light microscopy at 60X magnification under oil immersion. Images of total collagen were taken with an exposure time of 150 ms, in black and white (contrast setting 5, sharpness setting 15) with a green interference filter and a density filter in order to maximize contrast. Fields with the largest stained areas were selected for analysis. Images of fibrillar collagen were taken in the same field by adding a polarizing lens in front of the light source causing polarization retardance by birefringent fibrillar collagen fibres\textsuperscript{3, 281}. The image was captured with an exposure time of 25 ms, in black and white, with a density filter. Approximately 5 images were taken per animal. Total collagen images were colour inverted (such that stained areas appeared white). Threshold measurements were taken at a brightness value of 175 (out of 255). Pixels having a brightness of at least 175 were considered to be positively stained for collagen (rather than background staining). Fibrillar collagen images were brightness leveled via the “histogram stretch” method to increase the brightness of stained areas (polarized light images did not show background staining, thus increasing levels did not increase the appearance of background elements). Threshold measurements were taken at a brightness value of 175 (out of 255). Pixels having a brightness of at least 175 were considered to be positively stained for fibrillar collagen. The ratio of fibrillar collagen to total collagen was calculated as the ratio of the number of pixels in the fibrillar collagen image with a brightness of at least 175 to the number of pixels in the total collagen...
image with a brightness of at least 175. All image processing and analysis was done via automated scripts in order to eliminate investigator bias.

Commentary: Fibrillar collagens (primarily collagen I and III) are the most abundant in the heart. They form long bundles or “fibrils” which provide mechanical strength and support for cardiac tissue. Non fibrillar collagens (primarily collagen IV and VI, although there are many others) do not form fibrils. Their production is increased when tissue is damaged and they may help to organize fibrillar collagen arrangements (although their precise role is unclear) \(^{282}\).

The threshold of 175 is arbitrary; in the total collagen image, the value was selected to ensure that only positively stained pixels passed the threshold and background elements did not. In the fibrillar collagen image, the histogram stretch on the brightness levels ensured that all visible stained areas were very bright and they easily passed the 175 threshold.

**Tight vs. total collagen**

PSR stained sections of 14 day formalin fixed, paraffin embedded LAA tissue were analyzed by light microscopy at 60X magnification under oil immersion with an exposure time of 150 ms. Images were taken in black and white (contrast setting 5, sharpness setting 15) with a green interference filter in order to maximize contrast. Fields with the largest stained areas were selected for analysis. Approximately 5 images were taken per animal. Images were colour inverted (such that stained areas appeared white). Threshold measurements were taken at 2 brightness values, 185 and 215 (out of 255). Pixels having a brightness of at least 185 were considered to be positively stained for collagen (rather than background staining), pixels having a brightness of at least 215 were considered to be part of a tightly packed collagen bundle (tightly packed collagen bundles stain more darkly, and therefore appear brighter in the colour inverted images).
The ratio of tightly packed collagen to total collagen was calculated as the ratio of the number of pixels with a brightness of at least 215 to the number of pixels with a brightness of at least 185. All image processing and analysis was done via automated scripts in order to eliminate investigator bias.

Commentary: The idea of “tightly packed” vs. “loosely packed” collagen is not discussed in the literature as far as I have been able to determine, however similar concepts have been proposed (i.e. patchy vs. diffuse). Distribution of fibrosis has been found to affect arrhythmogenesis in cardiomyopathic models. Although our definition of tight and loose does not coincide directly with the published definition of patchy and diffuse, the concept is similar and potentially important.

The thresholds of 185 and 215 are arbitrary numbers; the lower value was selected to ensure that only positively stained pixels passed the threshold and background elements did not. The high value was selected so that a reasonable proportion of the brightest pixels passed the threshold. The maximum pixel brightness for the images appeared to be around 230 out of 255.

**Statistical analysis**

Statistical comparisons were performed using one-way analysis of variance with a Tukeys post hoc or Fishers least significant differences post test. A Kruskal-Wallis (non-parametric) test was used to confirm analysis of variance results where stated. Error bars on all charts indicate SEM unless otherwise indicated.

**Data presentation**
Each of the projects presented in the next section represent stages in the evolution of a single study. As such, graphs and tables presented in one project may contain data from previous projects in order to provide context and aid in interpretation of data.
Section Four: Projects

Project One - Effect of chronic, prophylactic PUFA administration on AF vulnerability and gene expression in a model of established atrial cardiomyopathy

Rationale

Our group has previously shown that prophylactic n3 PUFAs reduce the incidence and persistence of AF and atrial fibrosis in the SAVP model of atrial cardiomyopathy in dogs. We designed this experiment to further characterize the effects of SAVP on atrial electrophysiology, gene expression, and histology. Additionally, this project tested the effects of prophylactic n3 PUFAs in the SAVP model.

Objectives

1. Use a genome-wide approach (mRNA microarray) to identify gene expression profiles involved in AF vulnerability, and see whether they are altered by n3 PUFA therapy
2. Characterize the effects of SAVP and n3 PUFAs on atrial electrophysiology, hypertrophy, and fibrosis
3. Verify that supplemented n3 PUFAs are integrated into atrial tissue
4. Determine the potential relevance of experimental findings by comparison with human AF RNA microarray data

Hypothesis

AF inducibility is related to structural remodeling, and prophylactic n3 PUFAs will reduce AF vulnerability and expression of structural remodeling related genes.
Methods

Groups

Thirty-one dogs were randomized into 3 groups as follows:

1. Twelve dogs were paced using SAVP at 220 bpm for 14 days (Paced 14 days, No PUFAs)
2. Twelve dogs were paced using SAVP at 220 bpm for 14 days, and were supplemented with oral n3 PUFAs (460 mg EPA+380 mg DHA) daily for 21 days, beginning 7 days before the start of pacing (Paced 14 days, PUFAs 21 days) or (prophylactic PUFAs)
3. Seven dogs were unpaced, unsupplemented, and served as controls (Unpaced, No PUFAs)

All methods were performed as described in Section Three; any modifications specific to this project are listed below.

QT-RT-PCR

Primers for the following genes of interest were obtained from Applied Biosystems (Foster City, CA). JAM3 (Cf02650628_m1), tissue inhibitor of metalloproteinases 1 (TIMP1) (Cf02621937_g1), CD99 (Cf02657856_m1), Smad7 (Cf00998193_m1), epidermal growth factor (EGF) (Cf02622123_m1), protein kinase B (Akt1) (Cf02654390_m1), myosin heavy chain (MHC) β (Cf02633916_m1), MHC-α (Cf02624757_m1). Primer for Smad6 was obtained from ACGT Corp (Toronto, ON) GAC GAG TAC AAG CCA CTG GA (forward) CTC AGA CGC CAG CAT GTC T (reverse). 18S was used as the housekeeping gene in all instances.

Western blot
Antibodies used for Western blot in this project were Cell Signaling rabbit polyclonal to extracellular signal related kinase (ERK) 1 and 2 (p42/p44) 9102 (Cell Signaling, Danvers, MA) at 1:1000 dilution, mouse monoclonal to phosphorylated ERK 1 and 2 9106s at 1:1000 dilution, rabbit polyclonal to Akt 9272 at 1:1000 dilution, and rabbit polyclonal to phosphorylated Akt 9271 at 1:1000 dilution. Secondary antibodies were IgG (H+L) HRP conjugates at 1:5000 dilution for 1 hour at room temperature.

Results

Note that the dogs used in this project were initially studied under the leadership of Dr. Gabriel Laurent, who was a member of the Dorian lab from 2005-2008. His results from these dogs were published in 2008. Results that have been presented here are either new work that was performed after I took over leadership of this project, or represent reanalysis or recalculation of Dr. Laurent’s previous data based on new methodology (described in Section Three). An effort has been made to cite Dr. Laurent’s work where appropriate.

Electrical

**AF inducibility**

Inducibility and persistence of AF are presented in Figure 11. No AF incidents were observed in Unpaced, No PUFAs dogs. AF incidence and persistence increased significantly with pacing; this increase was significantly attenuated by prophylactic PUFA administration (Paced 14 days, PUFAs 21 days group). Significant effects of both pacing and PUFAs were seen in the number of episodes of AF induced, and the average length of each episode.

Molecular
**PUFA integration**

Plasma levels of PUFAs for dogs in this project have already been reported. Integration of PUFAs into atrial tissue phospholipids was assessed by gas-liquid chromatography; results are presented in Figure 12. There was no difference between the Unpaced, No PUFAs and the Paced 14 days, No PUFAs groups in terms of EPA+DHA levels in tissue. PUFA supplementation for 21 days lead to a highly significant 35% increase EPA+DHA in tissue phospholipids (P<0.01).

**RNA microarray**

Twelve total arrays were run corresponding to 4 Unpaced, No PUFAs dogs, 4 Paced 14 days, No PUFAs dogs and 4 Paced 14 days, PUFAs 21 days dogs. Microarray data will be made available for download on the NCBI GEO DataSets website (http://www.ncbi.nlm.nih.gov/geo/). 42,860 canine ESTs were measured; after RMA normalization and quality filtration 37,504 ESTs remained. A total of 6,467 ESTs were identified as significantly changed via multiclass SAM analysis; FDR 4.6% (delta 0.51). Figure 13 shows an unsupervised hierarchical clustering (complete clustering distance metrics) of significant ESTs. The software correctly sorted dogs into their respective treatment groups using raw mRNA values in a blinded fashion, indicating a strong treatment effect of both pacing and PUFAs. Assessment of gene expression values suggested that, in several areas, treatment with PUFAs returned gene expression towards baseline values seen in unpaced animals.

To determine the effect of pacing on gene expression we compared the expression profile of Unpaced, No PUFAs animals to Paced 14 days, No PUFAs animals. Of the 6,467 significantly changed ESTs mentioned above, 1,180 ESTs (representing 878 unique gene sequences) were deemed to be highly significantly changed specifically by pacing (≥1.5 fold increase/decrease between Paced 14 days,
No PUFAs and Unpaced, No PUFAs animals). To elucidate the molecular features of pacing on atrial gene expression, we used Ingenuity to search for enrichment in predicted functions. Table 10 shows an abridged list of the top significant (P<0.01) predicted functional enrichments as reported by the Ingenuity software. Overrepresented functions included fibrosis, hypertrophy, cell death and inflammation. To determine the effect of PUFAs on gene expression in paced animals we compared the expression profile of Paced 14 days, PUFAs 21 days animals to Paced 14 days, No PUFAs animals. Significantly changed ESTs were deemed to be important to our understanding of the PUFA effect on AF vulnerability in susceptible animals (susceptibility being defined by the differences between Unpaced, No PUFAs and Paced 14 days, No PUFAs animals). Of the 6,467 significantly changed ESTs mentioned above, 222 ESTs (representing 151 unique gene sequences) were deemed to be highly significantly changed by PUFA treatment (≥1.5 fold increase/decrease between Paced 14 days, PUFAs 21 days and Paced 14 days, No PUFAs animals). Table 11 shows an abridged list of the top significant predicted functional enrichments of genes modulated by PUFAs as reported by the Ingenuity software. Overrepresented functions included fibrosis, hypertrophy, cell death, inflammation and lipid metabolism. The similarity between these and the predicted functional enrichments listed previously, suggest PUFAs modulate genes involved in the same functions as those deemed to be important to the development of AF vulnerability induced by pacing.

An analysis of all significant ESTs yielded 17 ESTs related to fibrosis, 50 related to hypertrophy, 41 related to cell death and 22 related to inflammation. Each group of ESTs was hierarchically clustered with an unsupervised algorithm as described above. The results are presented in Figure 14. As with the large hierarchical cluster above, the dogs were correctly sorted into their respective treatment groups with a high degree of
accuracy in the case of fibrosis, hypertrophy, and cell death, indicating a strong treatment effect of both pacing and PUFA supplementation on genes related to those functions. Specifically a reversing effect of PUFAs back towards the unpaced phenotype was evident. Pacing demonstrated an effect on inflammatory genes, but no reversing effect of PUFA supplementation could be seen.

**Human microarray**

To validate our findings using human data we performed analyses on a publicly available microarray dataset obtained from NCBI GEO. Two-class SAM analysis yielded ESTs that showed significant differences between patients in sinus rhythm vs. persistent AF. The 1,180 ESTs significantly changed by pacing (see above) were converted into analogous human ESTs. Thirty-four unique instances of overlap were noted between the significant human ESTs and the significant, converted dog ESTs; 27 of which could be identified by Ingenuity Pathway Analysis. Twelve of these genes had altered regulation as a result of PUFA supplementation as well, the most significant of which were MAPK1 (ERK1/2), EDN1 (endothelin-1) and CPNE8 (copine-8). Results are presented in Table 12.

**QT-RT-PCR**

Confirmation of mRNA levels for selected genes from the microarray as well as other selected structural remodeling related genes was performed via QT-RT-PCR; results are presented in Tables 13 and 14 along with corresponding microarray data. A significant effect of pacing was seen on Smad6, MHC-α and β (P<0.05). A significant effect of PUFA supplementation was seen on Smad7, TIMP1, MHC-α, CD99, EGF and Akt1 (P<0.05). Significant group differences for all genes in the table were also found via Kruskal-Wallis test (P<0.05) with the exception of MHC-α (P=0.11). Where microarray data were discordant with PCR data, PCR data were taken as authoritative.
Complete concordance between microarray results and QT-RT-PCR is almost never observed in published studies.  

**Western blot**

Levels of total and phosphorylated (activated) ERK isoforms 1 and 2 (p42, p44) were determined via Western blot; results are presented in Figure 15. Protein levels for total ERK 1 and 2 increased 45% (P<0.01) between the Unpaced, No PUFAs and Paced 14 days, No PUFAs group. Supplementation with PUFAs reduced total ERK 1 and 2 by 23% (P>0.05) as compared to the SAVP-No PUFAs group. Pacing increased levels of activated ERK by 45% over unpaced levels, while PUFA supplementation reduced ERK activation by 30% as compared to Paced 14 days, No PUFAs dogs (P>0.05). These findings confirmed microarray results for ERK. No significant differences were found between groups when the ratio of activated ERK to total ERK was calculated.

Levels of total and phosphorylated Akt were determined via Western blot and presented in Figure 15. Protein levels for total Akt were similar in the unpaced and Paced 14 days, No PUFAs groups, but PUFA supplementation decreased total Akt by 42% (P<0.05) vs. the unpaced group; this is concordant with PCR data (see above). There was a trend towards decreased activation of Akt (a 49% decrease) in the Paced 14 days, PUFAs 21 days group as compared to the Paced 14 days, No PUFAs group (P>0.05). There also appeared to be a trend towards decreased activation of Akt in the unpaced group as compared to the Paced 14 days, No PUFAs group if the one outlier is removed. No significant differences were found between groups when the ratio of activated Akt to total Akt was calculated, although there was a trend towards a decreased proportion of activated of Akt with PUFA supplementation (P>0.05).  

