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Dedication Page

To my husband who offered me an unconditional love and support throughout my study, my son who is the light of my life, and to my parents and siblings who waited long for this to happen and were a great source of motivation
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**List of Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>AA</td>
<td>Arachidonic Acid</td>
</tr>
<tr>
<td>ACE</td>
<td>Angiotensin Converting Enzyme</td>
</tr>
<tr>
<td>AF</td>
<td>Atrial Fibrillation</td>
</tr>
<tr>
<td>AngII</td>
<td>Angiotensin II</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>ANP</td>
<td>Atrial Natriuretic Peptide</td>
</tr>
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<td>Asn</td>
<td>Asparagine</td>
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<tr>
<td>BNP</td>
<td>Brain Natriuretic Peptide</td>
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<tr>
<td>Bp</td>
<td>Blood Pressure</td>
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<td>Calcium</td>
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<td>CAD</td>
<td>Coronary Artery Disease</td>
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<td>cGMP</td>
<td>Cyclic Guanosine Monophosphate</td>
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<td>COL</td>
<td>Collagen</td>
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<td>Control</td>
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<tr>
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<td>C-Reactive Protein</td>
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<td>Connective Tissue Growth Factor</td>
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<td>Connexin 43</td>
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<tr>
<td>CYP450</td>
<td>Cytochrome P450 Super Family</td>
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<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
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<td>ECE</td>
<td>Endothelin Converting Enzyme</td>
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<tr>
<td>EGF</td>
<td>Epidermal Growth Factor</td>
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</table>
ENABLE  Endothelin Antagonist Bosentan for Lowering Cardiac Events in Heart
ENCORE Enrasentan Clinical Outcomes Randomized Trial of Endothelin Antagonism Failure
ENPEP Amino Peptidase Gene
ERK Extracellular Signal Regulated Kinases
ERP Effective Refractory Period
ET-1 Endothelin-1
ETAR Endothelin Receptor Type A
ETBR Endothelin Receptor Type B
FAME Fatty Acid Methyl Ester
FC Fold Change
FN Fibronectin
FO Fish Oil
GAPDH Glyceraldehyde 3-Phosphate Dehydrogenase
GPCR G Protein Couple Receptor
Ga/q Guanine Binding Protein Type Alpha/q
HDAC Histone Deacetylase
HF Heart Failure
HIF-α Hypoxia Inducible Factor Alpha
HSP Heat Shock Protein
HTN Hypertension
HUFA High unsaturated Fatty Acid (20-22 Carbon)
IL6 Interleukin 6
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>iLAD</td>
<td>Indexed Left Atrial Diameter</td>
</tr>
<tr>
<td>iLAV</td>
<td>Indexed Left Atrial Volume</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible Nitric Oxide Synthase</td>
</tr>
<tr>
<td>IP3</td>
<td>Inositol 1, 4, 5 Trisphosphate</td>
</tr>
<tr>
<td>IP3R</td>
<td>Inositol 1, 4, 5 Trisphosphate Receptor</td>
</tr>
<tr>
<td>IQR</td>
<td>Inter Quartile Range</td>
</tr>
<tr>
<td>Jab1</td>
<td>Jun activation binding protein 1</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-Terminal Kinases</td>
</tr>
<tr>
<td>KCNN-3</td>
<td>Potassium Small Conductance Calcium-Activated Channel, Subfamily N, Member 3</td>
</tr>
<tr>
<td>LA</td>
<td>Left Atria</td>
</tr>
<tr>
<td>LAA</td>
<td>Left Atrial Appendage</td>
</tr>
<tr>
<td>LRIT3</td>
<td>Leucine Rich Repeat Immunoglobulin Like Domain and Transmembrane Domain Containing Protein 3</td>
</tr>
<tr>
<td>LV</td>
<td>Left Ventricle</td>
</tr>
<tr>
<td>LVEF</td>
<td>Left Ventricular Ejection Fraction</td>
</tr>
<tr>
<td>Lys</td>
<td>Lysine</td>
</tr>
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<td>MAPK</td>
<td>Mitogen Activated Protein Kinase</td>
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<td>Myeloperoxidase</td>
</tr>
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<td>MR</td>
<td>Mitral Valve Regurgitation</td>
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<td>mtDNA</td>
<td>Mitochondrial Deoxyribonucleic acid</td>
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<td>MVD</td>
<td>Mitral Valve Disease</td>
</tr>
<tr>
<td>NCX</td>
<td>Sodium Calcium Exchanger</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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</tr>
<tr>
<td>NHE</td>
<td>Sodium Hydrogen Antiporter</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide Adenine Dinucleotide Phosphate</td>
</tr>
<tr>
<td>NFAT</td>
<td>Nuclear Factor Activated T-Cell</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric Oxide</td>
</tr>
<tr>
<td>NpCx43</td>
<td>Non Phosphorylated Connexin 43</td>
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<tr>
<td>PDGF</td>
<td>Platelet Derived Growth Factor</td>
</tr>
<tr>
<td>PG</td>
<td>Prostaglandins</td>
</tr>
<tr>
<td>PHTN</td>
<td>Pulmonary Hypertension</td>
</tr>
<tr>
<td>PIP2</td>
<td>Phosphatidyl Inositol 4,5 Bisphosphate</td>
</tr>
<tr>
<td>PITX2</td>
<td>Paired Like Home Domain Transcription Factor</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein Kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein Kinase C</td>
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<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>Pm</td>
<td>Permanent</td>
</tr>
<tr>
<td>POAF</td>
<td>Post Operative Atrial Fibrillation</td>
</tr>
<tr>
<td>PPAR</td>
<td>Peroxisome Proliferator-Activated Receptors</td>
</tr>
<tr>
<td>Ps</td>
<td>Persistent</td>
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<td>PUFA</td>
<td>Polyunsaturated Fatty Acid</td>
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<td>PV</td>
<td>Pulmonary Vein</td>
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<td>PVI</td>
<td>Pulmonary Vein Isolation</td>
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<td>Px</td>
<td>Paroxysmal</td>
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<td>RA</td>
<td>Right Atria</td>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>RAAS</td>
<td>Renin-Angiotensin-Aldosterone System</td>
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<tr>
<td>REACH</td>
<td>Research on Endothelin Antagonism in Chronic Heart Failure</td>
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<tr>
<td>RMMSD</td>
<td>Square Root of the Mean of the Sum of the Squares of the Differences Between Adjacent NN Intervals</td>
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<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>RV</td>
<td>Right Ventricle</td>
</tr>
<tr>
<td>RyR</td>
<td>Ryanodine Receptor</td>
</tr>
<tr>
<td>SDANN</td>
<td>Standard Deviation of the Averages of NN Intervals in all 5-minute Segments Of an Entire 24 Hour Recording</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulfate</td>
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<tr>
<td>SERCA</td>
<td>Sarcoplasmic Reticulum Ca(^{2+}) ATPase</td>
</tr>
<tr>
<td>SMC</td>
<td>Smooth Muscle Cell</td>
</tr>
<tr>
<td>SNP</td>
<td>Single Nucleotide Polymorphism</td>
</tr>
<tr>
<td>SOCE</td>
<td>Store Operated Ca(^{2+}) Entry</td>
</tr>
<tr>
<td>SR</td>
<td>Sinus Rhythm</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth Factor Beta</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor Necrosis Factor Alpha</td>
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<tr>
<td>TRP</td>
<td>Transient receptor Potential Protein</td>
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<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
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<td>VSMC</td>
<td>Vascular Smooth Muscle Cell</td>
</tr>
<tr>
<td>VTP</td>
<td>Ventricular Tachypacing</td>
</tr>
<tr>
<td>ω3</td>
<td>Omega 3 Fatty Acid</td>
</tr>
<tr>
<td>ω6</td>
<td>Omega 6 Fatty Acid</td>
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</table>
Atrial fibrillation (AF), the most commonly encountered arrhythmia, is responsible for significant morbidity and mortality. Structural, electrical, and hereditable factors promote AF. Endothelin-1 (ET-1), a potent vasoconstrictor peptide and mitogen, also affects cardiac myocytes. In vitro, ET-1 enhances myocyte Ca\textsuperscript{2+} transients, and promotes myocyte hypertrophy and interstitial fibrosis. In translational studies based on human atrial tissues and relevant experimental models, I tested the hypothesis that atrial ET-1 content is related to atrial remodeling, AF risk and AF persistence.

