

## **n-3 Polyunsaturated Fatty Acids Alter Expression of Fibrotic and Hypertrophic Genes in a Dog Model of Atrial Cardiomyopathy**

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**Short title:** n-3 PUFAs alter expression of remodeling genes

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**Disclosures**

None

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## **Abstract**

**Background:** We have previously shown that omega-3 polyunsaturated fatty acids (PUFAs) reduce vulnerability to AF. The mechanisms underlying this effect are unknown.

**Objective:** A genome wide approach was used to identify gene expression profiles involved in a new model of AF vulnerability, and to determine whether they were altered by PUFA therapy.

**Methods:** Thirty-six dogs were randomized evenly into 3 groups. Two groups were paced using simultaneous atrioventricular pacing (SAVP) at 220 bpm for 14 days to induce atrial enlargement, fibrosis and susceptibility to AF. One group was supplemented with oral PUFAs (850 mg/day) for 21 days, commencing 7 days before the start of pacing (SAVP-PUFAs); the other received no PUFAs (SAVP-No PUFAs). The remaining dogs were unpaced, unsupplemented controls (CTRL). Atrial tissue was sampled at the end of the protocol. Gene expression was analyzed in 4 dogs randomly selected from each group (n=12) via microarray. Results were confirmed with QT-RT-PCR and histology on all 36 dogs.

**Results:** Microarray or QT-RT-PCR results showed SAVP-No PUFAs dogs had significantly increased mRNA levels of Akt, EGF, JAM3, MHC $\alpha$ , CD99, and significantly decreased levels of Smad6 compared to CTRL dogs. QT-RT-PCR showed that PUFA supplementation was associated with significant down-regulation of Akt, EGF, JAM3, MHC $\alpha$  and CD99 levels compared to the SAVP-No PUFAs dogs.

**Conclusion:** The effect of PUFAs on these fibrosis, hypertrophy and inflammation related genes suggests that in this model, PUFA mediated prevention of AF may be due

to attenuation, at the genetic level, of adverse remodeling in response to mechanical stress.

**Word count:** 251 words

**Key words:** atria, fibrillation, PUFA, omega-3, remodeling, collagen, hypertrophy

**Abbreviations:**

Atrial fibrillation (AF)

Simultaneous atrioventricular pacing (SAVP)

Polyunsaturated fatty acids (PUFAs)

Eicosapentaenoic acid (EPA)

Docosahexaenoic acid (DHA)

Left atrial appendage (LAA)

Expression sequence tag (EST)

Robust-Multi Array Average (RMA)

Significance Analysis of Microarrays (SAM)

False discovery rate (FDR)

Hematoxylin and Eosin (H+E)

Extracellular signal related kinase (ERK1/2)

Myosin heavy chain (MHC)

Epidermal growth factor (EGF)

Protein kinase B (Akt1)

Rapid atrial pacing (RAP)

Rapid ventricular pacing (RVP)

Renin angiotensin system (RAS)

Extracellular matrix (ECM)

## Introduction

Atrial fibrillation (AF) is the most common arrhythmia and its prevalence is projected to rise more than 250% over the next few decades<sup>1</sup>. It is associated with substantial morbidity and mortality, primarily as a result of increased stroke risk<sup>2</sup>. The presence of an arrhythmogenic substrate due to mechanical and structural remodeling of the atria underlies most AF<sup>2</sup>. Current therapies for AF are limited in their efficacy, may cause adverse effects, and most do not treat the substrate which contributes to AF vulnerability<sup>3</sup>. Whilst experimental animal models such as pacing induced LV dysfunction in dogs may result in vulnerability to AF<sup>4</sup>, the application of this model to the majority of patients with AF is limited because of the resulting severe heart failure. Our group has created an experimental canine model of atrial pressure overload leading to atrial cardiomyopathy without overt LV failure. This simultaneous atrioventricular pacing model (SAVP) increases left atrial pressure and volume, causes structural and mechanical remodeling of the atria, conduction slowing and heterogeneity, and increased vulnerability to AF<sup>4-5</sup>.

Marine derived omega-3 polyunsaturated fatty acids (PUFAs) are termed “essential” fatty acids. In patients with heart failure, dietary supplementation with the PUFAs eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) reduces mortality, cardiac related hospitalizations; in post-MI patients they reduce all cause and sudden cardiac death<sup>6-7</sup>. The precise mechanism behind the beneficial effect of PUFAs is unknown, but hypotheses include antiarrhythmic<sup>8</sup>, antiinflammatory<sup>9</sup>, vascular/endothelial protective<sup>10</sup>, and/or lipid normalizing<sup>11</sup> properties.

We have previously shown that prophylactic PUFA administration prevents AF inducibility in the SAVP model, with a reduction in atrial fibrosis<sup>12</sup>. The objective of this study was to further characterize the effects of SAVP on the atria, and the effects of PUFAs in the SAVP model, via gene expression microarray, histological analyses, and confirmation of selected genes of interest with QT-RT-PCR.