**TNF-α level**
Inflammation caused by pacing was assessed by measuring TNF-α levels in Unpaced, No PUFAs dogs and Paced 14 days, No PUFAs dogs; results are presented in Figure 16. No difference was noted between the groups, 112±56 pg/mL (unpaced) vs. 108±28 pg/mL (paced 14 days) (P>0.05). Levels of inflammatory TNF-α level at 14 days of pacing were not different than in unpaced dogs.

**Caspase-3 activity**

Induction of apoptosis was quantified by measuring caspase-3 activity in 3 randomly chosen dogs per group; results are presented in Figure 16. Pacing appeared to significantly increase caspase-3 activity; this increase was not attenuated by PUFAs.

**Histology**

*Myocyte cross sectional area*

Cellular hypertrophy was quantified by measuring myocyte cross sectional area; results are presented in Figure 17. A significant 141% increase in size was observed with pacing alone (P<0.001); PUFAs significantly attenuate the pacing induced increase in area (55% decrease as compared to Paced 14 days, No PUFAs; P<0.01).

*Collagen area fraction*

Fibrosis was quantified by measurement of collagen area fraction; results are presented in Figure 17. Pacing significantly increased the percentage of tissue composed of collagen. There was a non-significant 19% decrease in fibrosis with PUFA administration (P>0.05).

*Fibrillar vs. total collagen*

A semi-quantitative measurement of fibrillar collagen isoforms was made with polarized light microscopy; results are presented in Figure 18 as a ratio of fibrillar to total collagen. Pacing significantly decreases the volume of fibrillar collagen isoforms
(as detected by our method) as a proportion of total collagen (31% reduction in the ratio of fibrillar to total collagen; P<0.05). This increase in non-fibrillar collagen was slightly, but not significantly, attenuated by PUFAs (12% larger ratio for Paced 14 days, PUFAs 21 days dogs as compared to Paced 14 days, No PUFAs dogs; P>0.05).

**Tight vs. total collagen**

A semi-quantitative measurement of collagen architecture was made specifically attempting to assess density of collagen packing; results are presented in Figure 18. Pacing non-significantly decreases the density of collagen bundles (8% decrease in the ratio of tight to total collagen; P>0.05). PUFA supplementation non-significantly increased the density of collagen bundles by 12% over non-PUFA supplemented dogs (P=0.06, Paced 14 days, No PUFAs vs. Paced 14 days, PUFAs 21 days).

**Discussion**

To my knowledge, this is the first study to take a genome-wide approach to looking at the effects of PUFAs on atrial cardiomyopathy and AF. The results of this project demonstrate that long term pacing (14 days) induces significant vulnerability to AF (incidence and maintenance). Prophylactic PUFA administration significantly reduces AF vulnerability despite the long term pacing. Both pacing induced AF vulnerability, and its attenuation by PUFAs, may potentially be the result of changes in gene expression.

Laurent et al. showed that PUFA levels are significantly enriched in plasma phospholipids in the PUFA supplemented dogs from this project. Results presented here show that PUFAs are also significantly integrated into atrial tissue phospholipids, thus it is reasonable to speculate that supplemented PUFAs are actively metabolized in the myocytes.
The microarray is a hypothesis generating tool which yields a broad understanding of the effects of pacing and PUFAs. Pacing induced significant changes in the expression of thousands of genes, underscoring the fact that pacing constitutes a considerable insult to the myocardium. A microarray study performed in a similar dog pacing model of congestive heart failure (CHF) and AF showed nearly 3,000 genes were significantly altered by pacing despite the use of more stringent statistical methods than the ones used here. PUFAs were also seen to significantly affect hundreds of genes, indicating that PUFAs either have a highly upstream effect on cellular signaling, or a multitude of individual downstream effects. This is interesting due to the fact that PUFAs are not generally thought to affect gene expression (except in relation to fatty acid metabolism), but rather to have acute effects (on ion channels for example).

Analysis was focused on the overlap between genes affected by pacing and genes affected by PUFAs. This was based on the hypothesis that the mechanism of PUFA mediated attenuation of AF should be indicated by genes that are regulated in one direction by pacing and the opposite direction by PUFAs. Functional clustering revealed that structural remodeling related genes (fibrotic, hypertrophic, inflammatory and cell death related genes) were regulated in such a manner. This is consistent with the fact that structural remodeling is known to be associated with AF incidence.

Microarray results were generally confirmed with PCR and protein analysis indicating significant changes in regulation or expression of structural remodeling related molecules, like Smad6, MHC-α, MHC-β and ERK, due to pacing. PUFAs altered the regulation or expression of structural remodeling molecules like Smad7, Timp1, Akt, EGF, JAM3, MHC-α and CD99 in the opposite direction. Several of these molecules such as EGF, Akt, and ERK are powerful remodeling factors with multiple downstream effects (see Figure 2).
Histological evidence also confirmed that fibrosis and hypertrophy occur in significant amounts after long term pacing, and that they are attenuated by PUFA administration. There may also be differences in terms of collagen architecture between paced dogs and paced dogs which received PUFAs that contribute to the increased AF burden. The more diffuse collagen arrangement seen in the paced only dogs has been associated with an increase in wave fractionation and conduction abnormalities in a sheep CHF AF model ⁹⁸.

Despite suggestions from the microarray, no effect of pacing was seen on inflammation at 14 days of pacing. It is probable that pacing did induce inflammation early on that is resolved by day 14. It appears that long term pacing increases apoptosis, but no attenuating effect of PUFAs was noted. This result is discussed in more detail in the next project.

It could be considered a limitation of this project that no control group using PUFA supplemented, unpaced dogs was included. However, our primary interest is in genes which are affected by both pacing and PUFAs. Therefore we elected not to include a group of dogs that received PUFAs only, given that the genes modified in that group would not necessarily be related to AF inducibility. A full discussion of the entire study can be found in Section Five.

**Interpretation**

Overall, this project suggests that long term (14 days) pacing induces AF vulnerability by inducing structural remodeling (hypertrophy and fibrosis). Prophylactic PUFAs attenuate AF vulnerability potentially via impeding the development of structural remodeling at the genetic level.
Next questions

1. If PUFAs administered prior to the start of atrial injury attenuate AF vulnerability via impeding structural remodeling, are PUFAs effective if given post-remodeling?

2. Do PUFAs have the ability to reverse existing structural remodeling?
Figure 11 – AF vulnerability
Vulnerability of dogs to an AF incident (irregular atrial tachyarrhythmia lasting more than 120 seconds) or persistent AF (AF incident lasting more than 600 seconds).

Panel A shows mean percentage of burst pacing attempts resulting in AF. Panel B shows mean duration of AF as a proportion of the number of burst pacing attempts. ** P<0.01 compared to Unpaced, No PUFAs; # P<0.05 compared to Paced 14 days, No PUFAs.
Figure 12 – Phospholipid PUFA integration
Incorporation of PUFAs into atrial tissue at the end study. ** P<0.01 compared to Unpaced, No PUFAs.
Figure 13 – RNA microarray
Panel A summarizes how the 6,467 ESTs focused on in the analysis were derived from the original 43,035 ESTs. Panel B is an unsupervised hierarchical cluster. The 12 rows of 6,467 coloured bars represent all significant ESTs for each of the 12 dogs analyzed. Bar colour indicates EST mRNA expression level; green (low), black (medium), red (strong). Dendrogram on the right depicts the relationship between individuals. Dendrogram scale is calculated via the “distance” method (line length inversely proportional to relatedness). CTRL indicates Unpaced, No PUFAs; HF indicates Paced, No PUFAs; PUFA indicates Paced, PUFAs individuals. Evidence of PUFA supplementation reversing the paced phenotype toward an unpaced phenotype is seen in 3 areas where the bar colours in the unpaced dogs match the colours in the PUFA dogs, but are opposite in colour to the paced only dogs (boxed in yellow).

Panel B is reprinted from Heart Rhythm, vol 7(4), A Ramadeen, G Laurent, CC dos Santos, et. al., n-3 Polyunsaturated Fatty Acids Alter Expression of Fibrotic and Hypertrophic Genes in a Dog Model of Atrial Cardiomyopathy, 520-528, Copyright (2010), with permission from Elsevier.
Figure 14 – Unsupervised functional hierarchical clustering of RNA microarray expression values
RNA microarray expression values from ESTs found to be significantly different between at least 2 groups were separated into functional categories and hierarchically clustered via an unsupervised algorithm. Colour indicates EST mRNA expression level; green (low), black (medium), red (strong). Dendrogram on the right indicates most closely related individuals. Dendrogram scale is calculated via the “distance” method (line length inversely proportional to relatedness). CTRL indicates Unpaced, No PUFAs; HF indicates Paced, No PUFAs; PUFA indicates Paced, PUFAs individuals. Panel A includes 17 fibrosis related ESTs, panel B includes 50 hypertrophy related ESTs, panel C includes 41 cell death related ESTs, and panel D includes 22 inflammation related ESTs.

Figure 14 is reprinted from Heart Rhythm, vol 7(4), A Ramadeen, G Laurent, CC dos Santos, et al., n-3 Polyunsaturated Fatty Acids Alter Expression of Fibrotic and Hypertrophic Genes in a Dog Model of Atrial Cardiomyopathy, 520-528, Copyright (2010), with permission from Elsevier.
Table 10 – Effect of pacing alone on gene regulation – Functional analysis

<table>
<thead>
<tr>
<th>Gene Functions</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cardiovascular Disease (Rupture, hypertrophy, injury, thrombosis, apoptosis, necrosis)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Organismal Injury and Abnormalities (Fibrosis, necrosis, neutrophilia, leukocytosis, injury)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Cellular Movement (Migration of connective tissue cells, fibroblasts, neutrophils, lymphocytes)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Cell Death (Apoptosis, necrosis and anoikis of multiple cell types)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ECM and cytoskeletal organization, disruption</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Migration, proliferation of muscle cells, contraction, formation and remodeling of bone</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Activation, movement, adhesion of immune/inflammatory cells, chemotaxis and homing</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Table 10 is reprinted from Heart Rhythm, vol 7(4), A Ramadeen, G Laurent, CC dos Santos, et. al., n-3 Polyunsaturated Fatty Acids Alter Expression of Fibrotic and Hypertrophic Genes in a Dog Model of Atrial Cardiomyopathy, 520-528, Copyright (2010), with permission from Elsevier.

Table 11 – Effect of PUFAs on gene regulation of paced dogs – Functional analysis

<table>
<thead>
<tr>
<th>Gene Functions</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immune disorders, cell death of immune/inflammatory cells</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Muscular hypertrophy, fibrosis and disease, arthritis</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Cell to Cell Signaling (Migration of connective tissue cells, fibroblasts, neutrophils, lymphocytes)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Lipid Metabolism (Biosynthesis, metabolism and modification of lipids)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Growth, proliferation, hypertrophy of multiple cell types (Immune/inflammatory and muscle cells)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Coagulation of fluids and clotting</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Cardiac Dysfunction</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Cardiac Fibrosis</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Cardiac Enlargement</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

Table 11 is reprinted from Heart Rhythm, vol 7(4), A Ramadeen, G Laurent, CC dos Santos, et. al., n-3 Polyunsaturated Fatty Acids Alter Expression of Fibrotic and Hypertrophic Genes in a Dog Model of Atrial Cardiomyopathy, 520-528, Copyright (2010), with permission from Elsevier.
Table 12 – Genes with significantly altered regulation in human persistent AF and dog atrial cardiomyopathy

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Gene title</th>
<th>Function</th>
<th>Expression fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>C9orf45</td>
<td>chromosome 9 open reading frame 45 spermatid perinuclear RNA binding protein</td>
<td>Double-stranded RNA binding, cell differentiation</td>
<td>≤0.67</td>
</tr>
<tr>
<td>CAMK2D</td>
<td>calcium/calmodulin-independent protein kinase (CaM kinase) II delta</td>
<td>protein kinase activity, regulation of cell growth</td>
<td>≥1.50</td>
</tr>
<tr>
<td>CPNE8</td>
<td>Copine VIII</td>
<td></td>
<td>≤0.67</td>
</tr>
<tr>
<td>CXXC5</td>
<td>CXXC finger 5</td>
<td>DNA binding</td>
<td>≥1.50</td>
</tr>
<tr>
<td>EDN1</td>
<td>endothelin 1</td>
<td>hormone activity, activation of PKC, cell-cell signaling</td>
<td>≤0.67</td>
</tr>
<tr>
<td>FRMD4A</td>
<td>FERM domain containing 4A</td>
<td>cytoskeletal protein binding</td>
<td>≥1.50</td>
</tr>
<tr>
<td>LGR4</td>
<td>leucine-rich repeat-containing G protein-coupled receptor 4</td>
<td>protein-hormone receptor activity</td>
<td>≥1.50</td>
</tr>
<tr>
<td>MAPK1</td>
<td>Mitogen-activated protein kinase 1</td>
<td>MAP kinase activity, serine/threonine kinase activity, cell cycle, chemotaxis, induction of apoptosis</td>
<td>≥1.50</td>
</tr>
<tr>
<td>MED28</td>
<td>Mediator complex subunit 28</td>
<td>actin binding, regulation of transcription</td>
<td>≥1.50</td>
</tr>
<tr>
<td>RBJ</td>
<td>rab and DnaJ domain containing</td>
<td>heat shock protein binding</td>
<td>≥1.50</td>
</tr>
<tr>
<td>SLC44A2</td>
<td>Solute carrier family 44, member 2</td>
<td>regulation of I-kappaB kinase/NF-kappaB cascade</td>
<td>≥1.50</td>
</tr>
</tbody>
</table>

Table 12 is reprinted from Heart Rhythm, vol 7(4), A Ramadeen, G Laurent, CC dos Santos, et. al., n-3 Polyunsaturated Fatty Acids Alter Expression of Fibrotic and Hypertrophic Genes in a Dog Model of Atrial Cardiomyopathy, 520-528, Copyright (2010), with permission from Elsevier.
### Table 13 – Analysis of mRNA levels via QT-RT-PCR and RNA microarray