In human left atria (LA), ET-1 protein was increased in AF patients and was associated with AF persistence and atrial size. Microarray analysis revealed that ET-1 mRNA abundance was correlated with expression of genes involved in fibrosis and hypertrophy. The SNP rs2200733 (4q25) is associated with increased AF risk. In lone AF patients, plasma ET-1 level was associated with the risk allele of this SNP.

Heart failure (HF) creates a substrate for AF. In a canine HF model due to ventricular tachypacing (VTP), atrial ET-1 was elevated prior to and during the development of HF.
During VTP, the atria and pulmonary veins (PVs) showed parallel increases in ET-1, IP3R-I protein and fibrosis that were markedly higher and earlier than increases in the ventricles. ET-1 protein was present in cardiac myocytes and fibroblasts. ET-1 may have both paracrine and autocrine effects that promote electrical and structural remodeling.

In a canine cardiac surgery model, we assessed the relationship of the ET-1 system and omega-3 fatty acid treatment on inflammation and AF inducibility following cardiac surgery. Following surgery, systemic inflammatory markers and ET-1 protein in the LA and PVs were increased. Increased LA ET-1 was associated with downregulation of ET-1 receptors but upregulation of IP3Rs, suggesting enhanced ET-1/IP3 signaling. Three weeks of dietary ω3-fatty acid supplement reduced inflammation and ET-1 protein levels and eliminated AF inducibility. Together, these studies clearly demonstrate that atrial ET-1 is associated with atrial fibrosis, atrial size and AF persistence. Available treatments for AF are suboptimal; these studies suggest that dietary ω3-fatty acids and/or ET-1 antagonists may attenuate AF development or progression. Additional studies are needed to test this intriguing hypothesis.

Key words: Endothelin-1, Atrial Fibrillation, Inositol Trisphosphate.
Chapter 1

Endothelin-1 Signaling in Cardiac Disease

1.1. Introduction

Despite the progress that has been made in the last few years to reduce the incidence of cardiovascular disease, more than 500,000 patients are diagnosed with heart disease annually, with a significant number of them dying due to complications of atrial arrhythmia. Among common cardiovascular diseases are hypertension (HTN), heart failure (HF), valvular and coronary artery diseases. These diseases share common risk factors such as diet, lifestyle, smoking and presence of concomitant cardiac disease. Other risk factors, including medical and genetic elements, are also crucial for the development of atrial arrhythmia.

1.2. The Problem of Atrial Fibrillation

Atrial fibrillation (AF) is the most common form of cardiac arrhythmia. AF affects more than 3 million Americans, and more than 12 million Americans are projected to have AF by 2050. AF is characterized by rapid atrial electrical activation, typically at rates of ~400 beats per minute, that quickly leads to loss of atrial contractility, Figure 1-1. Major complications associated with AF include thrombosis and impaired cardiac function, resulting in increased risk of HF, stroke and mortality. AF can occur in patients...
without evident heart disease (lone atrial fibrillation)^4, however, it is more frequently associated with organic cardiac diseases such as HTN, HF, valvular diseases, and cardiac myopathy^2.

Intriguingly, 400,000-500,000 patients/year with cardiovascular disease undergo cardiac surgery; 20%-50% of these develop AF in the days following surgery, with the incidence modified by patient age and type of surgery. Inflammatory pericarditis is a critical modulator of post-operative AF (POAF)^5. POAF is associated with increased hospital length of stay, increased hospital costs and increased mortality^5. The same mechanisms that underlie POAF development may also contribute to the promotion of AF in older patients. However, the mechanisms underlying POAF are not yet fully characterized.

Current pharmacologic strategies to control AF rely mainly on pharmacological blocking of ion channel activity and slowing electrical conduction. However, these interventions have been largely unsuccessful, with a majority of patients having recurrent AF within a year of treatment^6. Due to the poor efficacy of current medications, treatment efforts have increasingly focused on anticoagulants to reduce risk of stroke, and invasive cardiac procedures to eliminate AF (eg., the Maze procedure^7 and ablation/isolation of ectopic triggers in the pulmonary veins^8). The high recurrence rate of AF reflects in part our poor understanding of the mechanisms and causes of atrial electrical and structural remodeling that initiate and maintain AF.

The fundamental mechanisms underlying AF are complex and multiple, including electrical, metabolic, inflammatory, and structural factors^6. Several studies have suggested that perturbations in Ca^{2+} handling and atrial fibrosis are primary modulators of atrial arrhythmia development and progression^6. Neurohormonal activation occurs in
multiple cardiac settings and has been suggested as a potential mediator of AF development, by promoting atrial electrical and structural remodeling\(^9\). Among the most critical neurohormones are catecholamines, angiotensin-II and endothelin-1.
Figure 1-1: The electrical activity in normal and fibrillating atria

The normal electrical impulses in the heart are generated by the sinoatrial node (SA) that triggers atrial and ventricular contraction. In atrial fibrillation, the SA node electrical activity competes with electrical impulses that originate in other parts of the atria and pulmonary veins, leading to conduction of irregular impulses and impaired contractility. From: heartstrong.files.wordpress.com/2009/06/atria
1.3. Endothelin-1 Signaling

Endothelins (ETs) are a family of 3 isoforms of 21-amino acid vasoactive peptides that act on muscle, endothelial cells and connective cells\textsuperscript{10}. ET was originally isolated from the conditioned media of cultured porcine aortic endothelial cells\textsuperscript{11}. In the vascular endothelium, ET-1 is the principal long lasting vasoconstrictor isoform, and pathophysiologically is the most important isoform\textsuperscript{11, 12}. The peptide ET-1 is the most potent vasoconstrictor discovered yet\textsuperscript{10}. ET-1 is produced as a pro-peptide that requires processing by several enzymes. The precursor pro-endothelins are cleaved by furin-4 convertase of the constitutive secretory pathway, to produce the inactive intermediates, big ETs. The mature ET-1 peptide is generated from its inactive intermediate, big ET-1, through the action of endothelin-converting enzymes (ECE)\textsuperscript{10}. ECE are a family of novel zinc-binding metalloproteinases that include several isoforms. ECE-1 is a highly glycosylated type II integral membrane and neutral metalloprotease which present in two different isoforms, ECE-1-\(\alpha\) and ECE-1-\(\beta\). Both isoforms have similar tissue distribution and enzymatic properties. A homolog, designated ECE-2, has been also characterized\textsuperscript{13}. ECE-2 is less abundant than ECE-1 but is involved in ET-1 processing. Of note, changes in ET-1 levels seen in various pathological conditions may reflect changes in expression, or in the activity of ECEs. For example, expression of ECE-1 in atherosclerotic plaques and localization in macrophages and areas of inflammation in patients with coronary artery diseases (CAD)\textsuperscript{14} suggests that increased ET-1 in atherosclerosis may be driven by enhanced ECE activity.
ET-1 appears to be mainly released through constitutive secretory pathways\textsuperscript{10}, however, it can be also stored in vesicles. Mature ET-1 is degraded in the vascular system by neutral endopeptidases, produced mainly by the kidney.