## **Methods**

Detailed Methods are available in the online data supplement

### **Study Design**

The design has previously been described in detail<sup>12</sup>. The protocol was approved by the Animal Care Committee of St. Michael's Hospital, Toronto (ON, Canada). The investigation conforms to the *Guide for the Care and Use of Laboratory Animals*, US National Institutes of Health (NIH Publication N°.85-23, revised 1996). In brief, 36 mongrel dogs were randomized into 3 groups:

- 1) 12 dogs paced at 220 bpm for 14 days, supplemented with oral PUFAs (460mg EPA, 380mg DHA daily) for 21 days, commencing 7 days before the start of pacing (SAVP-PUFAs)
- 2) 12 dogs paced for 14 days, receiving no PUFAs (SAVP-No PUFAs)
- 3) 12 dogs unpaced and unsupplemented, serving as controls (CTRL)

On day 14 of pacing left atrial appendage (LAA) tissue samples were taken.

### **Phospholipid Analysis**

Lipids were extracted from frozen atrial tissue according to the method of Bligh and Dyer<sup>13</sup>. Phospholipid content was measured by gas-liquid chromatography. Sufficient quantities of tissue for this assay were not available for all dogs.

### **RNA Microarray**

RNA microarray was performed on LAA tissue at the Microarray Facility at The Center for Applied Genomics (TCAG, Hospital for Sick Children, Toronto, ON). One array was used per dog, 4 dogs per treatment group (n=12 total) (GeneChip Canine Genome 2.0 Array, Affymetrix, Santa Clara, CA). Data was normalized with the Robust-Multi Array Average (RMA) package in Bioconductor software (Bioconductor v2.1, [www.bioconductor.org](http://www.bioconductor.org)). After quality filtering, expression sequence tags (ESTs) were analyzed with the Significance Analysis of Microarrays (SAM) algorithm (SAM v3.02, <http://www-stat.stanford.edu/~tibs/SAM/>). Hierarchical clustering was performed with JMP statistical software (JMP v7.01, SAS Institute Inc). Significant ESTs were sorted according to intensity value fold change between treatment groups. Lists of ESTs significantly different between groups were analyzed separately by Ingenuity Pathway Analysis software (Ingenuity Systems, Redwood City, CA, <http://www.ingenuity.com/>). Gene lists were manually curated to determine function with the public databases PubMed (<http://www.ncbi.nlm.nih.gov/pubmed/>), SOURCE (<http://source.stanford.edu/cgi-bin/source/sourceSearch>), and the Ingenuity private database.

A public, normalized microarray dataset of humans with AF was downloaded from NCBI Gene Expression Omnibus (GEO) DataSets<sup>14</sup>. Two-class SAM analysis was performed. Significantly altered dog ESTs between the SAVP-No PUFAs and the CTRL

groups were converted to corresponding human ESTs with the Affymetrix

“canine\_v2\_hgu133\_plus\_complex\_match”

(<http://www.affymetrix.com/analysis/index.affx>). Significantly altered human ESTs were compared to converted dog ESTs to determine instances of overlap.

### **QT-RT-PCR**

QT-RT-PCR was performed with LAA tissue using standard techniques.

### **Caspase-3 Activity**

Caspase-3 assay was performed on LAA tissue with EnzChek Caspase-3 Assay Kit #1 (Molecular Probes, Eugene, OR) according to manufacturer’s instructions.

### **Cardiac Myocyte Cross Sectional Area**

Hematoxylin and Eosin (H+E) stained LAA tissue was analyzed at 40X magnification.

Approximately 25 images were taken per animal. Images presenting large collections of myocytes cut in cross section were selected for further analysis. Myocytes were judged to be cut in cross section if circular in shape, with a well defined membrane and large, circular, darkly staining nuclei appearing near the center of the cell. Images were analyzed with ImageJ software (Rasband, W.S., ImageJ, National Institutes of Health, Bethesda, Maryland, USA, <http://rsb.info.nih.gov/ij/>). All analysis was done with the investigator blind to the group association of the images.

### **Statistical Methods**

Statistical comparisons were performed using one way ANOVA with a Tukey’s post-hoc or Fisher’s least significant differences post test. A Kruskal-Wallis (non-parametric) test was used to confirm ANOVA results where stated.

## Results

### PUFA integration

Integration of omega-3 PUFAs into tissue phospholipids is presented in Table 1. No difference is seen between the CTRL group and the SAVP-No PUFAs group. After 3 weeks of PUFA supplementation, a 24% increase is seen in the level of total omega-3 fatty acids in the SAVP-PUFAs group compared to the SAVP-No PUFAs group ( $p < 0.01$ ). Groups were found to be significantly different for all variables via Kruskal-Wallis test as well ( $p < 0.05$ ).

### Microarray

42,860 canine ESTs were measured; after RMA normalization and quality filtration 37,504 ESTs remained. Multiclass SAM analysis was performed to identify the characteristic expression patterns of treatment groups (phenotypes). A total of 6,467 ESTs were identified as significantly changed, false discovery rate (FDR)  $< 4.6\%$  (delta 0.51). We allowed the FDR to rise so that we could later compare AF related gene expression pattern changes in the dogs with those documented in humans. Figure 1 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 SAVP and PUFAs. Assessment of gene expression values suggest that treatment with PUFAs returns gene expression to baseline values seen in CTRL animals.