<table>
<thead>
<tr>
<th></th>
<th>Unpaced, No PUFAs</th>
<th>Paced 14 days, No PUFAs</th>
<th>Paced 14 days, PUFAs 21 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Smad7 (P)</td>
<td>1.03±0.23</td>
<td>1.32±0.86</td>
<td>0.35±0.20†</td>
</tr>
<tr>
<td>Smad7 (M)</td>
<td>100±3</td>
<td>83±4**</td>
<td>96±9†</td>
</tr>
<tr>
<td>Smad6 (P)</td>
<td>1.08±0.44</td>
<td>0.29±0.19**</td>
<td>0.49±0.30</td>
</tr>
<tr>
<td>Smad6 (M)</td>
<td>96±15</td>
<td>80±9</td>
<td>107±13†</td>
</tr>
<tr>
<td>Timp1 (P)</td>
<td>1.04±0.30</td>
<td>1.30±0.26</td>
<td>0.44±0.14††</td>
</tr>
<tr>
<td>Timp1 (M)</td>
<td>7275±2219</td>
<td>3463±1453*</td>
<td>5459±639</td>
</tr>
<tr>
<td>Akt1 (P)</td>
<td>1.04±0.31</td>
<td>1.14±0.36</td>
<td>0.45±0.16†††</td>
</tr>
<tr>
<td>Akt1 (M)</td>
<td>128±37</td>
<td>197±34*</td>
<td>171±17</td>
</tr>
<tr>
<td>EGF (P)</td>
<td>1.01±0.13</td>
<td>1.08±0.36</td>
<td>0.44±0.18††</td>
</tr>
<tr>
<td>EGF (M)</td>
<td>486±33</td>
<td>786±172*</td>
<td>719±174</td>
</tr>
<tr>
<td>JAM3 (P)</td>
<td>1.05±0.36</td>
<td>1.27±0.43</td>
<td>0.66±0.40†</td>
</tr>
<tr>
<td>JAM3 (M)</td>
<td>91±3</td>
<td>124±8***</td>
<td>107±2†</td>
</tr>
<tr>
<td>MHCα (P)</td>
<td>1.13±0.57</td>
<td>2.76±1.43*</td>
<td>1.09±0.54†</td>
</tr>
<tr>
<td>MHCα (M)</td>
<td>22710±588</td>
<td>21860±762</td>
<td>23230±1833</td>
</tr>
<tr>
<td>MHCβ (P)</td>
<td>1.05±0.35</td>
<td>0.38±0.18***</td>
<td>0.15±0.07</td>
</tr>
<tr>
<td>MHCβ (M)</td>
<td>618±140</td>
<td>476±41</td>
<td>397±60</td>
</tr>
<tr>
<td>CD99 (P)</td>
<td>1.03±0.26</td>
<td>1.24±0.39</td>
<td>0.63±0.26†</td>
</tr>
<tr>
<td>CD99 (M)</td>
<td>1172±201</td>
<td>1769±258*</td>
<td>1699±333</td>
</tr>
</tbody>
</table>

(P) = QT-RT-PCR values (presented in ΔΔCt units)
(M) = RNA microarray values (presented in normalized fluorescent intensity units)
*P<0.05, **P<0.01, ***P<0.001 compared to Unpaced, No PUFAs
†P<0.05, ††P<0.01, †††P<0.001 compared to Paced 14 days, No PUFAs

Table 13 is reprinted from Heart Rhythm, vol 7(4), A Ramadeen, G Laurent, CC dos Santos, et al., n-3 Polyunsaturated Fatty Acids Alter Expression of Fibrotic and Hypertrophic Genes in a Dog Model of Atrial Cardiomyopathy, 520-528, Copyright (2010), with permission from Elsevier.

### Table 14 – mRNA levels via QT-RT-PCR and RNA microarray expressed as fold change compared to Unpaced, No PUFAs group

<table>
<thead>
<tr>
<th></th>
<th>Unpaced, No PUFAs</th>
<th>Paced 14 days, No PUFAs</th>
<th>Paced 14 days, PUFAs 21 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Smad7 (P)</td>
<td>1.00</td>
<td>1.28</td>
<td>0.34</td>
</tr>
<tr>
<td>Smad7 (M)</td>
<td>1.00</td>
<td>0.83</td>
<td>0.96</td>
</tr>
<tr>
<td>Smad6 (P)</td>
<td>1.00</td>
<td>0.27</td>
<td>0.45</td>
</tr>
<tr>
<td>Smad6 (M)</td>
<td>1.00</td>
<td>0.83</td>
<td>1.11</td>
</tr>
<tr>
<td>Timp1 (P)</td>
<td>1.00</td>
<td>1.25</td>
<td>0.42</td>
</tr>
<tr>
<td>Timp1 (M)</td>
<td>1.00</td>
<td>0.48</td>
<td>0.75</td>
</tr>
<tr>
<td>Akt1 (P)</td>
<td>1.00</td>
<td>1.10</td>
<td>0.43</td>
</tr>
<tr>
<td>Akt1 (M)</td>
<td>1.00</td>
<td>1.54</td>
<td>1.34</td>
</tr>
<tr>
<td>EGF (P)</td>
<td>1.00</td>
<td>1.07</td>
<td>0.44</td>
</tr>
<tr>
<td>EGF (M)</td>
<td>1.00</td>
<td>1.62</td>
<td>1.48</td>
</tr>
<tr>
<td>JAM3 (P)</td>
<td>1.00</td>
<td>1.21</td>
<td>0.63</td>
</tr>
<tr>
<td>JAM3 (M)</td>
<td>1.00</td>
<td>1.36</td>
<td>1.18</td>
</tr>
<tr>
<td>MHCα (P)</td>
<td>1.00</td>
<td>2.44</td>
<td>0.96</td>
</tr>
<tr>
<td>MHCα (M)</td>
<td>1.00</td>
<td>0.96</td>
<td>1.02</td>
</tr>
<tr>
<td>MHCβ (P)</td>
<td>1.00</td>
<td>0.36</td>
<td>0.14</td>
</tr>
<tr>
<td>MHCβ (M)</td>
<td>1.00</td>
<td>0.77</td>
<td>0.64</td>
</tr>
<tr>
<td>CD99 (P)</td>
<td>1.00</td>
<td>1.20</td>
<td>0.61</td>
</tr>
<tr>
<td>CD99 (M)</td>
<td>1.00</td>
<td>1.51</td>
<td>1.44</td>
</tr>
</tbody>
</table>

(P) = QT-RT-PCR values (presented as fold change in ΔΔCt units compared to Unpaced, No PUFAs group)
(M) = RNA microarray values (presented as fold change in normalized fluorescent intensity units compared to Unpaced, No PUFAs group)
Figure 15 – ERK and Akt Western blot
Panels A–C show Western blot results for total, activated (phosphorylated), and the ratio of activated:total extracellular signal related kinase (ERK) isoforms 1 and 2. Panels D–F show Western blot results for total, activated, and the ratio of activated:total protein kinase B (Akt) isoform 1. *P<0.05, **P<0.01 compared to Unpaced, No PUFAs.
Figure 16 – Inflammation and apoptosis
Panel A shows TNF-α protein levels in atrial tissue measured by ELISA. Panel B shows levels of active caspase-3 in atrial tissue. *P<0.05 compared to Unpaced, No PUFAs, ns = not significant.
Figure 17 – Hypertrophy and fibrosis
Panel A shows mean cross sectional area of atrial myocytes. 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. Panel B shows mean collagen area fraction of atrial tissue sections. *P<0.05, ***P<0.001 compared to Unpaced, No PUFAs; ##P<0.01 compared to Paced 14 days, No PUFAs.
Figure 18 – Collagen architecture
Panel A shows semi-quantitative determination of the ratio of fibrillar collagen to total collagen in atrial tissue. Mean ratios for each group are plotted. Illustrative images for each group are shown to the right of the graph (from the top, Unpaced, No PUFAs; Paced 14 days, No PUFAs; Paced 14 days, PUFAs 21 days). Images were created by merging 2 images of the same field; fibrillar collagen is tinted green, collagen not identified as fibrillar is tinted red, all other areas are black. Panel B shows semi-quantitative determination of the ratio of tightly packed collagen to total collagen in atrial tissue. Mean ratios are plotted for each group. Illustrative images for each group are shown to the right of the graph (from the top, Unpaced, No PUFAs; Paced 14 days, No PUFAs; Paced 14 days, PUFAs 21 days). Images were created by colour inverting black and white PSR stained images and adjusting colour levels via the histogram stretch method in order to remove background and increase contrast.
*P<0.05 compared to Unpaced, No PUFAs; &P=0.06 compared to Paced 14 days, No PUFAs.
Project Two - Effect of chronic, post-injury, PUFA administration on AF vulnerability in a model of established atrial cardiomyopathy

Rationale

Previous results have demonstrated that prophylactic n3 PUFA supplementation reduces the incidence and persistence of AF, as well as atrial fibrosis and hypertrophy in the SAVP model of atrial cardiomyopathy in dogs. We designed this experiment to test whether n3 PUFAs would be effective in attenuating vulnerability to AF when started part way into the development of severe atrial cardiomyopathy (post-injury).

Objectives

1. Determine the efficacy of post-injury (after 7 days of pacing) n3 PUFAs in reducing AF vulnerability and atrial structural remodeling
2. Establish the extent of AF vulnerability and atrial cardiomyopathy existing at the time post-injury PUFAs are started (day 7 of 14 days of pacing)
3. Check for the presence of inflammation and determine relevant n3 PUFA effects
4. Determine levels of previously identified structural remodeling related gene targets (ERK, Akt, EGF, etc.)

Hypothesis

Post-injury n3 PUFAs will be less effective than prophylactic n3 PUFAs in reducing vulnerability to AF.

Methods

Groups
Twenty two dogs were randomized into 3 groups as follows:

1. Six dogs were paced using SAVP at 220 bpm for 7 days (Paced 7 days, No PUFAs)

2. Twelve dogs were paced using SAVP at 220 bpm for 14 days, and were supplemented with oral n3 PUFAs (460 mg EPA+380 mg DHA) daily for 7 days, beginning 7 days after the start of pacing (Paced 14 days, PUFAs 7 days) or (post-injury PUFAs)

3. Four dogs were sham operated (pacemaker implant), but unpaced, unsupplemented, and served as controls (Unpaced, No PUFAs)

Data from the Unpaced, No PUFAs group and the Paced 14 days, No PUFAs group from Project One were included in some of the results presented here in order to provide context and aid in interpretation.

All methods were performed as described in Section Three; any modifications specific to this project are listed below.

**QT-RT-PCR**

Primers for the following genes of interest were obtained from Applied Biosystems (Foster City, CA). JAM3 (Cf02650628_m1), TIMP1 (Cf02621937_g1), CD99 (Cf02657856_m1), Smad7 (Cf00998193_m1), EGF (Cf02622123_m1), Akt1 (Cf02654390_m1), MHC-β (Cf02633916_m1), MHC-α (Cf02624757_m1). Primers for the following genes of interest were obtained from ACGT Corp (Toronto, ON). Smad6 (forward) GAC GAG TAC AAG CCA CTG GA (reverse) CTC AGA CGC CAG CAT GTC T, Collagen I (forward) GTG TGT ACA GAA CGG CCT CA (reverse) TCG CAA ATC ACG TCA TCG, Collagen III (forward) ATA GAG GCT TTG ATG GAC GAA (reverse)
CCT CGC TCA CCA GGA GC, TGF-β (forward) CAA GGA TCT GGG CTG GAA GTG GA (reverse) CCA GGA CCT TGC TGT ACT GCG TGT. 18S was used as the housekeeping gene in all instances.

**CD18 stain**

CD18 staining was performed on OCT embedded, frozen sections of LAA tissue in this project, rather than 1 day formalin fixed, paraffin embedded tissue. Analysis was performed as previously described.

**TUNEL stain**

TUNEL staining was performed on OCT embedded, frozen sections of LAA tissue in this project, rather than 1 day formalin fixed, paraffin embedded tissue. Sections were analyzed by light microscopy at 40X magnification with an exposure time of 75ms. Sections showed evidence of cell shrinkage possibly from poor fixation. As such, a quantitative analysis of these slides was abandoned; instead, the slides were scanned in an unblinded fashion to look for trends between groups.

**Results**

**Electrical**

**AF inducibility**

Inducibility and persistence of AF are presented in Figure 19. The sham operated Unpaced, No PUFAs dogs from this project were compared to the non-sham operated Unpaced, No PUFAs dogs from Project One to see whether pacemaker implantation had an effect on AF inducibility. The percentage of burst pacing attempts resulting in an AF incident was 0.8±1.5 vs. 0±0, and 0±0 vs. 0±0 for persistent AF (Project Two unpaced vs. Project One unpaced); AF inducibility data were not
considered different between the groups and they were combined in subsequent analyses (7 dogs from Project One and 4 sham operated from Project Two).

AF incidence and persistence increased with pacing duration. Post-injury PUFAs (Paced 14 days, PUFAs 7 days group) did not significantly reduce the incidence or persistence of AF in terms of either the number of episodes induced, or the average length of each episode.

**AERP**

Mean AERP was measured in order to assess the level of electrical remodeling; data are presented in Figure 20. There was no effect of pacing or PUFA administration on AERP at 200 ms cycle length.

**Global conduction time**

Mean global atrial conduction time was measured at 400 ms cycle length to assess changes in conduction speed; data are presented in Figure 20. As above, the sham operated Unpaced, No PUFAs dogs from this project were compared to the non-sham operated Unpaced, No PUFAs dogs from Project One. Sham operated controls had a global atrial conduction time of 64±4 ms vs. 68±6 ms for non-sham operated controls (P>0.05); data from both unpaced groups were combined.

Conduction time increased significantly with pacing duration from 64±4 ms to 72±7 ms after 7 days, to 75±11 ms after 14 days (P<0.05). PUFA supplementation reduced conduction time (6% decrease with post-injury PUFAs); prophylactic PUFA supplementation significantly attenuated pacing induced conduction slowing (14% decrease vs. no PUFA supplementation, P<0.05).

**Echocardiography**
Mechanical function was assessed via echocardiography. Measurements were made of LA and LV end systolic and end diastolic volumes as well as EF; results are presented in Figure 21. In almost every case (except LVEDV), chamber volume increased significantly after 7 days of pacing (LASV +40%, LADV +48%, LVESV +97%); EF decreased severely after 7 days of pacing (-51%). Dogs that were paced for 14 days and supplemented with post-injury PUFAs showed similar levels of mechanical dysfunction to dogs paced for 7 day in all measurements. It is unclear whether the similarity between Paced 7 days, No PUFAs dogs and Paced 14 days, PUFAs 7 days dogs was due to a plateau effect of mechanical remodeling after 7 days of pacing, or a protective effect of post-injury PUFAs in preventing further remodeling.

Molecular

**PUFA integration**

Plasma phospholipid PUFA levels were measured by gas-liquid chromatography; results are presented in Figure 22. The figure also includes results for Paced 14 days, No PUFAs and Paced 14 days, PUFAs 21 days groups originally published by Laurent et al. 12. Unsupplemented dogs show no change in plasma PUFA levels between baseline and end study regardless of pacing duration. A >5 fold increase over Unpaced, No PUFAs dogs was seen when dogs were given PUFAs for 7 days; an additional 61% increase was observed in dogs given PUFAs for 21 days.