ETs affect cardiac myocyte contractile properties and stimulate myocyte growth and myofibrillogenesis through modulation of phospholipase C (PLC) coupling to inositol trisphosphate (IP\textsubscript{3}) and mitogen activated protein kinases (MAPK) pathways\textsuperscript{15} (Figure 1-2). Expression and vascular release of ET-1 are promoted by a variety of stimuli, including hypoxia\textsuperscript{16}, shear stress, stretch, and pressure overload, both in vitro\textsuperscript{17} and in vivo\textsuperscript{18}. Two G protein coupled receptors (GPCR), ETA and ETB, transduce the effects of ET-1. These receptors are expressed widely but heterogeneously in the heart and vascular system\textsuperscript{29}. Through these receptors, ET-1 activates G\textsubscript{q}-PLC with subsequent production of diacylglycerol (DAG) and IP\textsubscript{3}\textsuperscript{10}. Activation of the IP\textsubscript{3} signaling pathway promotes intracellular Ca\textsuperscript{2+} increases, whereas activation of PKC (via DAG and Ca\textsuperscript{2+}) has diverse effects on Ca\textsuperscript{2+} signaling and cellular proliferation. As a vasoconstrictor peptide, ET-1 is implicated in the etiology of systemic HTN and pulmonary artery HTN (PHTN)\textsuperscript{19-21}. ET-1 is also associated with other cardiac diseases such as HF and mitral valve disease\textsuperscript{22}. It seems plausible that attenuation of ET-1 signaling may reduce the incidence of HTN or HF and development of AF.

Systemic infusion of ET-1 in healthy individuals increases diastolic but not systolic blood pressure (BP) and is associated with impaired renal function\textsuperscript{23}. Intra-coronary infusion of ET-1 increased mean arterial pressure, early after-depolarizations and ventricular fibrillation\textsuperscript{24}. Transgenic mice over-expressing ET-1 have normal BP, but structural and functional alterations in the kidney which lead to a susceptibility to salt-induced
hypertension\textsuperscript{25}. Cardiac specific over-expression of ET-1 is associated with inflammation, fibrosis and dilated cardiomyopathy, followed by death\textsuperscript{26}. In mice, ET-1 knockout is lethal at early embryonic stage due to abnormal cardiac development and malformation of aortic arch and ventricular septum\textsuperscript{27}. These studies suggest that ET-1 is crucial for cardiac development, and that enhanced ET-1 promotes deleterious vascular and myocardial remodeling.
Figure 1-2: Endothelin-1 signaling in the heart

Figure 1-2 shows the primary intracellular pathways that are activated downstream of endothelin-1 (ET-1). Several stimuli increase ET-1 gene expression and release. ET-1 acts through two receptors, ETAR and ETBR. ETAR receptors are coupled to Goq heterotrimer G protein subunits which link to phospholipase C activation and other cellular signal-transduction cascades such as those mediated by inositol 1,4,5 trisphosphate (IP3), protein kinase C (PKC), and mitogen activated protein kinase (MAPK). Phosphorylation of membrane Na+/H+ antiporters increase intracellular pH and modulates cardiac myocyte myofilament Ca²⁺ sensitivity. Influx of Na⁺ can activate reverse mode activity of the Na⁺/Ca²⁺ exchanger (NCX), resulting in elevated intracellular Ca²⁺, positive inotropy, and arrhythmogenesis. Hydrolysis of phosphatidylinositol 4,5-bis-phosphate (PIP2) increases intracellular IP3, which subsequently promotes Ca²⁺ release through IP3 receptors (IP3Rs) that are located under the plasmalemma adjacent to ryanodine receptors (RyRs), or around the nucleus. Ca²⁺ release through the IP3Rs may also contribute to the inotropic and arrhythmogenic activity of ET-1, by sensitizing RyRs located there, whereas Ca²⁺ release through nuclear IP3Rs plays a role in regulation of hypertrophic and fibrotic gene transcription. Fibrotic and hypertrophic gene transcription pathways are also activated downstream of MAPK and PKC signaling pathways following diacylglycerol generation (DAG). During atrial fibrillation, ET-1 signaling may be enhanced, promoting increased intracellular Ca²⁺ and hypertrophic/fibrotic remodeling.
The molecular mechanisms that underlie AF and the relation of ET-1 signaling to these mechanisms are described in details in the following sections.

1.4. AF and Ca\textsuperscript{2+} Handling

During normal sinus rhythm, the sinoatrial node in the right atrium is the electrical impulse generator (pacemaker) in the heart responsible for initiation of the heartbeat, and triggering coordinated cardiac muscle contraction\textsuperscript{7}. AF often begins as a result of a collision of normal electrical activity with ectopic electrical beats originating in other regions of the atria. Ectopic activity most often originates from spontaneous pacemaker activity in the pulmonary veins (PVs) entering the left atrium\textsuperscript{28} (Figure 1-3A). The progressive decline in PV fibrillatory activity with ablation provides compelling evidence for the direct participation of the PVs in the development and maintenance of AF\textsuperscript{29}. While many studies have characterized the electrophysiologic remodeling that accompanies AF\textsuperscript{7}, the mechanisms that underlie atrial ectopy that initiates AF are still not clearly understood. The mechanisms proposed to explain the development and maintenance of AF include: rapidly discharging foci, leading to fibrillatory conduction; multiple re-entrant circuits; and/or multiple impulses traveling around an abnormal circuit. These mechanisms are not mutually exclusive, and likely all contribute to AF at different times in the same individual\textsuperscript{30, 31}, Figure 1-3B.
Figure 1-3: Origin and mechanisms of atrial electrical activity during AF

A) The left-hand panel shows a diagram of the sites of 69 foci triggering atrial fibrillation (AF) in 45 patients. Note the clustering of sources in the pulmonary veins (PVs), particularly in the superior PVs. Numbers indicate the distribution of foci in the PVs. The right-hand panel is a radiograph depicting the source and exit of the ectopic activity during AF (from Haissaguerre.M et al, 199828).

B) Mechanisms of AF. A: multiple wavelets that are fragmented by atrial electrical heterogeneity find excitable tissue and maintain continuous electrical activity. B: spontaneous and premature focal electrical complexes occur when there is electrical heterogeneity. C: a single circuit reentry that acts as a dominant generator of electrical activity. Ectopic foci or single reentry can trigger multiple reentry in the presence of a vulnerable substrate. From Nattel.S et al, 200230.
Many studies suggest that improper Ca\textsuperscript{2+} handling is an important arrhythmogenic factor and a candidate mechanism to underlie AF-generating ectopic foci.

Membrane depolarization during an action potential leads to activation of L-type voltage-gated Ca\textsuperscript{2+} channels. This Ca\textsuperscript{2+} influx leads to Ca\textsuperscript{2+} release via ryanodine receptors in the sarcoplasmic reticulum (SR). This process, Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release, is a critical event in cardiac myocyte excitation-contraction coupling\textsuperscript{32,33,34}.

During AF, profound changes in Ca\textsuperscript{2+} cycling occur in the atria. At the single channel level, increased single L-type Ca\textsuperscript{2+} channel activity, due to an increase of channel open probability has been observed in human AF and may be related to a reduction of cytosolic phosphatase 2A (PP2a) activity or to impaired local PP2a interactions with the L-type Ca\textsuperscript{2+} channel in AF\textsuperscript{35}.