To determine the effect of SAVP on gene expression we compared the expression profile of CTRL animals to SAVP-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 SAVP treatment ( $\geq 1.5$  fold increase/decrease between SAVP-No PUFAs and CTRL animals). To elucidate the molecular features of SAVP treatment on atrial gene expression, we used Ingenuity to search for enrichment in predicted functions. Table 2 shows an abridged list of the top significant ( $p < 0.01$ ) predicted functional enrichments as reported by the Ingenuity software. Overrepresented functions include fibrosis, hypertrophy, cell death and inflammation. To determine the effect of PUFAs on gene expression in SAVP we compared the expression profile of SAVP-PUFAs animals to SAVP-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 CTRL and SAVP-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 ( $\geq 1.5$  fold increase/decrease between SAVP-PUFAs and SAVP-No-PUFAs animals). Table 3 shows an abridged list of the top significant predicted functional enrichments of genes modulated by PUFAs as reported by the Ingenuity software. Overrepresented functions include 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 SAVP.

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 2. As with the large hierarchical cluster above, the dogs are 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 SAVP and PUFA supplementation on genes related to those functions; specifically a reversing effect of PUFAs back towards the CTRL phenotype is evident. SAVP demonstrates an effect on inflammatory genes, but no reversing effect of PUFA supplementation can be seen.

### **Human Microarray**

To validate our findings using human data we performed analyses on a publicly available microarray dataset obtained from NCBI GEO<sup>14</sup>. Data was obtained from human atrial tissue samples taken from 30 patients undergoing open heart surgery for valve repair or coronary artery bypass grafting; 20 in sinus rhythm with no history of AF, and 10 with persistent AF (duration >3 months). We used two-class SAM analysis and then compared differentially expressed genes identified in these patients with the pattern of gene expression identified in our dogs. Analysis of human data yielded ESTs that showed significant differences between patients in sinus rhythm vs. persistent AF. The 1,180 ESTs significantly changed by SAVP treatment (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, extracellular signal related kinase), EDN1 (endothelin-1) and CPNE8 (copine-8). Results are presented in Table 4.

### **QT-RT-PCR**

In order to confirm trends observed in the microarray, we chose genes involved in fibrotic, hypertrophic, inflammatory and cell death pathways to quantify via QT-RT-PCR. Data is presented in Table 5 along with corresponding microarray data. A significant effect of SAVP was seen on Smad6, myosin heavy chains (MHC)  $\alpha$  and  $\beta$  ( $p < 0.05$ ). A significant effect of PUFA supplementation was seen on Smad7, TIMP1, MHC $\alpha$ , CD99, epidermal growth factor (EGF) and protein kinase B (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 $\alpha$  ( $p = 0.11$ ). Where microarray data was discordant with PCR data, PCR data was taken as authoritative. Complete concordance between microarray results and QT-RT-PCR is almost never observed in published studies<sup>15</sup>.

### **Caspase-3 Activity**

Induction of apoptosis was quantified by measuring active caspase-3 levels. SAVP increased active caspase-3 by 57% from  $11.8 \pm 6.5$   $\mu\text{mol/mL}$  in the CTRL group to  $26.7 \pm 3.7$   $\mu\text{mol/mL}$  in the SAVP-No PUFAs group ( $p < 0.05$ ); PUFAs had no attenuating effect on caspase-3 activity ( $28.8 \pm 7.6$   $\mu\text{mol/mL}$  NS).

### **Cardiac Myocyte Cross Sectional Area**

Cellular hypertrophy was identified by measurement of myocyte cross sectional area. Results presented in Figure 3, revealed a 40% ( $p < 0.01$ ) increase in size by SAVP alone, whereas a 15% increase in size was seen with SAVP and PUFAs (NS compared

to CTRL,  $p < 0.01$  compared to SAVP-No PUFAs). Groups were found to be significantly different via Kruskal-Wallis test as well ( $p < 0.05$ ).

## **Discussion**

This is, to our knowledge, the first study to take a genome wide approach to examine the effect of PUFA supplementation on gene expression in a model of atrial mechanical stress causing cardiomyopathy. Our previous study<sup>12</sup> demonstrated that prophylactic, chronic, low dose (<1g/day) PUFA supplementation attenuated structural remodeling (fibrosis) and AF vulnerability brought about by SAVP. Analysis of gene expression as well as histology showed that SAVP increases fibrosis, hypertrophy and apoptosis, while PUFAs attenuate fibrosis and hypertrophy. PUFAs significantly downregulate levels of key remodeling proteins EGF and Akt. These results suggest that PUFA mediated reduction in AF vulnerability in this model could be related to attenuation of the remodeling response at the genetic level.