Atrial tissue phospholipid PUFA levels were measured as well; results are presented in Figure 22. No difference was seen between unsupplemented groups. A significant 27% increase was seen in dogs receiving PUFAs for 7 days; dogs receiving PUFAs for 21 days showed an extra (non-significant) 6% increase in EPA+DHA in their tissue.
**QT-RT-PCR**

mRNA levels of selected structural remodeling genes were quantified via QT-RT-PCR; results are presented in Table 15. Collagen I, collagen III and TGF-β were measured in Project One dogs by Laurent et al. 12. Paced 14 days, No PUFAs dogs had a significant increase in collagen I and III levels as compared to unpaced dogs; this was seen as well in Paced 7 days dogs. Prophylactic PUFA administration reduces collagen levels to near unpaced values; this reduction was not seen with post-injury PUFAs. TGF-β levels were increased after 14 days of pacing; a similar increase was seen after 7 days of pacing as well. Pacing with prophylactic PUFA administration resulted in an increase in TGF-β above the level of pacing alone. Pacing with post-injury PUFA administration however, resulted in a reduction in TGF-β to near unpaced levels. Pacing for both 7 and 14 days increased expression of Smad7, JAM3, MHC-α and CD99. PUFA supplementation reduced expression of all these genes as compared to paced only groups. Prophylactic PUFAs resulted in expression levels lower than unpaced dogs, post-injury PUFAs did not reduce expression below unpaced levels. Pacing for 7 days lead to an increase in Timp1, Akt and EGF, however levels returned to baseline after 14 days of pacing. Pacing for 14 days with prophylactic PUFA administration reduced expression of these genes even more, to below unpaced levels. Pacing for 14 days with post-injury PUFAs maintained their expression near 7 day pacing levels. Both 7 and 14 days of pacing resulted in greatly depressed MHC-β levels. Neither prophylactic nor post-injury PUFAs had a significant effect on MHC-β levels.

**Western blot**

Levels of total and phosphorylated ERK (p42, p44) were determined via Western blot; results are presented in Figure 23. Total ERK levels were not changed by 7 days
of pacing, but were significantly decreased (70%) in the Paced 14 days, PUFAs 7 days group. It is unclear if the decrease was the result of continued pacing, or post-injury PUFA supplementation, or both; in Project One, PUFA supplementation was associated with decreased ERK. Activated ERK levels increased with pacing, after 7 days levels increased 97%; after 14 days of pacing (with post-injury PUFAs) activated ERK increased a further 26% (P>0.05). The ratio of activated to total ERK showed a strong trend towards increasing with increased pacing.

The levels of total and activated Akt were determined via Western blot and presented in Figure 23. Akt protein levels were increased by 30% after 7 days of pacing, but significantly reduced after 14 days of pacing with post-injury PUFAs. It is unclear whether the reduction in Akt was due to continued pacing, or PUFAs, or both. In Project One both PCR and protein data indicated that after 14 days of pacing Akt levels were not different than unpaced levels; PUFAs also reduced levels to lower than unpaced values. Activated Akt levels were not significantly different between groups, although there may be a trend towards increased activation with pacing. In Project One, prophylactic PUFA supplementation reduced the ratio of activated to total Akt below unpaced levels; no such effect was seen with post-injury PUFAs.

**Caspase-3 activity**

Induction of apoptosis was quantified by measuring caspase-3 activity; results are presented in Figure 24. Tissue from Project One dogs was reassayed along with tissue from Project Two dogs. The sham operated Unpaced, No PUFAs dogs from this project were compared to the non-sham operated Unpaced, No PUFAs dogs from Project One to see whether pacemaker implantation had an effect on caspase-3 activity. No significant difference was found between the groups, thus they were combined in subsequent analyses (Project Two 82±7 µmol/mL vs. 83±2 µmol/mL Project One,
P>0.05). As seen previously, Paced 14 days, No PUFAs dogs appeared to have elevated caspase-3 activity, however there was a decrease in active caspase-3 levels in the Paced 7 days, No PUFAs dogs as compared to Unpaced, No PUFAs dogs. Also, as seen previously, there did not appear to be any effect of PUFAs on caspase-3 activity.

**Histology**

**Positive CD18 stain count**

A semi-quantitative measurement of inflammation was made by analyzing CD18 stained tissues; results are presented in Figure 25. The 2 Unpaced, No PUFAs dogs that were used showed significant positive staining and evidence of myocarditis. Their medical records showed a severe eye infection in 1 case and evidence of an infected surgical incision in the second case. There was no difference in CD18 staining between any of the groups, Unpaced, No PUFAs dogs (1.2±0.4), Paced 7 days, No PUFAs (1.2±0.2), Paced 14 days, PUFAs 7 days (1.2±0.5).

**Positive CD45 cell count**

The level of inflammation was also assessed by a count of CD45 positive cells; results are presented in Figure 25. As above, there was no significant difference between any of the groups. The 2 highest scoring unpaced dogs were the same dogs with myocarditis mentioned above.

**Positive TUNEL stain analysis**

Apoptosis was qualitatively analyzed by examining TUNEL stained tissues. Of the 3 Unpaced, No PUFAs dog slides that were prepared, the 2 showing significant positive staining were the same dogs with myocarditis mentioned above. The remaining Unpaced, No PUFAs dog showed no positive staining. The Paced 7 days, No PUFAs
dogs showed clusters of positive staining in 4 of the 5 sections examined. Two of these showed staining primarily around the epi and endocardial surfaces; the other two showed staining in the interstitial area. One Paced 7 days, No PUFAs section showed virtually no staining at all. Paced 14 days, PUFAs 7 days dogs showed less staining than Paced 7 days dogs in general. One of the 5 Paced 14 days, PUFAs 7 days dogs showed significant positive staining, 2 others showed positivity around the epi and endocardial surfaces; the staining was diffuse and did not occur in clusters. Two Paced 14 days, PUFAs 7 days dogs did not show any staining at all.

**Myocyte cross sectional area**

Cellular hypertrophy was quantified by measuring myocyte cross sectional area; results are presented in Figure 26. The sham operated Unpaced, No PUFAs dogs from this project were compared to the non-sham operated Unpaced, No PUFAs dogs from Project One. Sham operated controls had a mean cross sectional area of 206±30 µm² vs. 168±44 µm² for non-sham operated controls (P>0.05). The groups were not considered different and were combined for subsequent analyses (6 dogs from Project One and 4 sham operated dogs). Pacing significantly increased cell size after 7 days; a further 20% increase was observed with an additional week of pacing (P<0.001). Post-injury PUFAs dogs showed a trend towards cell size reduction; a significant reduction was seen with prophylactic PUFAs (P<0.05).

**Collagen area fraction**

Fibrosis was quantified by measurement of collagen area fraction; results are presented in Figure 27. The sham operated Unpaced, No PUFAs dogs from this project were compared to the non-sham operated Unpaced, No PUFAs dogs from Project One to see whether pacemaker implantation had an effect on fibrosis. Sham operated controls had a mean collagen area fraction of 8.4±3.0% vs. 6.8±2.5% for non-sham
operated controls (P>0.05). The groups were not considered different and were combined for subsequent analyses (6 dogs from Project One and 4 sham operated dogs). Pacing increased the percentage of tissue composed of collagen (P<0.05 Unpaced, No PUFAs vs. Paced 14 days, No PUFAs via t-test; P=0.08 for one way ANOVA comparing 0, 7, 14 days of pacing groups). Post-injury PUFAs did not reduce fibrosis.

Discussion

This is the first study to my knowledge that has attempted to elucidate the effect of the timing of PUFA administration on AF and structural remodeling. The results of this project demonstrate that intermediate term pacing (7 days) induces conduction slowing and AF vulnerability, but to a lesser degree than long term pacing. Power calculation indicates a sample size of 20 per group is necessary to resolve a significant difference between the intermediate term and long term pacing group (with 80% power and P<0.05). Post-injury PUFAs show a trend towards reduction in AF inducibility and conduction slowing in long term pacing dogs, but it is not significant.

The lack of pacing effect on AERP indicates that the AF induced in this model is not related to electrical remodeling; data from Project One suggested that changes to gene expression resulting in structural remodeling (hypertrophy and fibrosis) were responsible for the AF vulnerability. Intermediate term pacing induces some structural remodeling: atrial enlargement, hypertrophy and fibrosis. This is supported by molecular data showing pacing induced increases in mRNA or protein levels of structural remodeling molecules like: collagens I and III, TGF-β, Smad7, Timp1, EGF, JAM3, CD99, activated ERK and Akt, and decreased levels of MHC-β.
PUFAs are significantly incorporated into both plasma and tissue after 7 days of supplementation; in fact, an additional 14 days of PUFA supplementation only results in a relatively small 6% increase in tissue phospholipids. Thus it seems reasonable to speculate that any acute effects of PUFAs should be clearly visible in the post-injury PUFA group. Post-injury PUFAs are not as effective at reducing AF vulnerability, conduction slowing, hypertrophy and fibrosis as prophylactic PUFAs in long term pacing dogs. However, post-injury PUFA dogs have AF inducibility and conduction time levels similar to those seen in intermediate term pacing dogs. Atrial enlargement, hypertrophy and fibrosis levels are also similar between the groups (Paced 14 days, PUFAs 21 days and Paced 7 days, No PUFAs groups). Levels of certain remodeling molecules are also not different between intermediate term paced dogs and post-injury PUFA dogs (collagen I, Timp1, Akt, EGF, MHC-β). Therefore, in this project, post-injury PUFAs do not demonstrate the ability to reverse structural remodeling, but they may arrest further development of structural remodeling.

Similarly to Project One, inflammation is not seen at the 7 day point of pacing; it is possible that early inflammation has been resolved by 7 days. Neither TUNEL nor caspase-3 analysis yield any consistent effect of pacing or PUFAs on apoptosis; this is similar to other animal and clinical studies which show disparate results on apoptosis in AF. A full discussion of the entire study can be found in Section Five.

Interpretation

Overall, this project seems to suggest that intermediate term pacing (7 days) induces an intermediate level of AF vulnerability due to the development of an intermediate level of structural remodeling (when compared to results from long term pacing dogs). Post-injury PUFAs do not appear to reverse existing AF vulnerability or
structural remodeling, but appear to arrest further development of atrial enlargement, hypertrophy and fibrosis.

Next questions

1. Given that the rise in AF vulnerability appears to be correlated with the rise in hypertrophy and fibrosis, is any appreciable AF inducible before hypertrophy and fibrosis become established?

2. Does the SAVP model result in inflammation which is resolved by day 7? If so, does this inflammation lead to AF vulnerability?

3. Do PUFAs have any relevant effects in the absence of pacing or significant structural remodeling?
Figure 19 – AF vulnerability
Vulnerability of dogs to an AF incident (irregular atrial tachyarrhythmia lasting more than 120 seconds) or persistent AF (AF incident lasting more than 600 seconds).

Panels A and B show mean percentage of burst pacing attempts resulting in AF.
Panels C and D show mean duration of AF as a proportion of the number of burst pacing attempts.

Panels A and C focus on the effect of pacing on AF, panels B and D focus on the effect of post-injury PUFAs on AF. *P<0.05, **P<0.01, ***P<0.001 compared to Unpaced, No PUFAs; ns = not significant.
Figure 20 – AERP and conduction time
Panels A and B show mean atrial effective refractory period measured from 5 atrial sites for each dog at 200 ms cycle length. Panels C and D show mean global atrial conduction time in sinus rhythm measured between the high right atrium and the left atrial appendage at 400 ms cycle length. Panels A and C focus on the effect of pacing, panels B and D focus on the effect of PUFA supplementation. In panel C \(*P<0.05\) compared to Unpaced, No PUFAs; in panel D \(*P<0.05\) compared to Paced 14 days, No PUFAs.
Figure 21 – Echocardiography
Panels A-D show percentage change in heart chamber sizes between pacemaker implantation and end study (left atrial systolic volume, left atrial diastolic volume, left ventricular end systolic volume and left ventricular end diastolic volume respectively). Panel E shows relative change in left ventricular ejection fraction. *P<0.05, **P<0.01, ***P<0.001 compared to Unpaced, No PUFAs.
Figure 22 – Phospholipid PUFA integration
Panel A shows change in integration of EPA and DHA into plasma phospholipids as a percent of total phospholipids. Panel B shows integration of EPA and DHA into atrial tissue phospholipids as a percent of total phospholipids. *P<0.05, **P<0.01, ***P<0.001 compared to Unpaced, No PUFAs.
Table 15 – Analysis of mRNA levels via QT-RT-PCR

<table>
<thead>
<tr>
<th></th>
<th>Unpaced, No PUFAs</th>
<th>Paced 7 days, No PUFAs</th>
<th>Paced 14 days, PUFAs 7 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen I</td>
<td>1.04±0.32</td>
<td>14±8*</td>
<td>20±13***</td>
</tr>
<tr>
<td>Collagen III</td>
<td>1.00±0.07</td>
<td>5.26±1.85***</td>
<td>26±15† ††</td>
</tr>
<tr>
<td>TGF-β</td>
<td>1.04±0.35</td>
<td>1.93±0.33**</td>
<td>1.06±0.77†</td>
</tr>
<tr>
<td>Smad7</td>
<td>1.04±0.32</td>
<td>4.66±2.47***</td>
<td>2.21±1.04††</td>
</tr>
<tr>
<td>Timp1</td>
<td>1.03±0.28</td>
<td>1.99±0.24**</td>
<td>1.94±0.82**</td>
</tr>
<tr>
<td>Akt1</td>
<td>1.00±0.07</td>
<td>1.98±0.73*</td>
<td>2.06±0.88**</td>
</tr>
<tr>
<td>EGF</td>
<td>1.03±0.27</td>
<td>1.90±0.49***</td>
<td>1.59±0.41**</td>
</tr>
<tr>
<td>JAM3</td>
<td>1.01±0.16</td>
<td>1.80±0.24***</td>
<td>1.32±0.33††</td>
</tr>
<tr>
<td>MHCα</td>
<td>1.02±0.23</td>
<td>1.98±1.12</td>
<td>1.80±1.54</td>
</tr>
<tr>
<td>MHCβ</td>
<td>1.01±0.16</td>
<td>0.31±0.18***</td>
<td>0.50±0.42**</td>
</tr>
<tr>
<td>CD99</td>
<td>1.04±0.31</td>
<td>2.26±1.17*</td>
<td>1.60±1.13</td>
</tr>
</tbody>
</table>

QT-RT-PCR values are presented in ΔΔCt units

*P<0.05, **P<0.01, ***P<0.001 compared to Unpaced, No PUFAs
†P<0.05, ††P<0.01, †††P<0.001 compared to Paced 7 days, No PUFAs
Figure 23 – ERK and Akt Western blot
Panels A-C show Western blot results for total, activated (phosphorylated), and the ratio of activated:total extracellular signal related kinase (ERK) isoforms 1 and 2. Panels D-F show Western blot results for total, activated, and the ratio of activated:total protein kinase B (Akt) isoform 1. **P<0.01 compared to Unpaced, No PUFAs; #P<0.05 compared to Paced 7 days, No PUFAs.
Panels A and B show levels of active caspase-3 in atrial tissue. Panel A focuses on the effect of pacing, panel B focuses on the effect of PUFA supplementation. In panel A, *P<0.05 compared to Unpaced, No PUFAs. In panel B, ***P<0.001 compared to Paced 14 days, No PUFAs and Paced 14 days, PUFAs 21 days.