In human and animal models of AF\textsuperscript{36,37}, whole cell L-type Ca\textsuperscript{2+} current densities are decreased by 60-70\%, possibly as an adaptive response to arrhythmia-induced Ca\textsuperscript{2+} overload. While animal studies suggest that Ca\textsuperscript{2+} channel expression is reduced in experimental AF\textsuperscript{38}, data on atrial tissues from AF patients showed no changes in dihydropyridine binding or alpha 1C mRNA expression\textsuperscript{39}. This suggests that the L-type Ca\textsuperscript{2+} current reduction in AF may not be related to a decrease in channel number, but rather to alterations in channel function.

Both the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger (NCX) and Na\textsuperscript{+}/H\textsuperscript{+} antiporter (NHE) are critical membrane proteins in cardiac myocytes, both are important in regulating intracellular Na\textsuperscript{+} and Ca\textsuperscript{2+} homeostasis, and both are implicated in the etiology of AF. Enhanced NHE activity increases intracellular Na\textsuperscript{+}, pH and myofilament Ca\textsuperscript{2+} sensitivity. On the other hand, activation of reverse mode NCX activity increases [Ca\textsuperscript{2+}], and Ca\textsuperscript{2+} overload promoting
arrhythmia. Triggered arrhythmia in superfused canine pulmonary veins was enhanced by increased Ca$^{2+}$ transient and increased NCX current$^{40}$. Activation of the NHE was also observed in short-term atrial electrophysiological remodeling$^{41}$.

Ryanodine receptor (RyR) expression is decreased in experimental AF, perhaps as an adaptive mechanism to reduce SR Ca$^{2+}$ leak$^{42}$. SR Ca$^{2+}$ leak due to increase RyR2 phosphorylation by protein kinase A (PKA) may play a role in the initiation and/or maintenance of AF$^{43}$.

Cardiac myocytes contain inositol 1,4,5 tris phosphate receptors (IP3R), which also contribute to Ca$^{2+}$ release from intracellular stores when activated by IP3. IP3 is generated by hydrolysis of phosphatidyl inositol 4,5-bisphosphate (PIP2) by PLC upon activation with GPCR agonists$^{44}$. Enhanced IP3 signaling increases the likelihood of spontaneous Ca$^{2+}$ release/overload. In addition, Ca$^{2+}$ release through plasmalemmal IP3Rs may sensitize RyRs. Catecholamines, angiotensin-II (Ang-II), and ET-1 are agonists of Gq-coupled GPCRs that can initiate Ca$^{2+}$ sparks, waves (a pro-arrhythmic condition), and spontaneous action potentials$^{45}$. These agonists are implicated in the generation of AF$^{45}$. Although these stimuli all activate the Gq-PLC signaling pathway and cause generation of IP3, the role of IP3Rs in cardiac pathophysiology remains controversial, with suggestions that they play only a minor role during cardiac Ca$^{2+}$ signaling or have a solely organelle-specific function$^{46}$. Recently, it has become apparent that IP3 signaling is involved in various cardiac pathologies$^{47}$. Interestingly, IP3 receptors are upregulated in AF, but functional studies examining their cellular effect are lacking$^{48}$. IP3R-I is the dominant isoform in human atria$^{48}$ and rat Purkinje fiber myocytes, whereas atrial and ventricular myocytes from most other species express predominantly IP3R-II
and to lesser extent IP3R-III\textsuperscript{49 34}. However, the physiological roles of IP3Rs still remain unclear\textsuperscript{34}.

Filling of SR Ca\textsuperscript{2+} stores is attributed to the activity of the sarcoplasmic reticulum Ca\textsuperscript{2+} ATPase (SERCA2a) and to proteins involved in store operated Ca\textsuperscript{2+} entry (SOCE). The transient receptor potential (TRP) channels are implicated in SOCE and can be activated by depletion of intracellular Ca\textsuperscript{2+} stores\textsuperscript{50}. These proteins have been shown to localize with IP3Rs and interact with NCX\textsuperscript{51}, suggesting a potential role of TRPs in excitation-contraction coupling\textsuperscript{52, 53}. Interestingly, TRP channels have been also implicated in AF\textsuperscript{54}, suggesting a role in atrial arrhythmogenesis.

1.4.1. Role of ET-1 in Modulating Ca\textsuperscript{2+} Signaling

IP3 generation causes release of intracellular Ca\textsuperscript{2+} stores from the SR via IP3Rs and promotes NCX current\textsuperscript{55}. IP3-mediated release of intracellular Ca\textsuperscript{2+} stores may contribute to the intracellular effects of ET-1\textsuperscript{47}. Elevated Ca\textsuperscript{2+} and arrhythmogenic Ca\textsuperscript{2+} release may lead to spontaneous activity of atrial myocytes and initiation of AF. Efforts to better understand the molecular mechanisms of ET-1 are potentially clinically important.

In cardiac hypertrophy and HF, IP3Rs are also upregulated, and expression of ET-1 and ET-1 receptors is altered\textsuperscript{56}. Plasma ET-1 levels are increased in patients with HTN\textsuperscript{57}, HF\textsuperscript{58}, and PHTN\textsuperscript{59}. In AF patients, plasma ET-1 is reported to be an independent predictor of AF recurrence following pulmonary vein isolation\textsuperscript{60}. Although plasma ET-1 levels are elevated in AF patients with structural heart disease\textsuperscript{61}, studies are lacking in which ET-1 expression is evaluated locally in fibrillating atria, particularly in lone AF. We have recently shown that atrial ET-1 is increased in AF patients with underlying cardiac disease and is associated with AF persistence (Chapter 2)\textsuperscript{62}. 
The rapid decrease of ET-1 levels after successful catheter ablation suggests that ET-1 expression may promote supraventricular tachycardia/AF\textsuperscript{15}. The effects of ET-1 on atrial arrhythmias, however, are less clear. In vitro studies on isolated atrial myocytes revealed that ET-1 provokes abbreviation of action potentials, atrial triggered activity and intracellular Ca\textsuperscript{2+} overload\textsuperscript{63, 64}. ET-1 antagonists prevented the acute arrhythmogenic effects of ET-1 on isolated atrial myocytes\textsuperscript{65}. However, these results are contrary to studies\textsuperscript{66, 67} showing that ET-1 also has potential anti-arrhythmic effects on the atria\textsuperscript{66, 67}.

In muscle studies from explanted failing hearts, the inotropic effect of ET-1 on isolated atrial trabeculae was much greater (+75\%) than on corresponding trabeculae from the left ventricle (+10\%)\textsuperscript{68}. Few studies support a negative inotropic response to ET-1\textsuperscript{69}. Although the factors responsible for these discordant findings are unclear, it appears that not only experimental procedures, but also differences in species or cardiac chamber studied which may be related to receptor abundance, the presence of concomitant heart disease, the degree of adrenergic stimulation and extent of coronary vasoconstriction may all modulate the response to ET-1\textsuperscript{10}. Most of the previous studies evaluated ET-1 impact on normal atrial myocytes isolated from various animal species, so further studies carefully evaluating the response of human fibrillating atrial myocytes are still warranted.

Atrial cells from IP3R-II deficient mice are significantly less prone to develop pro-arrhythmic disturbances in Ca\textsuperscript{2+} signaling with exogenous exposure to ET-1\textsuperscript{47}, suggesting that acute arrhythmogenic actions of ET-1 are mediated through intracellular Ca\textsuperscript{2+} release via IP3Rs. Similarly, atrial myocytes from transgenic mice overexpressing IP3R-II showed increased Ca\textsuperscript{2+} transients and arrhythmias in response to ET-1 stimulation\textsuperscript{70}.