### **Remodeling in AF**

Atrial structural and mechanical remodeling is a significant cause of AF. While AF frequently co-exists with congestive heart failure, many patients do not exhibit severe LV systolic dysfunction<sup>2</sup>. Experimental models of AF include vagal stimulation, rapid atrial pacing (RAP) and rapid ventricular pacing (RVP)<sup>16-17</sup>. Neither vagal stimulation nor RAP induce significant structural remodeling; RVP causes significant remodeling and has thus far been the standard model of cardiomyopathy induced AF<sup>4,17-18</sup>. However, RVP causes severe ventricular dysfunction, with ascites, pulmonary edema, renal failure and frequent mortality<sup>4</sup>. SAVP is a model of more selective atrial

cardiomyopathy. It produces an immediate increase in atrial pressure and a decrease in left atrial emptying function which lasts throughout the 2 weeks of pacing, resulting in atrial stretch and structural remodeling<sup>4-5</sup>.

After 14 days, SAVP is associated with significantly increased conduction heterogeneity, AF incidence (20±14% vs. 1±2% of burst attempts leading to AF, SAVP-No PUFAs vs. CTRL, p<0.01) and maintenance (1598 sec vs. 0 sec median AF episode duration, SAVP-No PUFAs vs. CTRL, p<0.01), and significantly decreased conduction velocity<sup>4-5,12</sup>. It is also associated with significantly increased atrial dimensions, decreased left atrial function, increased collagen turnover, fibrosis (11±6 vs. 1±0.2, collagen I mRNA level (arbitrary units), SAVP-No PUFAs vs. CTRL, p<0.05), hypertrophy and induction of apoptosis. This remodeling of both the structure and mechanical function of the atria is likely the cause of the increased vulnerability to AF. Microarray and PCR data strongly indicate that the regulation of fibrosis related genes like Smad6, and hypertrophy related genes like MHC $\alpha$  and  $\beta$  are affected by pacing along with other proremodeling factors such as EGF and Akt. Microarray and protein data indicate an effect of pacing on cell death related genes. Caspase-3 activity was significantly increased in SAVP animals suggesting a proapoptotic effect of pacing.

### **The effect of PUFAs on SAVP induced remodeling**

We have shown here and previously that PUFA supplementation increases levels of EPA and DHA in both plasma<sup>12</sup> and tissue phospholipids, and significantly decreases atrial electrical dysfunction despite SAVP (6±7% vs. 20±14% of burst attempts leading to AF, SAVP-PUFAs vs. SAVP-No PUFAs, p<0.01; 601 sec vs. 1598 sec median AF episode duration, SAVP-PUFAs vs. SAVP-No PUFAs, p<0.05). PUFA supplementation

also reduced collagen turnover and fibrosis ( $1 \pm 0.5$  vs.  $11 \pm 6$  collagen I mRNA level (arbitrary units), SAVP-PUFAs vs. SAVP-No PUFAs,  $p < 0.05$ )<sup>12</sup>. Microarray and PCR data suggest that PUFAs reverse the SAVP induced profibrotic and prohypertrophic phenotype by altering expression of Smad7, TIMP1, MHC $\alpha$ , EGF and Akt. Despite indication from the microarray that PUFAs were reversing the proapoptotic/pronecrotic phenotype in SAVP animals, we could not confirm that effect via caspase-3 activity. Cell death is a time dependant phenomenon and it is probable that our analysis at a single timepoint did not allow us to observe a PUFA effect on this parameter if it exists. The timing of our molecular findings is supported by prior studies. Janicki et al.<sup>19</sup> reviewed rat models of pressure/volume overload and noted 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. On day 14 of our study in our SAVP-No PUFAs dogs, we observe increased collagen I,III and TGF- $\beta$  mRNA, along with increased MMP9 activity<sup>12</sup>, coupled with reduced Smad6 mRNA content and increased TIMP1 expression. Thus we observed increased transcription of genes involved in both collagen synthesis and degradation. To our surprise, PUFA treated animals demonstrated reduced expression of genes involved in the inhibition of MMP activity and collagen turnover, such as Smad7, which plays a role in the inhibition of TGF- $\beta$  activity. Whilst at first this appears contradictory, in the context of reduced collagen synthesis in PUFA treated animals, this likely relates to an overall reduction in collagen turnover.

One possible explanation for the antiremodeling effect of PUFAs is that they interfere with the initial compensatory hypertrophy/ECM degradation phase. As a result, they

may alter transcription of genes involved in both collagen synthesis and degradation. For example, collagen levels as well as MMP9 levels and activity are low after PUFAs in this model<sup>12</sup>. As such, there is no need for negative feedback regulation of counterregulatory genes like Smad7 and TIMP1. These effects may be mediated by inhibition of MAP kinase activity (ERK1/2 or p38) as first suggested by Sakabe et al.<sup>18</sup>, or by inhibition of key signaling molecules like EGF or Akt as seen in this study. PUFAs may act on upstream components of the renin angiotensin system (RAS), and may be similar in action to other RAS inhibitors like angiotensin converting enzyme inhibitors, angiotensin receptor blockers and matrix metalloproteinase inhibitors. These compounds have been shown to prevent the development/maintenance of AF in both animal<sup>20-22</sup> and human studies<sup>23-25</sup>. They have been shown to prevent or even reverse arrhythmogenic substrate development, specifically fibrosis<sup>20-22</sup>. The exact mechanism by which PUFAs mediate their antiremodeling effect is however still speculative, and requires further investigation.