Figure 24 – Caspase-3 activity
Figure 25 – Inflammation (CD18 and CD45)
Panel A shows mean grade of CD18 stained atrial tissue sections. A representative image of CD18 stained sections from each group is shown to the right of the graph (from left to right, Unpaced, No PUFAs; Paced 7 days, No PUFAs; Paced 14 days, PUFAs 7 days). Panel B shows mean number of positive cells in images of CD45 stained atrial tissue. A representative image of CD45 stained sections from each group is shown to the right of the graph (from left to right, Unpaced, No PUFAs; Paced 7 days, No PUFAs; Paced 14 days, PUFAs 7 days).
Figure 26 – Hypertrophy
Panels A and B show mean cross sectional area of atrial myocytes. 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. Panel A focuses on the effect of pacing, panel B focuses on the effect of PUFA supplementation. In panel A, ***P<0.001 compared to Unpaced, No PUFAs. In panel B, *P<0.05 compared to Paced 14 days, No PUFAs.
Figure 27 – Fibrosis
Panels A and B show mean collagen area fraction of atrial tissue sections as a percentage of the Unpaced, No PUFAs group. Panel A focuses on the effect of pacing, panel B focuses on the effect of PUFA supplementation.
Project Three - AF vulnerability, and the effect of prophylactic PUFA administration in a model of short term atrial stretch

Rationale

Previous results have demonstrated that the degree of AF vulnerability in the SAVP model in dogs is correlated with the degree of structural remodeling. It is unknown whether SAVP results in inflammation at any point, and whether this has any effect on AF vulnerability. It is also unknown whether n3 PUFAs have any effects on the atria in the absence of structural remodeling in the SAVP model. We designed this experiment to test whether AF vulnerability exists at a time when inflammation is estimated to be at peak levels, and before structural remodeling has become established. Additionally we looked to see whether n3 PUFAs have any effects on atrial electrophysiology, histology or gene expression early in the remodeling process.

Objectives

1. Establish the extent of AF inducibility and atrial cardiomyopathy which exists after 2 days of SAVP (especially with regard to inflammation)

2. Determine the effect of n3 PUFA supplementation after 2 days of SAVP with regard to AF vulnerability, inflammation, and structural remodeling

3. Determine whether n3 PUFAs have relevant effects in the absence of pacing

4. Determine whether placebo fat supplementation yields a different outcome in a group made to mimic the Paced 14 days, No PUFAs group from Project One with regard to AF vulnerability, atrial histology or gene expression

Hypothesis
Significant AF vulnerability will not be present after 2 days of SAVP due to the absence of significant structural remodeling.

**Methods**

**Groups**

Twenty five dogs were randomized into 4 groups as follows:

1. Six dogs were paced using SAVP at 220 bpm for 2 days, and were supplemented with oral olive oil (2.5 mL) daily for 9 days, beginning 7 days before the start of pacing (Paced 2 days, Olive oil 9 days)

2. Six dogs were paced using SAVP at 220 bpm for 2 days, and were supplemented with oral n3 PUFAs (460 mg EPA+380 mg DHA) daily for 9 days, beginning 7 days before the start of pacing (Paced 2 days, PUFAs 9 days)

3. Eight dogs were paced using SAVP at 220 bpm for 14 days, and were supplemented with oral olive oil (2.5 mL) daily for 21 days, beginning 7 days before the start of pacing (Paced 14 days, Olive oil 21 days)

4. Five dogs were sham operated, but unpaced, and were supplemented with oral n3 PUFAs (460 mg EPA+380 mg DHA) daily for 21 days

Data from all groups in all previous projects were included in some of the results presented here in order to provide context and aid in interpretation. These groups were the Unpaced, No PUFAs group, Paced 7 days, No PUFAs group, Paced 14 days, No PUFAs group, Paced 14 days, PUFAs 7 days group, and Paced 14 days, PUFAs 21 days group.
All methods were performed as described in Section Three; any modifications specific to this project are listed below.

**QT-RT-PCR**

Primers for genes of interest were obtained from ACGT Corp (ACGT Corp, Toronto, ON). TGF-β (forward) CAA GGA TCT GGG CTG GAA GTG GA (reverse) CCA GGA CCT TGC TGT ACT GCG TGT, TNF-α (forward) GGC TGA GCC GAC GTG CCA AT (reverse) AGG GCA CCC TTGG CCC TTG A. rpl13a was used as the housekeeping gene in both instances.

**CD18 stain**

CD18 staining was performed on 1 day formalin fixed, paraffin embedded tissue in this project. Analysis was performed as described above.

**TUNEL stain**

TUNEL stained sections of 1 day formalin fixed, paraffin embedded LAA tissue were analyzed by light microscopy at 40X magnification with an exposure time of 75ms. The entire specimen was scanned and images were taken of every area where positive staining was suspected. All images were carefully examined in a blinded fashion. Cells were considered positively stained if they had staining (brown) in the nucleus. Positively stained cells were identified as being cardiomyocytes or “other” (fibroblasts, inflammatory cells, etc.). The entire specimen was photographed again at 2X magnification with an exposure time of 500ms and the cross sectional area of the specimen was calculated.

**Results**

**Electrical**

**AF inducibility**
Inducibility and persistence of AF are presented in Figures 28 and 29. The Paced 14 days, Olive oil 21 days dogs from this project were compared to the Paced 14 days, No PUFAs dogs from Project One to see whether placebo supplementation affected AF inducibility. They were not found to differ in terms of AF incidence or persistence either in terms of number of episodes of AF induced, or mean episode duration. Therefore, data from the groups were combined in subsequent analyses (12 dogs from Project One and 5 olive oil supplemented from Project Three).

AF incidence and persistence increased with pacing duration, both in terms of number of AF episodes induced per burst pacing attempt, and mean length of episode induced per burst pacing attempt. However, no significant AF inducibility was evident after 2 days of pacing by any measure.

Unpaced, PUFAs 21 days dogs were compared to the combined Unpaced, No PUFAs dogs from Project Two to see whether PUFA supplementation affected AF inducibility in unpaced dogs. When “AF” was defined as any irregular atrial tachyarrhythmia >60 seconds, supplementation with PUFAs significantly increased inducibility of AF. However, if our normal definition of AF was used (irregular atrial tachyarrhythmia >120 seconds), there was a non-significant difference between the groups (P=0.54). A similar trend was seen when comparing Paced 2 days, PUFAs 9 days dogs to Paced 2 days, Olive oil 9 days dogs. PUFA supplemented dogs had a trend towards greater “AF” inducibility (if AF is anything >60 seconds) than olive oil dogs; the trend was much less evident when the more stringent definition of AF was used (AF is anything >120 seconds) (P=0.29). A 1 second representative “AF” recording from an Unpaced, PUFAs 21 days dog is shown in contrast to a 1 second representative AF recording from a Paced 14 days, Olive oil 21 days dog. The Paced 14 days recording exhibits a far more irregular pattern and possibly a faster atrial rate.
Episodes of "AF" from Unpaced, PUFAs 21 days dogs and Paced 2 days, PUFAs 9 days dogs were very likely to be pace terminated.

**AERP**

Electrical remodeling was assessed via AERP measurement; data are presented in Figure 30. There was no effect of pacing, PUFA administration or olive oil administration on AERP at 200 ms cycle length.

**Global conduction time**

Conduction time was used as a surrogate of conduction speed. Mean global atrial conduction time at 400 ms cycle length is presented in Figure 30. Conduction time increased significantly with pacing duration (7% increase after 7 days, 15% increase after 14 days as compared to unpaced dogs), however no conduction slowing was evident after 2 days of pacing. Paced 14 days, No PUFAs dogs were compared to Paced 14 days, Olive oil 21 days dogs to see whether there was an effect of olive oil on conduction time; no effect was evident. Similarly, no effect of PUFAs was seen on conduction time in the Unpaced or the Paced 2 days groups.

**Echocardiography**

Changes in mechanical function were assessed with echocardiography. Measurements were made of LASV, LADV, LVESV, LVEDV and EF; results are presented in Figure 31. Chamber volumes increased with increased pacing duration (7 days LASV +40%, LADV +48%, LVESV +97%, LVEDV +32%; 14 days LASV +73%, LADV +72%, LVESV +99%, LVEDV +52%); LVESV increased significantly after only 7 days of pacing. There were no significant increases in any chamber size after 2 days of pacing. EF decreased significantly after only 2 days of pacing, no further significant decrease occurred with increased pacing.
Unpaced, PUFAs 21 days dogs were compared to Unpaced, No PUFAs dogs from Project Two to see whether PUFA supplementation altered chamber size or mechanical function in the absence of pacing. No significant differences were found between the groups for any measurement [(%Δ LASV) -5±17 vs. -3±6 (P>0.05); (%Δ LADV) -1±14 vs. -7±15 (P>0.05); (%Δ LVESV) -7±12 vs. 11±40 (P>0.05); (%Δ LVEDV) -5±11 vs. -6±15 (P>0.05); (%Δ EF) 5±20 vs. 5±11 (P>0.05)]. Paced 2 days, PUFAs 9 days dogs were compared to Paced 2 days, Olive oil 9 days dogs. No significant differences were found between the groups for any measurement and both groups were similar to the unpaced groups (except in the case of EF where both Paced 2 days groups showed a similar, significant decrease) [(%Δ LASV) -7±15 vs. -16±10 (P>0.05); (%Δ LADV) 3±7 vs. -8±11 (P>0.05); (%Δ LVESV) 33±29 vs. 46±27 (P>0.05); (%Δ LVEDV) 7±9 vs. 8±11 (P>0.05); (%Δ EF) -40±13 vs. -32±18 (P>0.05)].

**Molecular**

**PUFA integration**

Plasma phospholipid PUFA levels were measured by gas-liquid chromatography; results are presented in Figure 32. The figure also includes results for Paced 14 days, No PUFAs and Paced 14 days, PUFAs 21 days groups originally published by Laurent et al. ¹². Results from Project Two indicated that pacing had no effect on phospholipid composition, therefore several paced groups have been combined in the No PUFAs group. A highly significant 1.4% (absolute) increase in EPA+DHA occurred in plasma after 7 days of PUFA supplementation (Paced 14 days, PUFAs 7 days group, P<0.001); an additional 0.69% increase (absolute) occurred after 21 days of supplementation (Unpaced, PUFAs 21 days and Paced 14 days, PUFAs 21 days groups combined).
Atrial tissue phospholipid PUFA levels were measured as well; results are presented in Figure 32. As above, unsupplemented groups have been combined. A very significant 25% (relative) increase in EPA+DHA in tissue phospholipids occurred after only 7 days of supplementation (Paced 14 days, PUFAs 7 days group, P<0.01). Only an additional 7% increase was observed with an extra 14 days of supplementation (Unpaced, PUFAs 21 days and Paced 14 days, PUFAs 21 days groups combined).

**QT-RT-PCR**

mRNA levels of selected structural remodeling genes were quantified via QT-RT-PCR; results are presented in Figure 33. New tissue samples from all previous projects were prepared and reassayed alongside tissue from Project Three so that values could be compared across all groups. TGF-β was increased significantly at 2 days of pacing, but was reduced dramatically by day 7 and 14 (although it remains approximately 3 fold higher at 14 days than in the unpaced group). Despite the low levels at 14 days of pacing, there was a trend towards further reduction in levels of TGF-β with prophylactic PUFA supplementation (P>0.05). Highly elevated expression at 2 days of pacing was attenuated (though not significantly) by prophylactic PUFAs (P=0.25).

TNF-α expression was elevated at 7 and 14 days of pacing, but not by 2 days. Both prophylactic and post-injury PUFAs significantly reduced expression at 14 days of pacing; prophylactic PUFAs yielded a greater reduction than post-injury.

**TNF-α level**

Inflammation was assessed by measuring TNF-α protein levels; results are presented in Figure 34. Tissue samples were prepared from Unpaced, No PUFAs and Paced 7 days, No PUFAs dogs from Project Two. These were analyzed alongside dogs from Project Three. Many of the dogs assayed had a TNF-α level below the detection threshold; these included 2 of the 4 Paced 2 days, Olive oil 9 days dogs, 5 of the 6
Paced 7 days, No PUFAs dogs, and 6 of the 6 Paced 14 days, Olive oil 21 days dogs. Although this was unexpected, the standard curve generated during the assay indicated the ELISA worked normally. The Unpaced, No PUFAs dog with a TNF-α level of 119, is the dog with the severe eye infection mentioned previously. Discounting the outlier in the unpaced group, it appears that TNF-α levels were elevated after 2 days of pacing, but were reduced after 7 days, and again after 14 days. Low n values precluded a proper statistical analysis. There was no significant difference detected between dogs paced 2 days and given olive oil vs. PUFAs; 3 of the 6 Paced 2 days, PUFAs 9 days dogs had TNF-α levels below the detectable threshold.

**Caspase-3 activity**

Induction of apoptosis was quantified by measuring caspase-3 activity; results are presented in Figure 34. Tissue from some Project Two dogs was reassayed along with tissue from Project Three dogs. From the previous projects it was determined that PUFAs do not have an effect on caspase-3 activity levels, therefore Unpaced, PUFAs 21 days dogs were used as controls. There did not appear to be an effect of pacing on caspase-3 activity; no change was seen after 2 days of pacing, after 7 days there was a significant decrease, but after 14 days there was a significant recovery in activity levels.

**Histology**

**Positive CD18 stain count**

A semi-quantitative measurement of inflammation was made by analyzing CD18 stained tissues; results are presented in Figure 35. Due to the presence of confounding infections in Unpaced, No PUFAs dogs (mentioned above), data from the Unpaced, PUFAs 21 days group are shown in the figure. A highly significant 179% increase in CD18 staining occurred after 2 days of pacing (P<0.01). By day 7 there was a
significant resolution of inflammation (82% reduction in staining); this resolution continued through day 14 (P<0.01). Prophylactic PUFA supplementation reduces CD18 staining at the 2 day timepoint; although not quite statistically significant, it is a strong trend (P=0.055).

**Positive TUNEL stain analysis**

Induction of apoptosis was quantified histologically with TUNEL staining; results are presented in Figure 35. As mentioned above, Unpaced, No PUFAs dogs are not presented in the figure due to the presence of infections in two dogs unrelated to pacing or PUFAs. There was a non-significant trend towards increased positive staining in Paced 2 days, Olive oil 9 days dogs that resolved with continued pacing (P=0.15). PUFAs appear to have no effect on the amount of positive stain observed; a comparison of Paced 2 days, Olive oil 9 days dogs to Paced 2 days, PUFAs 9 days dogs showed no difference between the groups (1.6±1.1 vs. 1.4±0.6 positive cells/mm², P>0.05).