Atrial myocytes express functional IP3Rs proteins that cause spontaneous Ca\textsuperscript{2+}-release
and modulate excitation contraction coupling. IP3R-II protein is the predominant isoform in rat atria and is expressed in the atria at levels 6 times higher than in the ventricles, and to a greater extent than IP3R-I expression.

IP3Rs also support the development of spontaneous, pro-arrhythmic Ca\(^{2+}\) transients and positive inotropy in ventricular myocytes following ET-1 stimulation. In ventricular myocytes, ET-1 increases Ca\(^{2+}\) release through IP3Rs present in the junctional SR and contributes to enhanced excitation-contraction coupling, a characteristic of hypertrophic myocytes. Due to their close proximity to RyRs in the SR, enhanced Ca\(^{2+}\) release from IP3Rs may serve to sensitize RyRs.

Defective regulation of inter-domain interactions within the RyR plays a key role in the pathogenesis of HF and cardiac diseases. Defective domain unzipping promotes diastolic SR Ca\(^{2+}\) leak, resulting in cardiac disease and arrhythmia. ET-1 can contribute to defective RyR domain interactions and hypertrophy in neonatal cardiac myocytes, suggesting that ET-1 may also modulate Ca\(^{2+}\) release via RyRs.

The role of ET-1 as a modulator of L-type Ca\(^{2+}\) channel activity is unclear. In guinea pig ventricular myocytes, ET-1 evoked early after depolarizations via prolongation of action potential plateau, which resulted from L-type Ca\(^{2+}\) current enhancement. Similarly, another study in rat ventricular myocytes documented that ET-1 increased the L-type Ca\(^{2+}\) current (recorded by perforated patch clamp) via ETAR/PKC/Ca\(^{2+}\)-calmodulin dependent pathways. In human atrial myocytes, ET-1 increased L-type Ca\(^{2+}\) current recorded by whole cell patch clamp. However, following activation with isoproterenol, ET-1 causes a decreased in the L-type Ca\(^{2+}\) current. These data suggest that ET-1 actions may be influenced by the phosphorylation state of the L-type Ca\(^{2+}\) channel, and by
intracellular Ca\(^{2+}\) stores. As ET-1 receptors can couple with both G\(_i\) and G\(_q\) coupled receptors, some of the different responses are attributable to the downstream signaling cascades. Other differences in ET-1 actions may be related to experimental condition (eg., use of ruptured versus perforated patch clamp technique), suggesting that the intracellular environment is critical for ET-1 signaling.

NCX\(^{75}\) and NHE\(^{76}\) activities are both influenced by ET-1 signaling. For example, the positive inotropic of ET-1 on cardiac myocytes is mediated by activation of the NHE\(^{76}\) resulting in increased sensitivity to Ca\(^{2+}\) and possibly activation of reverse mode NCX activity. Moreover, ET-1 also stimulates reverse mode NCX activity through intracellular Na\(^{+}\) and PKC dependent pathways\(^{75}\).

In smooth muscle cells, stimulation of TRP channels by neurohormones such as norepinephrine, Ang-II and ET-1 mediates functions including vasoconstriction, cell growth and proliferation. TRP channel activity can be triggered by intracellular Ca\(^{2+}\) depletion as well as by IP3 and DAG formation; both could be generated by ET-1\(^{51}\). Ca\(^{2+}\) release via IP3Rs activates entry of Ca\(^{2+}\) via plasma membrane TRP channels\(^{77}\). Recently, it has been shown that ET-1 induced interaction of TRPC3 and IP3R-I promotes Ca\(^{2+}\) release and vasoconstriction in cerebral arterial myocytes\(^{78}\). Interestingly, TRP channels are also expressed in cardiac myocytes (Appendix 1) and, when open, can modulate the resting membrane potential\(^{52}\).

Although emerging evidence suggests an important role of ET-1 in regulating Ca\(^{2+}\) cycling via multiple mechanisms, it is apparent that ET-1 coupled to IP3 signaling is the predominant pathway accounting for ET-1 effects on intracellular Ca\(^{2+}\) stores. Recently, it has been shown that ET-1 promotes local cytosolic\(^{79,\ 80}\) and perinuclear IP3
generation\textsuperscript{81}, promoting spontaneous Ca\textsuperscript{2+} release and cardiac myocyte hypertrophy. Together, these data suggest that ET-1 has a potentially important role as a modulator of the substrate for AF.

1.5. AF and Inflammation/Oxidative Stress

Inflammation and oxidative stress are modulators of the substrate for atrial arrhythmia. A role for inflammation in AF can be inferred from the common association of AF with inflammatory conditions like pericarditis and myocarditis\textsuperscript{82}. Impaired atrial contractility leads to pro-inflammatory changes (platelet adhesion, cytokine production, etc.). The presence of monocytes and leukocytes in the atria of AF patients\textsuperscript{83} also suggests a critical role of inflammation in the etiology of AF. Increased C-reactive protein (CRP)-complement 4B complex levels are associated with increased risk of POAF, and peak incidence of POAF coincides with peak elevation of CRP\textsuperscript{84}. Elevated levels of interleukin-6 (IL-6) are also independent predictors of stroke and death in high risk AF patients, linking abnormal inflammation with risk of thromboembolism\textsuperscript{85}. Interestingly, a polymorphism of the IL-6 gene promoter influences the development of POAF\textsuperscript{86}.

Inflammation is related to oxidative stress. Our lab was the first to show the evidence of increased oxidative stress in the atria of AF patients\textsuperscript{87}. We also showed that rapid atrial pacing was associated with increased nitrotyrosine abundance and decreased Ca\textsuperscript{2+} current\textsuperscript{88}, suggesting an important role of reactive oxygen species (ROS)/oxidative stress in AF development and in the progression of AF. Importantly, use of glutathione\textsuperscript{88} and ascorbic acid\textsuperscript{89}, a water-soluble antioxidant, attenuated atrial electrical remodeling. Studies have also documented increased ROS production in AF. Enhanced ROS generation via nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase)
activity is associated both with persistent AF\textsuperscript{90} and with the development of POAF\textsuperscript{91}. ROS are also produced by the mitochondria, and AF is associated with mitochondrial injury and dysfunction\textsuperscript{92}.

Myocardial injury promotes the production of inflammatory cytokines including tumor necrosis factor alpha (TNF-\(\alpha\)). TNF-\(\alpha\) is associated with apoptosis, oxidative stress and mitochondrial dysfunction\textsuperscript{93}. Intriguingly, increased tissue and serum TNF-\(\alpha\) levels in a canine ventricular tachypacing model of HF are associated with decreased activity of mitochondrial complexes III and V, mitochondrial DNA damage and oxidative stress promoting lipid peroxidation and impaired mitochondrial function\textsuperscript{94}. Similarly, in a goat model of AF, atrial tissues showed signs of mitochondrial dysfunction\textsuperscript{92}, decreased oxidative phosphorylation, and increased proton leak\textsuperscript{95}. This was associated with increased ROS from the mitochondria. Oxidative stress can promote a cycle of oxidative damage in which the mitochondrial dysfunction progresses to a state of impaired energy production, myocardial cell dysfunction and cell death.

1.5.1. \textit{Role of ET-1 in Promoting Inflammation/Oxidative Stress}

During ischemia and inflammatory responses, a dramatic shift in tissue metabolism and acidosis can occur, resulting in tissue hypoxia. Hypoxia is a potent stimulant of ET-1 gene expression. Activation and nuclear translocation of hypoxia inducible factor-alpha (HIF-\(\alpha\)) promotes ET-1 gene transcription\textsuperscript{16}.