### **PUFAs as antiremodeling agents**

Although there exist many hypotheses to explain the beneficial effects of PUFAs on cardiovascular disease, our key finding of an antiremodeling effect of PUFAs can be demonstrated at the genetic, protein, histological and functional levels by our studies as well as others. Our current and previous study<sup>12</sup> show mRNA expression data to indicate that PUFAs attenuate SAVP induced remodeling. Similarly, a microarray study in a dog model of RVP by Cardin et al. showed atrial ECM related gene expression was increased<sup>17</sup>. The same group demonstrated that PUFAs attenuate pacing induced fibrosis<sup>18</sup>. PUFA mediated reductions in remodeling related protein levels such as

collagen I and IV, Akt, ERK1/2 and TGF- $\beta$  have been shown in a model of rat aortic banding<sup>26</sup>, rat diabetic nephropathy<sup>27</sup> and the dog RVP model<sup>18</sup>. Histological evidence of the antifibrotic and antihypertrophic effect of PUFAs in the SAVP model were demonstrated in our current and previous study<sup>12</sup>. Similar findings are reported in the rat and dog RVP models<sup>18,26-27</sup>.

PUFAs, when given acutely at high doses, may exert a direct antiarrhythmic effect in vitro<sup>28</sup> and in vivo<sup>8</sup>, perhaps via ion channel altering mechanisms. However, they do not prevent VT or VF in randomized human studies of patients with ICDs<sup>29</sup>. Other actions of PUFAs such as heart rate<sup>30</sup> and blood pressure reduction<sup>10</sup>, reduction of inflammation<sup>9</sup> and improved lipid profiles<sup>11</sup> cannot satisfactorily explain our observations in this model; none of these studies involved AF or modeled mechanical stress.

### **Clinical studies with PUFAs**

The results of this study may help reconcile seemingly contradictory results of clinical studies of PUFA supplementation on cardiovascular disease<sup>31</sup>. In patients supplemented with PUFAs the DART<sup>32</sup>, GISSI-Prevenzione<sup>6</sup>, JELIS<sup>33</sup>, and GISSI-Heart Failure<sup>7</sup> trials noted significant decreases in cardiovascular endpoints. These results can be contrasted with findings from other trials including DART-2<sup>34</sup>, and some ICD trials<sup>29</sup> which did not note a benefit with PUFAs. These findings can be reconciled if PUFAs are considered antiremodeling 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 antiremodeling 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. 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<sup>35</sup>.

### **Limitations**

Due to the complexities of working with a large animal in vivo model we are unable to establish direct mechanistic links between PUFA supplementation, signaling molecules and cellular effects in this study. However, this hypothesis-generating study provides a starting point for more detailed in vitro mechanistic studies. Additionally, the mechanical damage response is a dynamic process that begins at the moment of injury and involves both injury repair cascades and negative feedback loops. We studied only a single timepoint at the 14 day mark of these processes. Finally, there are other potentially important mechanisms which were not studied that may mediate the PUFA effect, specifically the inhibition of inflammation.

### **Clinical Implications**

Currently, treatment of AF aims to control symptoms and reduce risk of stroke. Little data exists to support therapies aimed at prevention. We have previously shown that prophylactic, chronic, low dose PUFA supplementation was associated with a reduction in AF vulnerability in this model. Genetic and histological analyses revealed an association between PUFA supplementation and attenuation of atrial remodelling, highlighting several key remodeling genes. A comparison with data from humans in persistent AF demonstrated the relevance of some of these genes to human disease. These findings support the idea that PUFA therapy may prevent the development of an

arrhythmogenic substrate in the atria. If this is true, it may suggest that PUFAs may be most effective in disorders with a remodeling component such as post-MI, and hypertension, and may be less effective in scenarios where remodeling is not relevant to disease pathology. They also suggest that PUFAs would be most effective when given prophylactically rather than after remodeling had already occurred. These findings require confirmation in human studies as a potential therapeutic strategy against the development of AF.

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## Figure Legend

**Figure 1** – Unsupervised hierarchical clustering of RNA microarray expression values  
RNA microarray expression values from 6,467 ESTs found to be significantly different between at least 2 treatment groups were hierarchically clustered via an unsupervised algorithm. The 12 rows of 6,467 colored bars represent all significant ESTs for each of the 12 dogs analyzed. Bar color indicates EST mRNA expression level and the scale runs from green (low expression) to black (medium expression) to red (strong expression). The 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, unsupplemented individuals, HF indicates SAVP-No PUFAs individuals (heart failure); PUFA indicates SAVP-PUFAs individuals. Evidence of PUFA supplementation reversing the SAVP phenotype back toward a CTRL phenotype is clearly seen in 3 areas where the bar colors in the CTRL dogs match the colors in the SAVP-PUFAs dogs, but are opposite in color to the SAVP-No PUFAs dogs (boxed in yellow).