**Myocyte cross sectional area**

Cellular hypertrophy was quantified by measuring myocyte cross sectional area; results are presented in Figure 36. A 53% increase was seen after only 2 days of pacing; continued pacing significantly increased cell size with a further 80% increase at day 7, and an additional 20% increase by day 14. Unpaced, PUFAs 21 days dogs were compared to the combined Unpaced, No PUFAs dogs from Project Two to see whether PUFA supplementation affected cell size in unpaced dogs. Unpaced, No PUFAs dogs had a mean cross sectional area of 191±39 µm² vs. 151±42 µm² for PUFA supplemented animals (P>0.05); thus PUFAs were not considered to affect cross sectional area in the absence of pacing. The Paced 14 days, Olive oil 21 days dogs were compared to the Paced 14 days, No PUFAs dogs from Project One to see if olive
oil supplementation affected cell size. Interestingly, olive oil fed dogs had a 181±51% increase in cell cross sectional area over Unpaced dogs vs. a 261±33% increase for non-olive oil fed dogs (P<0.05). The reason for this effect of olive oil is not clear.

**Collagen area fraction**

Fibrosis was quantified by measurement of collagen area fraction; results are presented in Figure 36. The Unpaced, PUFAs 21 days group was compared to the Unpaced, No PUFAs group from Project Two to see if PUFAs had any effect on collagen deposition in the absence of pacing. Unpaced, PUFAs 21 day dogs had a collagen area fraction of 5.5±0.5% vs. 7.4±2.7% for Unpaced, No PUFAs dogs (P>0.05). PUFAs were not considered to have any effect in the absence of pacing and all unpaced groups were combined.

Paced 14 days, Olive oil 21 days dogs were compared to the Paced 14 days, No PUFAs dogs from Project One to see if olive oil had any effect on fibrosis. Olive oil fed dogs had a mean increase in collagen area fraction of 194±76% over unpaced dogs versus a 151±33% increase for non-olive oil fed dogs (P<0.05). Olive oil was not considered to have any effect on fibrosis, and the groups were combined (8 dogs from Project One and 7 olive oil fed dogs from Project Three). There was a significant effect of pacing on collagen deposition; no increase in fibrosis was observed after 2 days of pacing, however a 51% increase occurred after 7 days, and a further 27% increase occurred after 14 days. The combined Paced 14 days, No PUFAs group was compared to the PUFA treated groups to see whether PUFAs could attenuate pacing induced fibrosis. Both post-injury and prophylactic PUFAs reduced fibrosis (P=0.11 for one way ANOVA Paced 14 days, No PUFAs vs. Paced 14 days, PUFAs 7 days vs. Paced 14 days, PUFAs 21 days). Prophylactic PUFAs significantly reduced fibrosis as compared to no PUFAs (P<0.05 via t test).
Discussion

To my knowledge, this is the first study to look at the effect of PUFA supplementation at multiple timepoints during the development of atrial cardiomyopathy and AF vulnerability. Results from this project suggest that short term pacing (2 days) produces significant inflammation, but no significant conduction slowing or AF vulnerability. Prophylactic PUFA administration may be anti-inflammatory, but has no effect on conduction time or AF vulnerability in short term pacing dogs.

Data from Projects One and Two suggest that AF vulnerability increases with increasing structural remodeling; data from this project further strengthens that theory. In most cases, as pacing duration increases, sequential increases in atrial enlargement, hypertrophy and fibrosis mirror sequential increases in conduction slowing and AF vulnerability (specifically moving from 2 days of pacing to 7 days, and again from 7 days to 14 days).

Histological evidence demonstrates significant inflammation occurs at 2 days of pacing. This is supported by molecular data showing TGF-β mRNA upregulation (which may be initiating resolution), and elevated TNF-α protein levels (an inflammatory molecule). Measurement of TNF-α mRNA levels only shows upregulation after the 2 day timepoint; the reason for this discrepancy between mRNA and protein measurements is unclear, however it should be noted that mRNA and protein are distinct and it is not necessarily true that TNF-α mRNA is being translated at the 7 day timepoint despite elevated mRNA levels. The lack of AF inducibility at 2 days indicates that the AF in this model is not related to inflammation. This is consistent with data from other studies which suggest inflammation on its own is not sufficient to induce AF.  

40
Prophylactic PUFA supplementation in short term pacing dogs shows a strong trend towards attenuation of inflammation. This anti-inflammatory effect of PUFAs is consistent with molecular data showing a trend towards reduction of TGF-β mRNA by PUFAs at 2 days. Also in support is data showing that both post-injury and prophylactic PUFAs reduce TGF-β and TNF-α expression as compared to unsupplemented animals during long term pacing. Attenuation of inflammation early on may contribute to the prevention of subsequent hypertrophy and fibrosis; this is one potential mechanism for the anti-remodeling effect of PUFAs. It is noteworthy that the measurement of TGF-β mRNA levels in the Paced 14 days, PUFAs 21 days group in this project is not in agreement with a measurement performed in the same group of dogs in a previous study. In that study, TGF-β expression was not decreased by prophylactic PUFAs. The reason for this discrepancy is not clear, however in this project the housekeeping gene was rpl13a which showed more consistency between samples than the 18S used previously. This could contribute to the different result seen in the other study.

It is also noteworthy that in the absence of structural remodeling (at 2 days of pacing), prophylactic PUFA administration showed a trend towards increased vulnerability to “experimental AF” (depending on how “AF” was defined). The differences between supplemented and non-supplemented groups were small and generally non-significant, but at a minimum it seems reasonable to speculate that PUFAs, at this dosage and in this model, do not act like AADs.

As in previous projects, data concerning apoptosis are unclear. TUNEL results seem to suggest elevated levels of apoptosis with short term pacing, but caspase-3 activity did not confirm that finding. A full discussion of the entire study can be found in Section Five.
**Interpretation**

Overall, this project suggests that short term pacing (2 days) does not induce significant AF vulnerability due to the absence of structural remodeling. Prophylactic PUFAs may be anti-inflammatory, and prevention of inflammation may play a role in attenuating subsequent hypertrophy and fibrosis.

**Next questions**

1. Is EPA the particular n3 PUFA responsible for producing the effects that have been observed throughout all the projects thus far, or is it DHA?
2. Are EPA and DHA more effective when given in combination?
Figure 28 – AF vulnerability (effect of pacing)
Vulnerability of dogs to an AF incident (irregular atrial tachyarrhythmia lasting more than 120 seconds) or persistent AF (AF incident lasting more than 600 seconds). Panel A shows mean percentage of burst pacing attempts resulting in AF. Panel B shows mean duration of AF as a proportion of the number of burst pacing attempts. *P<0.05, ** P<0.01 compared to Unpaced, No PUFAs.
Figure 29 – AF vulnerability (effect of supplementation)
Vulnerability of dogs to an AF incident (irregular atrial tachyarrhythmia lasting more than 120 seconds) or persistent AF (AF incident lasting more than 600 seconds). Panels A-C show mean percentage of burst pacing attempts resulting in AF.
Panel A compares 14 day paced dogs with no supplement vs. dogs that received olive oil. Panel B compares unpaced dogs that received no supplement vs. dogs that received PUFAs. Panel C compares 2 day paced dogs that received olive oil vs. dogs that received PUFAs. *P<0.05 compared to Unpaced, No PUFAs, ns = not significant. Panels D and E show representative 1 second tracings of arrhythmia resulting from burst pacing. The tracing in panel D is from an Unpaced, PUFAs 21 days dog and shows rapid, but regular atrial activity; the tracing in panel E is from a Paced 14 days, Olive oil 21 days dog and shows rapid, irregular atrial activity.
Figure 30 – AERP and conduction time
Panel A shows mean atrial effective refractory period at 200 ms cycle length. The left segment compares unpaced dogs with and without PUFAs, the center segment compares paced 2 days dogs with olive oil and with PUFAs, the right segment compares paced 14 days dogs with and without olive oil. Panels B-E show mean global atrial conduction time in sinus rhythm measured between the high right atrium and the left atrial appendage at 400 ms cycle length. Panel B focuses on the effect of pacing, panels C, D and E compare paced 14 days dogs with and without olive oil, unpaced dogs with and without PUFAs, and paced 2 days dogs with olive oil and PUFAs. *P<0.05 compared to Unpaced, No PUFAs; ns = not significant.
Figure 31 – Echocardiography

Panels A-D show percentage change in heart chamber sizes between pacemaker implantation and end study (left atrial systolic volume, left atrial diastolic volume, left ventricular end systolic volume and left ventricular end diastolic volume respectively). Panel E shows relative change in left ventricular ejection fraction.

*P<0.05, **P<0.01, ***P<0.001 compared to Unpaced, No PUFAs.
Figure 32 – Phospholipid PUFA integration
Panel A shows change in integration of EPA and DHA into plasma phospholipids as a percent of total phospholipids. Panel B shows integration of EPA and DHA into atrial tissue phospholipids as a percent of total phospholipids. **P<0.01, ***P<0.001 compared to No PUFAs.
Figure 33 – TGF-β and TNF-α mRNA levels via QT-RT-PCR
Panels A-C show TGF-β mRNA levels, panels D-E show TNF-α mRNA levels. Panels A and D focus on the effect of pacing, panels B and E focus on the effect of PUFA supplementation. Since TGF-β levels are elevated after 2 days of pacing, Panel C compares 2 day pacing dogs with and without PUFAs. In panels A and D *P<0.05 compared to Unpaced, No PUFAs, #P<0.05 compared to Paced 2 days, PUFAs 9 days. In panel E *P<0.05, **P<0.01 compared to Paced 14 days, No PUFAs. ns = not significant.
Figure 34 – Inflammation and apoptosis (TNF-α and caspase-3)
Panel A shows TNF-α protein levels in atrial tissue measured by ELISA. Panel B shows levels of active caspase-3 in atrial tissue. **P<0.01 compared to all other groups.
Figure 35 – Inflammation and apoptosis (CD18 and TUNEL)

Panels A and B show mean grade of CD18 stained tissue sections. Panel A focuses on the effect of pacing. Given that CD18 staining is significantly increased at 2 days, panel B compares 2 day pacing dogs with and without PUFAs. The line in panel B represents the level of staining in Unpaced, PUFAs 21 day dogs. \( P=0.055 \).

Panels C and D show mean number of positively stained TUNEL cells per mm\(^2\) in TUNEL stained tissue sections. Panel C shows the effect of pacing. Given that TUNEL staining is elevated at 2 days of pacing, panel D compares 2 day pacing dogs with and without PUFAs. \( \text{ns} = \text{not significant} \).
Figure 36 – Hypertrophy and fibrosis
Panel A shows mean cross sectional area of atrial myocytes. 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. Panels B and C show mean collagen area fraction of atrial tissue sections. **P<0.01, ***P<0.001 compared to Unpaced, No PUFAs; †P<0.05 compared to Paced 14 days, No PUFAs via t test.
Project Four - Effect of chronic, prophylactic, EPA or DHA alone on AF vulnerability in a model of established atrial cardiomyopathy

Rationale

Previous results have demonstrated that the n3 PUFAs EPA and DHA, when supplied in combination, attenuate AF vulnerability and atrial structural remodeling. We designed this experiment to test whether prophylactic administration of EPA alone, or DHA alone, could attenuate vulnerability to AF in the SAVP model in dogs.

Objectives

1. Determine AF vulnerability in EPA only dogs and DHA only dogs
2. Assess differences between EPA and DHA groups in terms of atrial enlargement, hypertrophy and fibrosis
3. Determine if supplemented EPA and DHA are integrated into plasma and tissue

Hypothesis

DHA will be more effective than EPA in reducing AF vulnerability.

Methods

Groups

Twelve dogs were randomized into 2 groups as follows:

1. Six dogs were paced using SAVP at 220 bpm for 14 days, and were supplemented with oral n3 PUFAs (1.0 g EPA) daily for 21 days, beginning 7 days before the start of pacing (Paced 14 days, EPA 21 days)
2. Six dogs were paced using SAVP at 220 bpm for 14 days, and were supplemented with oral n3 PUFAs (1.0 g DHA) daily for 21 days, beginning 7 days before the start of pacing (Paced 14 days, DHA 21 days)

Data from the Unpaced, No PUFAs group and the Paced 14 days, No PUFAs group from Project One, as well as the Paced 14 days, Olive oil 21 days group from Project Three were included in some of the results presented here in order to provide context and aid in interpretation.

All methods were performed as described in Section Three.

Results

Electrical

AF inducibility

Inducibility and persistence of AF are presented in Figure 37. Significant AF inducibility and persistence occurred with pacing in EPA supplemented dogs (similar to the level seen in Paced 14 days, No PUFAs dogs), but not in DHA supplemented dogs. This was evident looking at both the number of episodes of AF induced per burst pacing attempt, and the mean duration of AF episodes induced per burst pacing attempt.

AERP

Mean AERP was calculated in order to assess electrical remodeling; data are presented in Figure 38. There was no effect of pacing or different PUFA administration on AERP at 200 ms cycle length.

Global conduction time
Mean global atrial conduction time was measured at 400 ms cycle length to assess changes in conduction speed; data are presented in Figure 38. The Paced 14 days, No PUFAs group demonstrated significant conduction slowing with pacing (15% increase in conduction time as compared to unpaced; P<0.05). EPA and DHA supplementation do not show significant conduction slowing, however they did not completely abolish pacing induced conduction slowing either (approximately 10% increase in conduction time for both EPA and DHA supplemented as compared to unpaced dogs; P>0.05).

**Echocardiography**

Mechanical function was analyzed via echocardiography. Measurements were made of LASV, LADV, LVESV, LVEDV and EF; results are presented in Figure 39. Chamber volumes increased with pacing in all EPA and DHA supplemented dogs; significant increases were only seen in ventricular chambers (LVESV and LVEDV). There appeared to be a trend towards greater enlargement in EPA supplemented dogs over DHA supplemented dogs (EPA dogs LASV +82% vs. DHA dogs LASV +28% [P>0.05]; EPA dogs LADV +95% vs. DHA dogs LADV +23% [P>0.05]). EF decreased significantly with EPA (-46%), a non-significant decrease was observed in DHA supplemented dogs (-27%).

**Molecular**

*PUFA integration*

Plasma phospholipid PUFA levels were measured by gas-liquid chromatography; results are presented in Figure 40. The figure also includes results for all dogs from Projects One, Two and Three which were not supplemented with PUFAs combined
together in the No PUFAs group. Compared to unsupplemented dogs, the level of EPA in plasma increased significantly in the EPA supplemented group (0.33% [absolute], $P<0.05$). A non-significant 0.1% (absolute) increase in EPA was seen in the DHA supplemented group ($P>0.05$). Again compared to unsupplemented dogs, the level of DHA in plasma increased significantly in the DHA supplemented group (2.7% [absolute], $P<0.001$). A non-significant 0.4% (absolute) increase in DHA was seen in the EPA supplemented group ($P>0.05$).

Atrial tissue phospholipid PUFA levels were measured as well; results are presented in Figure 40. As above, unsupplemented groups have been combined. A very significant 45% (relative) increase in EPA in tissue phospholipids was seen in the EPA supplemented group ($P<0.01$). EPA levels in the DHA supplemented group were not different than the unsupplemented group. A highly significant 76% (relative) increase in DHA was seen in the DHA supplemented group ($P<0.001$). DHA levels in the EPA supplemented group were not different than the unsupplemented group.