Although ET-1 is predominantly expressed in endothelial cells, it is also expressed in human macrophages and leukocytes\textsuperscript{96}, suggesting a role for ET-1 in local inflammatory processes. In ischemic cardiomyopathy hearts, macrophages located in infarcted regions produce ET-1 and may contribute to ET-1 mediated cardiac effects via paracrine
actions\textsuperscript{14}. Elevated plasma levels of ET-1 in coronary artery disease (CAD), and the
detection of ET-1 in coronary atherosclerotic plaques, where it appears to localize with
macrophages and other inflammatory markers\textsuperscript{96} also supports a role of ET-1 in the
inflammatory processes associated with atherosclerosis.

Additional evidence supporting a role for ET-1 in promoting inflammation stems from
the finding that transgenic mice with conditional, cardiac-restricted ET-1 overexpression
exhibit massive myocardial inflammation, followed by dilated cardiomyopathy and
death\textsuperscript{26}. These mice showed increased inflammatory cell surface markers and increased
cell infiltrates such as macrophages and T cells\textsuperscript{26}. In addition to cardiac hypertrophy,
interstitial fibrosis and enlargement of the atria and ventricles, ET-1 overexpression was
also associated with increased inflammatory cytokines (TNF, IL6) and increased NF-κB
nuclear translocation in cardiac myocytes\textsuperscript{26}. Use of a non-selective ET-1 antagonist
(mixed ETAR/ETBR antagonist), but not a selective ETAR antagonist, improved survival
of ET-1-transgenic mice, suggesting a more important pathological role of ETBR
signaling in mediating cardiac inflammation and mortality.

ET-1 has been shown to increase production of the inducible form of nitric oxide
synthase (iNOS), promoting cardiac myocytes hypertrophy\textsuperscript{97}. Cytokine production
stimulates iNOS gene expression, suggesting that ET-1, via induction of various
cytokines, may modulate myocardial iNOS expression. The critical balance of nitric
oxide (NO) and ET-1 is vital in normal physiology and is disrupted in pathologic
conditions during inflammatory processes\textsuperscript{98}.

ET-1 mediates ROS production by increasing NADPH oxidase activity and expression\textsuperscript{99}.
The positive inotropic effect of ET-1 is mediated by mitochondrial ROS production,
subsequent to enhanced NADPH oxidase activity\textsuperscript{76}. Furthermore, ET-1 reduced mitochondrial function and integrity\textsuperscript{100}. Interestingly, mitochondrial injury is associated with enhanced ET-1 production suggesting that it may contribute to ET-1 activation during HF and ischemic heart diseases\textsuperscript{101}. It has been shown that ET-1 modulates volume-sensitive Cl\textsuperscript{-} current and osmotic swelling by stimulating ROS production from NADPH oxidase and mitochondria\textsuperscript{102}. Together, these data suggest that ET-1 can modulate oxidative stress and cellular inflammatory activities in various pathophysiological settings, by multiple mechanisms.

Interestingly, ET-1 also modulates calcineurin protein expression and activity, nuclear translocation of nuclear factor activated T cells (NFAT-c4) and cardiac hypertrophy\textsuperscript{103}. These effects were inhibited by peroxisome proliferators-activated receptor gamma (PPAR-\(\gamma\))\textsuperscript{103}. Similarly, ET-1 induced cardiac hypertrophy is inhibited by activation of PPAR-alpha, partly via blockade of c-Jun NH2-terminal kinase pathway\textsuperscript{104, 105}. These findings further link ET-1 signaling to both inflammation/hypertrophy and metabolic processes in the heart.

\subsection*{1.5.2. Relation of Omega 3 Fatty Acids to Inflammation and AF}

Fatty acids are the primary source of energy for the myocardium. They are also an important source for membrane biosynthesis, generation of signaling molecules, transcriptional and post-translational modification, and modulators of mitochondrial function. Thus, changes in cardiac lipid composition may have global effects on various cellular activities. Recent attention has focused on the therapeutic potential of dietary fish oil (FO) as an approach to prevent cardiovascular diseases\textsuperscript{1}. 
Omega-3 polyunsaturated fatty acids (ω3-PUFA) have anti-inflammatory properties, due in part to their competition with ω6-PUFA. The ω6-PUFA are the precursors of numerous inflammatory factors and prostaglandin (PG) formation. In vitro studies reveal that prostaglandin E2 (PGE2) increased HIF-α stability by enhancing its translocation from the cytosol to the nucleus. As HIF-α regulates ET-1 gene expression, these data suggest that FO may also reduce ET-1 expression. In vitro, FO reduced ET-1 mRNA expression and attenuated cardiac myocyte hypertrophy.

The ω3-PUFA also have antioxidant and antiarrhythmic properties. Reactive molecules such as free radicals and lipid peroxides may contribute to the formation and progression of pathological states. As antioxidants, ω3-PUFA may act as scavengers for ROS products, and reduce inflammation/oxidative stress through the induction of antioxidant enzyme activities. In a rat model of pressure overload-induced cardiac dysfunction, FO reduced production of TNF-α. Inhibition of cytokine production and antioxidant effects minimize ROS production, resulting in preservation of a reduced intracellular redox state and decreased post-translational modification of ion channels and fibrosis. Decreased ROS production may preserve mitochondrial ATP production, limit sodium loading, calcium overload, and ectopic activity.

Accumulating evidence from molecular and clinical studies suggests that the cardioprotective effects of FO result from a synergism between multiple, interactive mechanisms that involve anti-inflammatory and antithrombotic effects, modulation of cardiac ion channels, pro-resolving effects of lipid mediators, reduction of triglycerides, and an influence on downstream signaling pathways. In a large cohort of older people, increased consumption of FO was associated with less AF development, less fibrosis and
a reduction in connexin expression\textsuperscript{110}. Concordant with this, dietary administration of ω3-PUFAs in a canine HF model (ventricular tachypacing) was associated with improved hemodynamics, less interstitial fibrosis, and shorter episodes of induced AF\textsuperscript{111}. In this study, although PUFAs were effective in the setting of ventricular dysfunction, they did not prevent sustained AF in response to atrial tachypacing, suggesting that ω3-PUFAs may prevent AF development, rather than its progression. In addition, ω3-PUFAs may be more effective in attenuating AF associated with ventricular rather than atrial tachypacing.

FO can inhibit NF-κB signaling and down-regulate fatty acids synthesis gene expression (e.g. sterol element binding proteins) and up-regulate the genes involved in fatty acid oxidation (e.g. peroxisome proliferator activated receptor α, PPARα)\textsuperscript{112}. However, the roles and the mechanisms underlying FO-induced AF cardioprotection remain controversial\textsuperscript{113,114}. 
1.6. AF and Structural Remodeling

Fibrosis is a hallmark of atrial structural remodeling and a common feature of clinical AF\textsuperscript{115}. Fibrosis occurs as a reparative process to replace degenerating myocardial parenchyma with concomitant reactive fibrosis that leads to interstitial fibrosis\textsuperscript{115}. Cardiac myocytes are electrically coupled, mainly in an end-to-end fashion, by gap junctions. Increased fibrosis (extracellular matrix expansion between myocytes or bundles of myocytes) can physically separate myocytes, decreasing myocyte electrical coupling and creating a barrier to impulse propagation\textsuperscript{115}.