**Figure 2** – 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. Color scale runs from green (negative expression) to black (weak expression) to red (strong expression). 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, unsupplemented individuals; HF indicates SAVP-No PUFAs individuals (heart failure); PUFA indicates SAVP-PUFAs individuals. The top panel includes 17 fibrosis related ESTs, the second includes 50 hypertrophy related ESTs, the third includes 41 cell death related ESTs and the fourth includes 22 inflammation related ESTs.

**Figure 3 – Analysis of myocyte hypertrophy**

Paraffin embedded sections of left atrial appendage tissue were stained with H+E and analyzed via light microscopy under 40X magnification to measure myocyte cross sectional area. 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. Representative images with several cells matching the criteria are shown (\*\* $p < 0.01$ ).

**Table 1 – Left atrial tissue integration of PUFAs (% of total phospholipids).**

	<b>CTRL</b>	<b>SAVP-No PUFAs</b>	<b>SAVP-PUFAs</b>
	<b>(n=5)</b>	<b>(n=9)</b>	<b>(n=10)</b>
EPA	0.66±0.15	0.60±0.22	0.88±0.23*
DHA	1.89±0.32	1.87±0.34	2.54±0.25**
Total n3	4.63±0.52	4.25±0.62	5.27±0.60**

\*p<0.05 compared to SAVP-No PUFAs

\*\*p<0.01 compared to SAVP-No PUFAs

EPA – eicosapentaenoic acid, DHA – docosahexaenoic acid

**Table 2 – Effect of pacing alone on gene regulation – Functional analysis.**

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**Genes with  $\geq 1.5$  fold expression change: SAVP-No PUFAs vs. CTRL (878 genes analyzed)**

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

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**Table 3 – Effect of PUFAs on gene regulation of paced dogs – Functional analysis.**

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**Genes with  $\geq 1.5$  fold expression change: SAVP-PUFAs vs. SAVP-No PUFAs (151 genes analyzed)**

<b><i>Gene Functions</i></b>	<b><i>p value</i></b>
Immune disorders, cell death of immune/inflammatory cells	<0.01
Muscular hypertrophy, fibrosis and disease, arthritis	<0.01
Cell to Cell Signaling (Migration of connective tissue cells, fibroblasts, neutrophils, lymphocytes)	<0.01
Lipid Metabolism (Biosynthesis, metabolism and modification of lipids)	<0.01
Growth, proliferation, hypertrophy of multiple cell types (Immune/inflammatory and muscle cells)	<0.01
Coagulation of fluids and clotting	<0.01
Cardiac Dysfunction	<0.01
Cardiac Fibrosis	<0.01
Cardiac Enlargement	<0.01

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**Table 4 – Genes with significantly altered regulation in human persistent AF<sup>14</sup> and dog atrial cardiomyopathy.**

Gene symbol	Gene title	Function	Expression fold change	
			SAVP- No PUFAs vs. CTRL	SAVP- PUFAs vs. SAVP- No PUFAs
C9orf45	chromosome 9 open reading frame 45	Double-stranded RNA binding, cell differentiation	≤0.67	1.30
STRBP	spermatid perinuclear RNA binding protein			
CAMK2D	calcium/calmodulin-dependent protein kinase (CaM kinase) II delta	protein kinase activity, regulation of cell growth	≥1.50	0.82
CPNE8	Copine VIII		≤0.67	≥1.5
CXXC5	CXXC finger 5	DNA binding	≤0.67	1.41
EDN1	endothelin 1	hormone activity, activation of PKC, cell-cell signaling	≤0.67	≥1.5
FRMD4A	FERM domain	cytoskeletal protein binding	≥1.5	0.76

	containing 4A			
LGR4	leucine-rich repeat- containing G protein- coupled receptor 4	protein-hormone receptor activity	$\geq 1.5$	0.77
MAPK1	Mitogen-activated protein kinase 1	MAP kinase activity, serine/threonine kinase activity, cell cycle, chemotaxis, induction of apoptosis	$\geq 1.5$	$\leq 0.67$
MED28	Mediator complex subunit 28	actin binding, regulation of transcription	$\geq 1.5$	0.69
RBJ	rab and DnaJ domain containing	heat shock protein binding	$\geq 1.5$	0.88
SLC44A2	Solute carrier family 44, member 2	regulation of I-kappaB kinase/NF-kappaB cascade	$\geq 1.5$	0.92

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**Table 5 – Analysis of mRNA levels via QT-RT-PCR and RNA Microarray.**

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

(P) = QT-RT-PCR values (presented in  $\Delta\Delta\text{Ct}$  units)

(M) = RNA microarray values (presented in normalized fluorescent intensity units)

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\*p<0.05 compared to CTRL, \*\*p<0.01 compared to CTRL, \*\*\*p<0.001 compared to CTRL

†p<0.05 compared to SAVP-No PUFAs, ††p<0.01 compared to SAVP-No PUFAs, †††p<0.001 compared to SAVP-No PUFAs