**Histology**

*Myocyte cross sectional area*

Cellular hypertrophy was quantified by measuring myocyte cross sectional area; results are presented in Figure 41. Both EPA and DHA supplemented dogs showed significant increases in cell cross sectional area over unpaced dogs to a level nearly identical to that seen in Paced 14 days, Olive oil 21 days dogs. Neither EPA alone, nor DHA alone, exhibited any attenuation of cellular hypertrophy.

*Collagen area fraction*

Fibrosis was quantified by measurement of collagen area fraction; results are presented in Figure 41. Dogs supplemented with EPA or DHA did not have significantly
more fibrosis than unpaced dogs, however the variances in both EPA and DHA groups were quite large. Collagen area fraction was not different between Paced 14 days, No PUFAs dogs and EPA alone or DHA alone (P>0.05 for both t tests). DHA supplementation was associated with approximately 14% less fibrosis than EPA supplementation (P>0.05).

**Discussion**

To my knowledge, this is the first study to look at the effects of EPA and DHA separately in a model of atrial cardiomyopathy and AF vulnerability. Results from this project potentially suggest that DHA is more effective than EPA at attenuating long term pacing induced AF vulnerability and fibrosis.

DHA has a trend towards lower AF inducibility than both Paced 14 days, No PUFAs dogs and Paced 14 days, EPA 21 days dogs. Neither EPA nor DHA affect AERP, suggesting that neither EPA nor DHA have ion channel effects even at a 1.0 g/day dose (2-3 times the dose of EPA or DHA used in Projects One, Two and Three). DHA has a trend towards less atrial enlargement than EPA (though not ventricular enlargement). DHA also has a trend towards less fibrosis than EPA (though not hypertrophy).

The variance of the data in this project is quite large and it is underpowered to detect significant differences between the groups. Power calculation indicates a sample size of 31 per group is necessary to resolve a significant difference between the EPA and DHA group (with 80% power and P<0.05). However, it does underscore the fact that despite being molecularly similar, EPA and DHA may have (possibly substantially) different effects. This may be related to differences in metabolism and/or distribution of EPA and DHA in the body.
Integration data suggests that supplemented EPA and DHA are being incorporated into atrial tissue, however there does not appear to be significant conversion of supplemented EPA into DHA or vice versa. Given the lack of conversion, it seems reasonable that EPA supplementation would result in increased EPA metabolism only (similarly for DHA supplementation). EPA and DHA may be metabolized with differing efficiency, or produce products with differing properties in much the same way that arachidonic acid and EPA do \textsuperscript{109, 161}. EPA derived prostaglandins, thromboxanes and leukotrienes may suppress inflammation, as well as EPA derived class E Resolvins \textsuperscript{109, 161}. DHA derived class D Resolvins and Protectins have different mechanisms of suppressing inflammation \textsuperscript{161}. DHA, but not EPA, is also known to affect β-adrenergic function which has a whole host of downstream effects, potentially including effects on the TGF-β signaling pathway \textsuperscript{155, 115}.

EPA and DHA may also have different distributions in the body which affect their availability for metabolic activities. It has been suggested that DHA is efficiently concentrated in the heart whereas EPA is not well incorporated into cardiac phospholipids \textsuperscript{155}. It has also been suggested that DHA is incorporated primarily in the inner leaflet of the plasma membrane where it is perhaps more likely to be involved in signaling processes \textsuperscript{109}. Nevertheless, the metabolism and distribution of EPA and DHA are not well understood, and suggested mechanisms for differences in EPA and DHA activity remain speculative.

**Interpretation**

Prophylactic DHA supplementation is potentially more effective than EPA in reducing vulnerability to AF in long term pacing dogs.
Figure 37 – AF vulnerability
Vulnerability of dogs to an AF incident (irregular atrial tachyarrhythmia lasting more than 120 seconds) or persistent AF (AF incident lasting more than 600 seconds). Panel A shows mean percentage of burst pacing attempts resulting in AF. Panel B shows mean duration of AF as a proportion of the number of burst pacing attempts. Lines represent values for Paced 14 days, No PUFAs group. * P<0.05 compared to Unpaced, No PUFAs.
Figure 38 – AERP and conduction time
Panel A shows mean atrial effective refractory period measured from 5 atrial sites for each dog at 200 ms cycle length. Panel B shows mean global atrial conduction time in sinus rhythm measured between the high right atrium and the left atrial appendage at 400 ms cycle length. *P<0.05 compared to Unpaced, No PUFAs.
Figure 39 - Echocardiography
Panels A-D show percentage change in heart chamber sizes between pacemaker implantation and end study (left atrial systolic volume, left atrial diastolic volume, left ventricular end systolic volume and left ventricular end diastolic volume respectively). Panel E shows relative change in left ventricular ejection fraction. Dashed lines in panels represent values for Paced 14 days, Olive oil 21 days group. *P<0.05, **P<0.01, ***P<0.001 compared to Unpaced, No PUFAs.
Panels A and B show change in integration of EPA and DHA respectively into plasma phospholipids as a percent of total phospholipids. Panels C and D show integration of EPA and DHA respectively into atrial tissue phospholipids as a percent of total phospholipids. The dashed line represents the level of EPA or DHA in the Paced 14 days, PUFAs 21 days dogs from Project One (which received a combination of EPA+DHA).

*P<0.05, **P<0.01, ***P<0.001 compared to No PUFAs. In panel C ##P<0.01 compared to No PUFAs. In panel D ###P<0.01 compared to EPA 21 days.
Figure 41 – Hypertrophy and fibrosis
Panel A shows mean cross sectional area of atrial myocytes. 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. Panel B shows mean collagen area fraction of atrial tissue sections. Dashed lines in panels represent level of Paced 14 days, Olive oil 21 days group. *P<0.05 compared to Unpaced, No PUFAs.
Section Five: Discussion

In the four projects which comprise this study, we used SAVP at 220 bpm in dogs for 0, 2, 7 or 14 days to induce atrial cardiomyopathy and vulnerability to AF. We supplemented some dogs with ~1 g/day of oral PUFAs (EPA, DHA or both), either prophylactically or part way through the pacing protocol, in order to study the effect of PUFAs on pacing induced disease development.

To my knowledge, this is the first study to take a genome-wide approach to studying the effect of PUFA supplementation on gene expression in a model of atrial stretch induced cardiomyopathy. Also, I believe this is the first study to compare the effect of prophylactic and post-injury PUFAs in a model of AF, and to observe the effect of PUFAs on AF development at more than one timepoint.

Multiple lines of evidence including RNA microarray, mRNA levels, protein levels, histology, echocardiography, and electrophysiology generally agree that conduction slowing and AF vulnerability increase sequentially with increased pacing duration (short term to intermediate term to long term). This parallels increases in structural remodeling (atrial enlargement, hypertrophy, and fibrosis). Prophylactic PUFAs significantly attenuate conduction slowing, AF vulnerability, hypertrophy, and fibrosis. Post-injury PUFAs do not rescue conduction slowing or AF vulnerability, and do not reverse structural remodeling, but appear to arrest further development of these. Changes in the regulation and expression of key structural remodeling related molecules like EGF, Akt, TGF-β, and ERK were observed as a result of pacing. Many of these changes were attenuated or reversed by PUFAs, thus it is possible that PUFAs mediate their effects in this model via attenuation of the remodeling response at the genetic level.
Evidence from this study strongly suggests that the AF induced in this model is not related to electrical remodeling since no significant changes to AERP were observed in any experimental group. The AF does not seem to be directly related to inflammation either since at 2 days of pacing, when there is peak inflammation, there is little AF inducibility.

**Pacing induced disease development**

The timing of our molecular findings is supported by prior studies. Janicki et al. reviewed rat models of pressure/volume overload and noted that mechanical stress induced an initial compensatory hypertrophy/extracellular matrix (ECM) degradation phase, followed by a rapid fibrotic and continued hypertrophic phase that tapered off 14 days after the mechanical stress began. In our study, inflammatory molecules like CD99, JAM3 (both involved in leukocyte migration and adhesion), and TNF-α are upregulated significantly at the early timepoints (2 and 7 days of pacing), but return to unpaced levels by 14 days of pacing. Regulation of hypertrophic MHC-β is significantly altered by day 7, MHC-α levels are not significantly increased until day 14. Levels of collagens I and III, as well as fibrosis regulating Smad7 and Timp1 are significantly upregulated at day 7. Collagen continues to be upregulated at day 14, while Smad7 and Timp1 have declined (fibrosis inhibiting Smad6 is downregulated at day 14). A previous study by Laurent et al. also showed elevated matrix metalloproteinase (MMP9) activity (involved in ECM degradation) in paced dogs at 14 days. These findings (supported by the histological results) are consistent with the idea of a progressive development of disease involving early inflammation (which is resolved by day 7), and a developing hypertrophy and fibrosis (involving both collagen production and degradation i.e. collagen turnover) which may be tapering off by day 14.
Potential key signaling mediators of this cascade could include TGF-β, EGF, Akt, and ERK (see Figure 2). TGF-β is a powerful remodeling factor and is significantly upregulated at 2 days; it is known to play a role in transitioning tissue from an inflammatory state to a hypertrophic/fibrotic state. EGF is a potent mediator of mechanical stress induced signaling and is significantly upregulated at 7 days, but levels return to baseline by 14 days. Akt and ERK strongly promote hypertrophy and fibrosis. In general, the data support the idea that pacing increases regulation and expression of Akt and ERK (mRNA, protein or activated protein levels); levels of Akt (mRNA, protein and activated protein) appear to return to baseline by 14 days.

**PUFA effect on pacing induced disease development**

PUFAs are significantly integrated into both plasma and tissue after only 7 days. In tissue phospholipids, levels near the highest observed level in the whole study were observed after only 7 days of supplementation. Therefore, differences between prophylactic and post-injury PUFA supplementation are probably not related to a cumulative dose effect (dogs in the prophylactic PUFA group received more PUFA capsules) of PUFAs received during study, but rather due to the timing of the intervention. No acute effects of PUFAs were observed i.e. ion channel block, etc. Rather, detected actions of PUFAs were at the genetic level, altering expression of structural remodeling related genes. Prophylactic PUFAs significantly reduced expression of inflammatory genes (JAM3, CD99, TNF-α), also hypertrophic MHC-α, and fibrosis related Smad7 and Timp1 (also collagen I, III, and MMP9 activity). It may be considered contradictory that PUFAs reduce expression of fibrosis inhibiting genes like Smad7, however in the context of reduced collagen synthesis in PUFA treated animals, this finding likely relates to an overall reduction in collagen turnover.
PUFAs alter regulation of the potential key signaling mediators discussed above. Prophylactic PUFAs significantly reduce expression of EGF; mRNA and protein data generally suggest that PUFAs may reduce expression and activation of both ERK and Akt. Similarly, mRNA measurements suggest that PUFAs reduce TGF-β expression.

The observation of an anti-structural remodeling effect of PUFAs is consistent with observations made in other studies. A microarray study in a dog model of CHF and AF by Cardin et al. showed atrial ECM related gene expression was increased. The same group demonstrated that PUFAs attenuate pacing induced fibrosis. PUFA mediated reductions in remodeling related protein levels such as collagen I and IV, Akt, ERK, and TGF-β have been shown in a model of rat aortic banding, rat diabetic nephropathy, and the dog CHF model. Histological evidence of the anti-fibrotic and anti-hypertrophic effect of PUFAs has also been shown in these studies.

In this study, much emphasis has been placed on the ability of PUFAs to modulate the response to stress at the genetic level. That these findings may be of some relevance to human disease is suggested by the observation that there is overlap between genes found to be important in human AF and genes modified by both pacing and PUFAs in these dog projects (see Table 1).

**Potential alternative antiarrhythmic effects of PUFAs**

PUFAs given acutely at high doses may exert a direct antiarrhythmic effect *in vitro* and *in vivo*, perhaps via ion channel altering mechanisms. Inhibition of Na⁺ channels is the most commonly suggested mechanism. Individual cells were not studied in these projects, therefore we cannot say what effect PUFAs had on individual ion channels and action potential shape in the dogs. However, the integrated effect of PUFAs on the electrophysiology of the heart in this model does not support the idea that
the antiarrhythmic mechanism is Na\(^+\) channel blockade. Na\(^+\) channel blockers would be expected to slow conduction, however we observed an increase in conduction speed. That PUFAs do not act like direct AADs is supported by their failure to prevent ventricular tachycardia or ventricular fibrillation in human studies of patients with implantable cardioverter-defibrillators (ICDs)\(^{10}\). Other actions of PUFAs such as heart rate reduction or promotion of heart rate variability\(^{193-206}\), blood pressure reduction\(^{193, 198, 200, 204, 205, 207, 231}\), and improved lipid profiles\(^{193, 214, 218-237}\), may be true, but cannot satisfactorily explain our observations in this model of stretch induced vulnerability to AF.

**Potential mechanisms of PUFA attenuation of structural remodeling**

In general, this study suggests 2 potential mechanisms for PUFA mediated attenuation of structural remodeling. PUFAs may exhibit anti-inflammatory properties; prevention of inflammation could prevent subsequent hypertrophy and fibrosis. This is consistent with many published studies which suggest the mechanism of PUFA benefit in CVD is suppression or resolution of inflammation\(^{211, 214, 223, 233, 246, 248-251}\). However, post-injury PUFAs prevent worsening AF inducibility even though appreciable inflammation is not present at 7 days. This suggests a possibly direct effect of PUFAs on hypertrophy and fibrosis not related to inflammation.

Regardless of the specific mechanism, it seems evident from this study that PUFAs act like a “vaccine”. In order to prevent disease they must be present in the body before it begins; once the disease becomes established PUFAs may prevent further progression, but cannot reverse it.

**Relation to clinical studies**
The results of this study may help reconcile the seemingly contradictory results of clinical studies on PUFA supplementation in CVD \cite{9,256}. In patients supplemented with PUFAs, the DART \cite{187}, GISSI-Prevenzione \cite{175}, JELIS \cite{185}, and GISSI-Heart Failure \cite{288} trials noted significant decreases in cardiovascular endpoints. These results can be contrasted with findings from other trials including DART-2 \cite{176}, and the ICD trials mentioned above \cite{10} which did not note a benefit with PUFAs. These findings can be reconciled if PUFAs are considered anti-structural remodeling agents. Studies showing PUFA benefit primarily included post-MI or heart failure patients, in whom a significant remodeling component may be present, and followed patients for long enough to observe an anti-remodeling effect. The negative results in DART-2 and the ICD trials may be a consequence of the absence of important remodeling in the former, and the presence of irreversible remodeling and/or a lack of short term antiarrhythmic efficacy in the latter. Indeed, the most negative of the ICD trials had 60% of patients in New York Heart Association class III or IV at baseline. One of the few clinical trials dealing specifically with PUFAs and AF, the Cardiovascular Health Study, showed a 28-31% reduction in AF incidence in an elderly cohort of patients with high fish consumption, but without known atrial structural remodeling \cite{5}.