The volume and composition of the extracellular matrix is associated with AF persistence, suggesting that atrial fibrosis is an important component of a substrate for AF\textsuperscript{115}. Myofibroblasts, the cells responsible for extracellular matrix production, modulate conduction velocity, ectopic activity and provide stiffness to injured myocardium promoting heterogeneous conduction and arrhythmias\textsuperscript{116}. In an experimental animal model, ventricular tachypacing induced HF is associated with extracellular matrix remodeling\textsuperscript{117-121}. The resulting atrial interstitial fibrosis leads to conduction abnormalities and increased duration of AF episodes\textsuperscript{118, 119}. Atrial remodeling and fibrosis are also observed in patients with HF\textsuperscript{122, 123}. Atrial remodeling is responsible for the formation of a substrate that promotes atrial arrhythmias and AF vulnerability\textsuperscript{124, 125}.

Atrial fibrosis might result from a variety of cardiac insults that share common signaling pathways. The renin-angiotensin-aldosterone system (RAAS) is involved in myocardial fibrosis in hypertensive heart disease, HF, myocardial infarction, and cardiomyopathy\textsuperscript{126}. Increased Ang-II production in transgenic mice with cardiac-restricted angiotensin-converting enzyme (ACE) over expression causes marked atrial dilation with focal
fibrosis and AF\textsuperscript{127}. In this system, use of ACE inhibitors improved left ventricular function and attenuated cardiac fibrosis\textsuperscript{128, 129}. In the RALES study\textsuperscript{130}, spironolactone; an aldosterone receptor antagonist, led to a significant improvement of LV function and a reduction in cardiac fibrosis and sudden death in patients with severe HF. Although these studies provide evidence for a role of the RAAS in cardiac fibrosis, the therapeutic benefits obtained from ACE and aldosterone antagonists remain insufficient. This suggests that there are other important pathways that are still involved in atrial fibrosis that have not yet been fully elucidated. Cardiac and plasma ET-1 are increased in multiple cardiovascular disease settings\textsuperscript{10} and ET-1 is known to cause cardiac hypertrophy and fibrosis \textit{in vitro}\textsuperscript{97} and \textit{in vivo}\textsuperscript{26}. This suggests that, in addition to the RAAS system, ET-1 may be a potential mediator of hypertrophic and fibrotic signaling in the heart.

Ang-II is a well-characterized profibrotic molecule, with prominent downstream mediators like transforming growth factor (TGF-β1)\textsuperscript{126}. Following activation, TGF-β1 dimers bind to TGF-β1 type II receptors which recruit and phosphorylates type I receptors. The TGF-β1 type I receptor then recruits and phosphorylate several Smad proteins, resulting in their activation\textsuperscript{131}. Transgenic mice overexpressing TGF-β1 are associated with cardiac hypertrophy and interstitial fibrosis\textsuperscript{132}. Both TGF-β1 and Ang-II are more elevated in the atria than in the ventricles of failing hearts\textsuperscript{121}. Mice expressing a mutant TGF-β1 transgene in the heart show atrial but not ventricular fibrosis, suggesting that TGF-β1 receptors or other activators are differentially expressed in the atria\textsuperscript{133}. TGF-β1 activation following the stimulation of Ang-II receptors is a primary mediator of
cardiac fibrosis. Other potential mediators such as platelet-derived growth factor (PDGF) are also of interest. PDGFs are a family of dimeric isoforms that are generated by 4 genes: the classical PDGF A and B chains, and more recently identified C and D chains. PDGF is highly expressed in the myocardium throughout development and adulthood. The PDGFs stimulate proliferation, migration, and differentiation in several organs by binding to two receptor tyrosine kinases, PDGF α and β. The role of PDGFs in cardiac fibrosis has only recently been investigated. Transgenic mice with cardiac-specific PDGF overexpression show cardiac fibrosis followed by dilated cardiomyopathy and HF. In the latter study, PDGFD transgenic mice showed abnormal heart size, with notable atrial enlargement. This phenotype was more severe in PDGFD than in PDGFC transgenic mice. These studies raise the possibility of potential involvement of PDGF in atrial-selective hypertrophy and fibrosis. Interestingly, in a recent study, gene microarrays revealed a differential expression of PDGFs and their receptors between atrial and ventricular canine fibroblasts. This agrees with observations of greater fibrous tissue in normal and failed atria compared to ventricles, and strengthen a potential role of PDGF in atrial–ventricular remodeling differences. However, the upstream neurohormonal mediators of PDGF signaling in the heart are not fully known.

Mast cells are key mediators of allergic and immune responses. Interestingly, mast cells may be involved in AF pathogenesis and atrial fibrosis in pressure-overloaded mouse hearts. Experimental pressure overload induced mast cell infiltration, PDGFA expression, and atrial-selective fibrosis, resulting in increased AF susceptibility following atrial burst stimulation.
Connective tissue growth factor (CTGF) has also been shown to be increased in human AF, via Rac-1 dependent pathway\textsuperscript{141}. In this small study, the authors identified CTGF as an important mediator of atrial structural remodeling during AF. At the cellular level, Ang II activates CTGF via activation of Rac1 and NADPH oxidase, leading to up-regulation of connexin 43 (Cx43), N-cadherin, and interstitial fibrosis, contributing to atrial structural remodeling. In the same study, use of a specific small molecule inhibitor of Rac1, or use of simvastatin, completely prevented the AngII-induced up-regulation of CTGF and Cx43 in neonatal rat cardiomyocytes and fibroblasts. CTGF is increased in response to a variety of stimuli and GPCR agonists including Ang-II\textsuperscript{141} and ET-1\textsuperscript{142, 143}. In vitro, ET-1 promotes NADPH oxidase activity\textsuperscript{76} and CTGF expression\textsuperscript{142, 143}. However, the role of ET-1 in atrial structural remodeling has not yet been carefully examined.

As discussed above, IP3 production causes an increase in intracellular Ca\textsuperscript{2+}, which in turn activates calmodulin kinases and calcineurin (a phosphatase). Calcineurin activation leads to changes in gene expression by facilitating translocation of transcription factors from the NFAT family. These factors bind to nuclear DNA, together with other transcription factors from the hypertrophic pathways, to facilitate transcription-promoting hypertrophy\textsuperscript{80}.

1.6.1. Role of ET-1 in Promoting Myocardial Fibrosis and Hypertrophy

Mitogen activated protein kinases (MAPKs) are important modulators of cellular proliferation and tissue structure\textsuperscript{144}. GPCRs agonists acting through PKC activate the extracellular signal regulated kinases (ERKs). These agonists include Ang-II and ET-1, which also activate the Jun kinases (JNKs). During cellular stresses, GPCR agonists
induce phosphorylation of c-Jun, a critical early responsive transcription factor in cellular proliferation.