**Figure 1**  
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**Figure 1.**

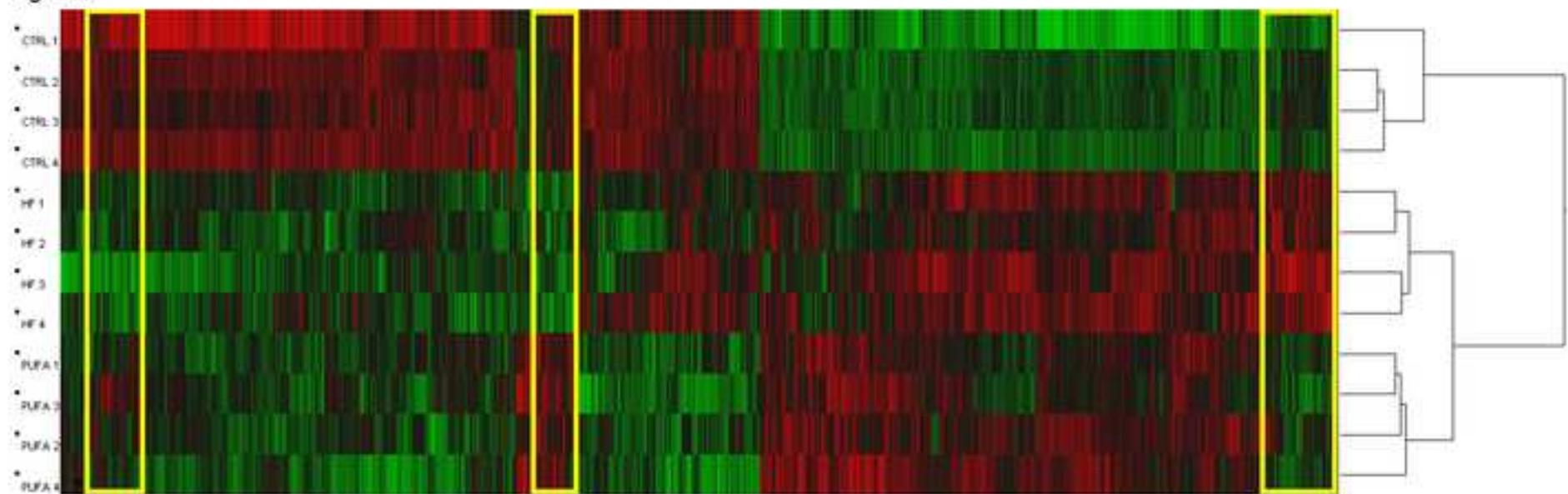


Figure 2.