**Limitations**

The limitations of this study have been mentioned throughout the description of the methods, however some of the most critical limitations are listed here. This study employed a large animal, *in vivo* approach. As such, disease progression is multifactorial, and not well controlled. Failing ventricles and endocrine signaling prevent this from being a model of purely atrial stretch induced cardiomyopathy. The response to mechanical injury is complex and dynamic, involving repair cascades and negative
feedback loops. Relatively few timepoints were analyzed and important observations may have been missed, especially in regard to activation of signaling molecules (which is highly transitory). In general, this study yields correlations rather than causations, but provides a starting point for more detailed, *in vitro* studies which may yield more mechanistic insights.

**Future studies**

We were unable to establish direct mechanistic links between PUFA supplementation, signaling molecules, and cellular effects. The use of cell culture and small animal studies, where more tools are available for mechanistic research is recommended. The mechanism behind the putative anti-inflammatory, anti-hypertrophic, and anti-fibrotic properties of PUFAs can be studied in the following ways:

1. Cell cultures of atrial myocytes stimulated to initiate inflammation can be treated with PUFAs to observe any anti-inflammatory response
2. Cell cultures of atrial myocytes that are cyclically stretched in order to produce cellular damage can be treated with PUFAs to observe any anti-remodeling response
3. Cell cultures of atrial fibroblasts can be treated with PUFAs to see if there are effects on maturation and/or ECM production
4. A rodent model of atrial stretch induced structural remodeling (aortic banding, fistula, etc.) will still allow an *in vivo* approach, but will allow more timepoints and experimental conditions to be studied.

The results of this study suggest that PUFAs need to be started before significant structural remodeling has occurred in order to be effective. Designing a clinical trial to test this in humans is difficult. The subjects would have to be healthy at baseline,
therefore it may be difficult to determine whether or not they ever would have developed AF in the absence of any intervention. Additionally, the requirement for a healthy baseline population would almost certainly mean their average age would be young, thus requiring a long follow up time in order to observe any significant treatment effect. One potential patient population for such a clinical trial would be young paroxysmal AF patients. They may be less likely to have significant structural heart disease, but they have a high risk of developing persistent AF. They could be supplemented with PUFAs and ideally would be followed until death.
Section Six: Conclusion

The results of this study suggest that, in the SAVP model of atrial cardiomyopathy and AF vulnerability, n3 PUFAs at a dose of ~1 g/day may reduce AF via attenuation, at the genetic level, of adverse remodeling in response to mechanical stress.
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LIST OF PUBLICATIONS AND ABSTRACTS

Published Refereed Papers


Published Non-Refereed Papers

Dorian P, A Ramadeen. 2008. Omega-3 Polyunsaturated Fatty Acids (Fish Oils) and Heart Disease – Clinical Benefit or Just a Fad? Cardiology Rounds (Division of Cardiology, St. Michael’s Hospital, University of Toronto). 13(7).

Accepted Abstracts


APPENDIX ONE – COMBINED PROJECT SUMMARY

A  Study groups and timelines

Mongrel dogs, 1-3 years, 20-30 kg

Unpaced, No PUFAs  Unpaced, PUFAs

Paced, No PUFAs
(some received olive oil placebo)

-7 days  0  2  7  14

Implant pacemaker  Commence Pacing  End study  End study  End study

Paced, PUFAs
(some received EPA, or DHA, or combination)

-7 days  0  2  7  14

Implant Pacemaker  Start prophylactic PUFAs

B  Methods

Electrophysiology
- AF inducibility, AERP, conduction time

Echocardiography
- Atrial dimensions, ventricular dimensions, ventricular function

mRNA level measurement
- Microarray, QT-RT-PCR

Protein level measurement
- Western blot, ELISA

Histology
- Cell size, collagen area, inflammatory cells, apoptotic cells

Experimental methods – Panels A and B show the experimental groups and general methods used in all projects in this study.
Phospholipid PUFA integration – Panels A and B show EPA and DHA integration into plasma and tissue phospholipids as a % of total phospholipids. **P<0.01, ***P<0.001 compared to No PUFAs.
AF vulnerability – Panels A and B show mean duration of AF as a proportion of the number of burst pacing attempts. Panel A shows the effect of pacing, panel B shows the effect of PUFA supplementation. AF incidence is defined as an irregular atrial tachyarrhythmia lasting more than 120 seconds, persistent AF is an AF incident lasting more than 600 seconds. In panel A *P<0.05, **P<0.01 compared to Unpaced, No PUFAs. In panel B *P<0.05 compared to Paced 14 days, No PUFAs via t test.
Conduction time – Panels A and B show mean global atrial conduction time in sinus rhythm measured between the high right atrium and the left atrial appendage at 400 ms cycle length. Panel A shows the effect of pacing, panel B shows the effect of PUFA supplementation. In panel A *P<0.05 compared to Unpaced, No PUFAs; in panel B *P<0.05 compared to Paced 14 days, No PUFAs.
Panel A is an unsupervised hierarchical cluster of microarray data. The 12 rows of 6,467 coloured bars represent all significant ESTs for each of the 12 dogs analyzed. Bar colour indicates EST mRNA expression level; green (low), black (medium), red (strong). Dendrogram on the right depicts the relationship between individuals. Dendrogram scale is calculated via the “distance” method (line length inversely proportional to relatedness). CTRL indicates Unpaced, No PUFAs; HF indicates Paced 14 days, No PUFAs; PUFA indicates Paced 14 days, PUFAs 21 days individuals. Evidence of PUFA supplementation reversing the paced phenotype toward an unpaced phenotype is seen in 3 areas where the bar colours in the unpaced dogs match the colours in the PUFA dogs, but are opposite in colour to the paced only dogs (boxed in yellow).

Panels B-E are unsupervised, functional hierarchical clusters of microarray data. Microarray expression values from ESTs found to be significantly different between at least 2 groups were separated into functional categories and hierarchically clustered via an unsupervised algorithm. Panel B includes 17 fibrosis related ESTs, panel C includes 50 hypertrophy related ESTs, panel D includes 41 cell death related ESTs and panel E includes 22 inflammation related ESTs. Panels A-E are reprinted from Heart Rhythm, vol 7(4), A Ramadeen, G Laurent, CC dos Santos, et al., n-3 Polyunsaturated Fatty Acids Alter Expression of Fibrotic and Hypertrophic Genes in a Dog Model of Atrial Cardiomyopathy, 520-528, Copyright (2010), with permission from Elsevier.
TGF-β and TNF-α QT-RT-PCR – Panels A-D show TGF-β and TNF-α mRNA levels via quantitative real time PCR. Panels A-B show TGF-β mRNA levels, panels C-D show TNF-α mRNA levels. Panels A and C focus on the effect of pacing, panels B and D focus on the effect of PUFA supplementation. In panels A and C *P<0.05 compared to Unpaced, No PUFAs, #P<0.05 compared to Paced 2 days, PUFAs 9 days. In panel D *P<0.05, **P<0.01 compared to Paced 14 days, No PUFAs. ns = not significant.
ERK and Akt Western blot – Panels A-C show Western blot results for total, activated (phosphorylated), and the ratio of activated:total extracellular signal related kinase (ERK) isoforms 1 and 2. Panels D-F show Western blot results for total, activated, and the ratio of activated:total protein kinase B (Akt) isoform 1. *P<0.05, **P<0.01 compared to Unpaced, No PUFAs.
Inflammation – Panels A and B show mean grade of CD18 stained tissue sections. Panel A focuses on the effect of pacing. Panel B compares 2 day pacing dogs with and without PUFAs. The line in panel B represents the level of staining in Unpaced, PUFAs 21 days dogs. **P<0.01 compared to Unpaced, No PUFAs; #P<0.05, ##P<0.01 compared to Paced 2 days, Olive 9 days; &P=0.055.
Hypertrophy – Panels A and B show mean cross sectional area of atrial myocytes. 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. Panel A focuses on the effect of pacing, panel B focuses on the effect of PUFA supplementation. In panel A, ***P<0.001 compared to Unpaced, No PUFAs. In panel B, *P<0.05 compared to Paced 14 days, No PUFAs.
Fibrosis – Panels A and B show mean collagen area fraction of atrial tissue sections as a percentage of the Unpaced, No PUFAs group. Panel A focuses on the effect of pacing, panel B focuses on the effect of PUFA supplementation. **P<0.01 compared to Unpaced, No PUFAs. *P<0.05 compared to Paced 14 days, No PUFAs via t test.
Summary -

- 90 dogs
- 12 groups
- 0, 2, 7, 14 days SAVP
- Prophylactic and post-injury PUFAs
- EPA vs. DHA

- Gene microarray, mRNA levels, protein levels, protein activity, histology, echocardiography, electrophysiology

- PUFAs attenuate AF inducibility and conduction slowing in the SAVP model
  - PUFAs affect expression of structural remodeling related genes
  - PUFAs may arrest, but do not reverse, progression of structural remodeling
  - PUFAs may be anti-inflammatory, but may also have direct effects on hypertrophy and fibrosis

Figure lists summary information for the entire study. From the top: number of dogs, experimental groups, experimental results collected, general conclusions.
Dog dietary information – Dogs were fed Pedigree dog chow regularly. For 2-3 days after surgery dogs were fed Gastro Formula as it was typically better tolerated during the recovery period. Gastro Formula contains a small quantity of mackerel which contains n3 PUFAs.
APPENDIX THREE – STUDY DOGS

Baseline characteristics of study dogs

**Group A**  14 days SAVP 220bpm, 21 days EPA+DHA 850 mg/day

<table>
<thead>
<tr>
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<tbody>
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<td>2</td>
<td>KENT</td>
<td>M</td>
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<td>9</td>
<td>GEORGES</td>
<td>M</td>
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<td>10</td>
<td>DRAGON</td>
<td>M</td>
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<tr>
<td>11</td>
<td>VERNITA</td>
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</tr>
<tr>
<td>12</td>
<td>HONEY BUNNY</td>
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**Group B**  14 days SAVP 220 bpm, 0 days PUFAs

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<td>12</td>
<td>COPPER</td>
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**Group C**  14 days SAVP 220 bpm, 7 days EPA+DHA 850 mg/day

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<td>BRUCE</td>
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<td>5</td>
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<td>MOLSON</td>
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<td>M</td>
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### Group D 7 days SAVP 220 bpm, 0 days PUFAs

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<td>1</td>
<td>BO</td>
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<td>2</td>
<td>SCOOTER</td>
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<td>3</td>
<td>DANE</td>
<td>M</td>
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<tr>
<td>4</td>
<td>MADDY</td>
<td>F</td>
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<td>5</td>
<td>BOB</td>
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<td>6</td>
<td>BUCK</td>
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### Group E 0 days SAVP, 0 days PUFAs (not sham operated)

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### Group F 0 days SAVP, 0 days PUFAs (sham operated)

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<td>AVA</td>
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<td>HARRIETTE</td>
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### Group G 14 days SAVP 220bpm, 21 days EPA 1g/day

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<td>CURT</td>
<td>M</td>
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<td>3</td>
<td>GOURD</td>
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<td>4</td>
<td>FLOWER</td>
<td>F</td>
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<td>5</td>
<td>BUTTERCUP</td>
<td>F</td>
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<tr>
<td>6</td>
<td>LUCAS</td>
<td>M</td>
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### Group H 14 days SAVP 220bpm, 21 days DHA 1g/day

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<tr>
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<td>MARTINI</td>
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<td>REMY</td>
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<tr>
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<td>BUSTER</td>
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<td>FOXY LADY</td>
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<tr>
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<td>FABIO</td>
<td>M</td>
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**Group J**  0 days SAVP, 21 days EPA+DHA 850mg/day

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<td>5</td>
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**Group K**  2 days SAVP 220bpm, 9 days EPA+DHA 850mg/day

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<th>Sex</th>
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<td>3</td>
<td>LEXIE</td>
<td>F</td>
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<tr>
<td>4</td>
<td>SOX</td>
<td>F</td>
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<td>5</td>
<td>PINTO</td>
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<td>POOPY (MISHA)</td>
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**Group L**  2 days SAVP 220bpm, 9 days Olive oil 1g/day

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<td>FANTASIA</td>
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<td>DRACULA</td>
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<td>6</td>
<td>BUNTY</td>
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APPENDIX FOUR – CONTRIBUTORS TO THIS STUDY

The work presented in this study involved contributions from several individuals. I, Andrew Ramadeen, planned and directed the entire study, however certain specific tasks were performed by other people. Their roles are listed below. All other experimental work was performed by me. I performed all data analyses presented in this study. All graphs, tables, and figures in this study have been created by me.

Animal care

Dogs were selected from the breeder by me, but day-to-day care of the animals was performed by the Vivarium staff at St. Michael's Hospital (Toronto, ON). Surgical perioperative work for both pacemaker implantation and end study, including sedation, anesthesia, surgery, and recovery was performed by myself and Dr. Xudong Hu (St. Michael's Hospital).

Tissue samples for all pathology and molecular analyses were taken and preserved by myself and Dr. Xudong Hu.

The dogs used in Project One were selected by Dr. Gabriel Laurent (St. Michael’s Hospital). Surgical perioperative work for those dogs was performed by Dr. Xudong Hu and Dr. Gabriel Laurent.

Echocardiography

Recording and measurement of echocardiographic parameters was performed by Dr. Hiroko Fuji (St. Michael’s Hospital) and Dr. Xudong Hu, in my presence. I oversaw the design of the echo protocol and decided what measurements should be taken. Dr. Howard Leong-Poi (St. Michael's Hospital) verified the accuracy of the echo
measurements at the conclusion of the study. Once the data was verified, I performed all data analysis.

**PUFA integration**

I took and prepared plasma and tissue samples for the analysis. In most cases, I performed the phospholipid extraction myself. However, gas-liquid chromatography was performed by Mr. Jerry Piekarski at the Lipid Analytical Lab (Guelph, ON). I received the data from the mass spectrometer and performed all subsequent analyses.

**RNA microarray**

I prepared tissue samples for microarray analysis along with Ms. Kerri Thai (St. Michael’s Hospital). The microarray was run at The Centre for Applied Genomics (Toronto, ON). I received the raw data, and was taught by Dr. Claudia dos Santos (St. Michael’s Hospital) how to perform data normalization, and use microarray analysis software. I performed all subsequent analyses.

**PCR and Western blot**

I prepared the tissue samples for molecular analyses. PCR and Western blotting was performed by Ms. Kerri Thai. I was present for the reactions in several cases.

**ELISA**

I prepared the tissue samples for ELISA. The ELISA reaction was performed by Dr. Qingping Feng at the University of Western Ontario (London, ON). I received the raw data and performed all subsequent analyses.

**Caspase-3 activity assay**

I performed the caspase-3 activity assay with the assistance of Dr. Shehla Izhar (St. Michael's Hospital).

**Histology**
All staining was performed by the pathology department at St. Michael's Hospital (Toronto, ON), The Centre for Phenogenomics (Toronto, ON), or Hospital for Sick Children (Toronto, ON) (see Section Three of Experimental Work). I performed all histological analyses presented in this study.