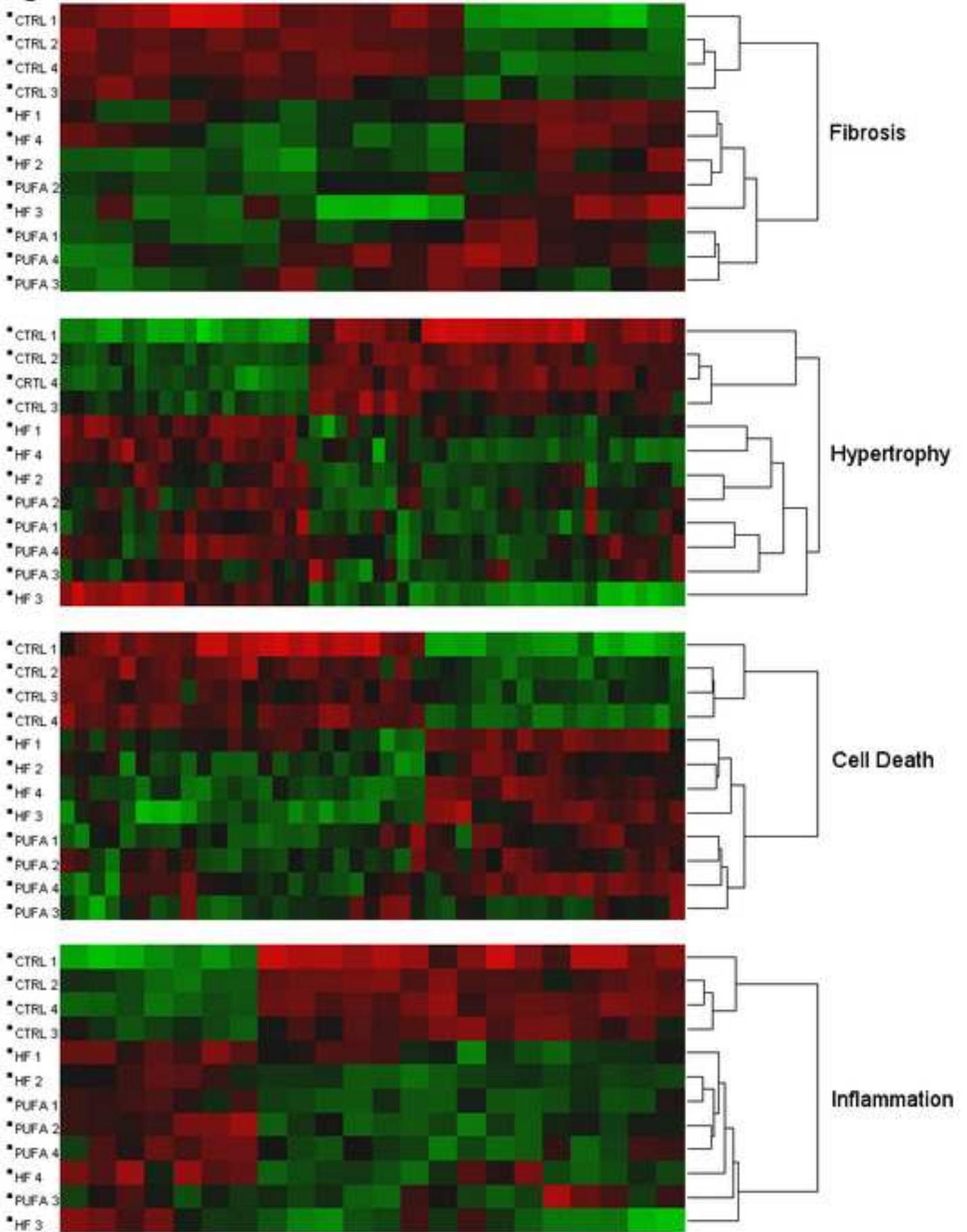
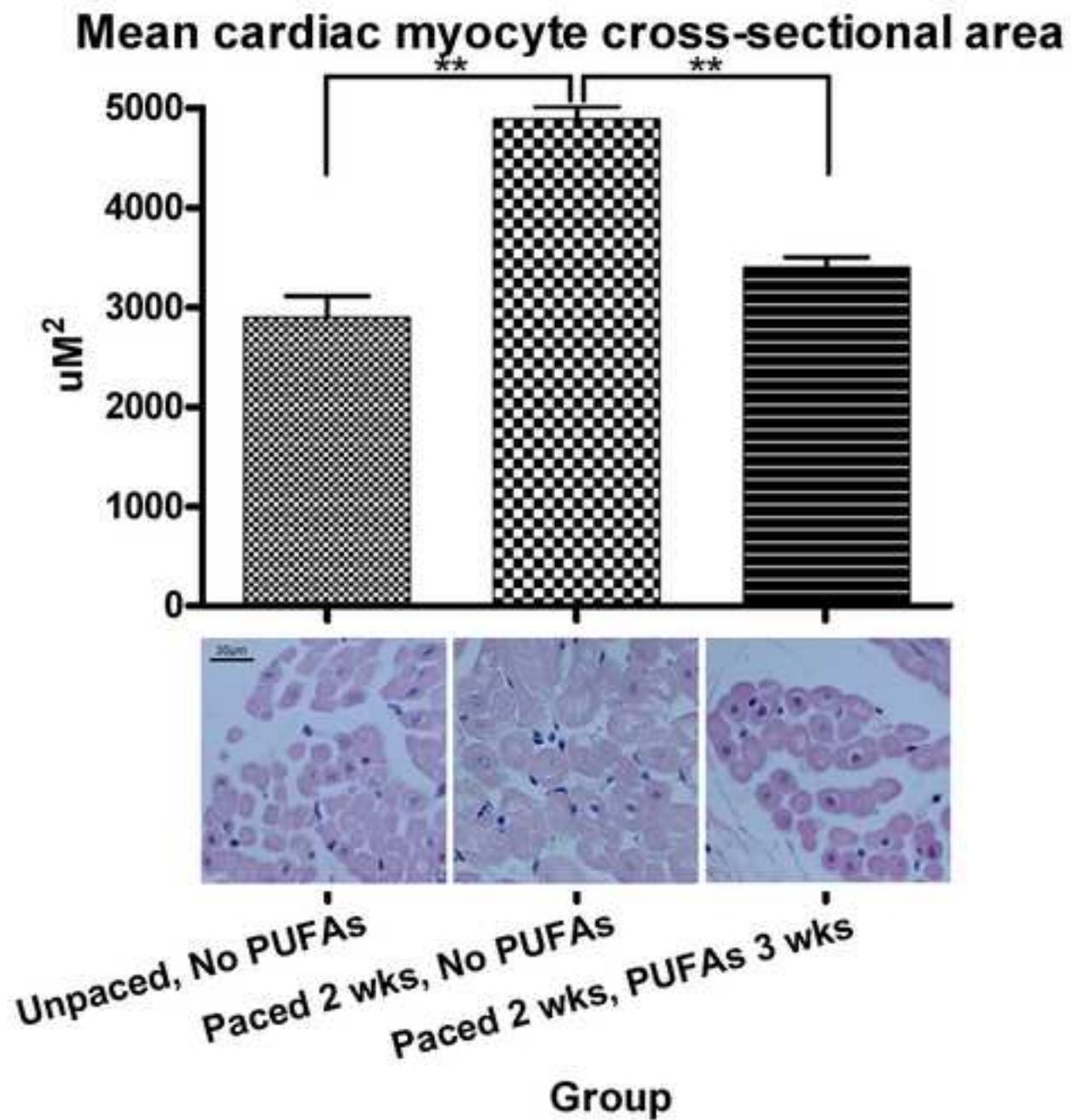


Figure 3.



**Supplementary Material**

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