ET-1 promotes myocyte hypertrophy and interstitial fibrosis. Early studies identified ET-1 as a strong mitogenic factor that stimulates DNA synthesis similar to known growth factors like PDGF and epithelial growth factor (EGF). However, the mechanisms underlying ET-1 actions were not understood. In vascular smooth muscle cells, ET-1 induced hypertrophy, proliferation, and synthesis of extracellular matrix proteins (eg., type I collagen and fibronectin (FN)). ET-1 also stimulates production of cytokines such as TNF-α, and growth factors including VEGF and basic fibroblast growth factor. ET-1 also strengthens the mitogenic activity of TGF-β and PDGF. In renal mesangial cells, ET-1 stimulates PDGF secretion. Recently, it has been shown that ET-1, independent of TGF-β and through activation of ERK/MAPK signaling pathway, stimulates CTGF in VSMCs. In cardiac myocytes, ET-1 stimulates CTGF production, promotes hypertrophy and fibrosis. ET-1 promotes induction of myofibroblasts through ETAR activation, via the rac-phosphoinositide-3 kinase (PI3K)/Akt pathway. In these studies, ET-1 was essential for the enhanced contractile phenotype of myofibroblasts by promoting up regulation of alpha smooth muscle actinin (α-SMA), erzin and paxillin in fibroblasts. One can speculate that use of ET-1 antagonists might reduce scar formation and fibrosis. Interestingly, transgenic mice overexpressing cardiac ET-1 develop cardiac fibrosis and enlargement of both atria and ventricles. The use of an ET-1 antagonist improved survival in these mice. In Chapter 2, we show that in human AF, atrial ET-1 mRNA is associated with the gene expression of cardiac collagen isoforms, as well as PDGFD and CTGF. Atrial ET-1 protein was also
associated with atrial fibrosis and size, suggesting that ET-1 may promote human structural remodeling via activation of the CTGF and the PDGF pathways. In addition to promoting cardiac fibrosis, ET-1 can also promote cardiac hypertrophy. In neonatal rat cardiac myocytes, ET-1 stimulated cardiac hypertrophy has been attributed to activation of PKC. ET-1 causes translocation of PKCε from cytosol to caveolae, where the ERKs are located. ET-1 also stimulates p38-MAPK activity through PKC activation. In addition to the PKC dependent pathway, ET-1 can induce hypertrophy through elevation of intracellular Ca²⁺ and activation of Ca²⁺ dependent kinases and calcineurin-dependent pathways. Furthermore, ET-1 modulates nuclear Ca²⁺ levels via perinuclear IP3 generation, leading to Ca²⁺ overload and activation of Ca²⁺-calmodulin kinase activity in the nuclei of cardiac myocytes. Increased activity of Ca²⁺/calmodulin kinases promoted histone deacytelase (HDAC) phosphorylation and transcription of genes involved in cardiac hypertrophy and fibrosis. HDAC inhibition reversed atrial arrhythmia vulnerability and fibrosis in cardiac hypertrophy independent of AngII, suggesting that additional neurohormones that phosphorylate HDACs may be critical in atrial structural remodeling and AF. ET-1 may be a strong candidate hormone for this role.

Heat-shock proteins (HSP) are a class of cytoprotective proteins released in response to a variety of stressors. Stimulation of HSPs has been shown to suppress AF following atrial tachycardia remodeling as well as HF-related structural remodeling. Human studies suggest a cardioprotective effect of HSPs against the progression from paroxysmal to persistent AF, and in the development of POAF. It is intriguing that ET-1 stimulates HSP70 expression in neonatal cardiac myocytes and may improve hypoxia tolerance.
HSP70 binds to and protects the microtubule network and limits myofibril disruption after ischemic myocardial stress \textsuperscript{166}.

1.7. Endothelin Receptor Densities in Cardiac Disease

ET-1 mediate its actions through binding to two GPCRs: ETAR and ETBR\textsuperscript{10}. These receptors are widely expressed in the myocardium. ETAR is the predominant isoform in the heart and present (relative to total ET receptors binding sites) in 91\% of atrial myocytes vs. 86\% of ventricular myocytes\textsuperscript{10}. ETAR is associated with vasoconstriction and increased intracellular Ca\textsuperscript{2+}\textsuperscript{10}, while ETBR receptors are commonly thought to mediate vasodilatation through nitric oxide production (NO) and are involved in ET-1 clearance. A recent study suggests that ETBR is also involved in sustained Ca\textsuperscript{2+} influx via PLC/G\textsubscript{q} signaling, in a manner similar to ETAR\textsuperscript{167}. It is possible that the activity, coupling and regulation of these receptors are tissue specific.

While ETAR is expressed mainly by vascular smooth muscles (SMCs), ETBRs are expressed by both SMCs and endothelial cells. Both receptors are also expressed in cardiac myocytes and fibroblasts\textsuperscript{10} (Appendix 2-3).

Systemic infusion of an ETAR-selective antagonist into healthy human subjects decreased BP and vascular resistance, suggesting that ETAR receptors mediate vasoconstriction\textsuperscript{168}. On the other hand, ETBR-deficient mice\textsuperscript{169} or infusion of an ETBR-selective antagonist\textsuperscript{170} is associated with increased peripheral resistance and increased BP, suggesting that ETBRs mediate vasodilatation. However, specific ETBR deletion in endothelial cells failed to increase BP\textsuperscript{171}, suggesting that ETBR-vasodilatory effect is primarily mediated by its expression in non-endothelial cells (eg., SMCs).
ET-1 peptide is internalized upon binding to ETBR. This process has also been shown to decrease the level of ET-1 mRNA\textsuperscript{172}. However, ETBR can be internalized and recycled in a ligand-independent manner and targeted to the lysosomes for degradation\textsuperscript{173}. ETAR is also internalized in a ligand-dependent manner. Upon internalization, ETAR follows a recycling pathway through the peri-centriolar recycling compartment and is then transported back to the plasma membrane\textsuperscript{173}.

In ventricular myocytes, ETAR is present mainly in the plasma membrane, and is less abundant in the cytosol and the nucleus. In contrast, ETBR is mainly cytosolic and is widely expressed in the nuclei, suggesting a possible role for ETBR as a modulator of gene transcription\textsuperscript{174}. In the nuclei of ventricular myocytes, both receptors show functional interaction with ET-1 followed by nuclear Ca\textsuperscript{2+} release\textsuperscript{174}. However, the localization and distribution of ET-1 receptors in atrial myocytes has not yet been characterized.

Several studies suggest that changes in ET-1 receptors expression predict the presence or progression of cardiac disease. In idiopathic dilated cardiomyopathy (IDCM) patients, ETAR is either increased or unchanged, but there is no change in ETBR expression\textsuperscript{175}. Similarly, ischemic heart disease is associated with an increase in ETAR abundance but no change in ETBR expression\textsuperscript{176}.

In human pulmonary HTN, upregulation of ETBR but not ETAR mRNA transcript levels and immunoreactive protein in the pulmonary arteries were observed\textsuperscript{177}.

In human HF, ventricular ETAR density is increased while ETBR is decreased\textsuperscript{178}. The ETAR, but not the ETBR protein change is accompanied by cognate regulation of mRNA level. Importantly, the expression of the receptors was not correlated with myocardial
ET-1 levels. Thus, it seems unlikely that HF related changes in ET-1 receptor proteins or mRNA expression result from homologous regulation by ET-1\textsuperscript{178}.

In a small study in lone AF patients, both ETAR and ETBR mRNA levels and protein expression of ETBR were shown to be downregulated\textsuperscript{179} while ET-1 expression was unchanged. In human AF with underlying cardiac disease, we did not observe marked changes in expression or localization of ETAR or ETBR proteins\textsuperscript{62}.

Changes in ET-1 receptors density might be a consequence rather than a cause of cardiac disease, and may be also influenced by agonist concentration and/or presence of concomitant cardiac disease. The biological significance of down- or upregulation of ET-1 receptors needs to be explored. There is a complex relationship between agonist thresholds and receptor subtypes. Changes in receptors expression may not be primarily mediated by ligand binding interactions.

1.8. Genetics of AF

Recent genetic studies, triggered by recognition of a hereditary component of AF, have begun to identify AF-predisposing genes. A greater understanding of the genetics of AF may provide insights into novel signaling pathways, diagnostic testing and therapeutic targets for AF. Genetic variants linked to AF have been found for a variety of ion channels, circulating hormones and cytokines.

The candidate gene approach was first used to assess genetic determinants of disease and is still used to test the association of specific genetic variants with AF. Several candidate gene studies have characterized mutations in ion channels that may participate in AF; however, these mutations seem to be relatively rare causes of AF\textsuperscript{180}. Among the evaluated candidate genes are genes encoding potassium channel subunits\textsuperscript{181} that are
associated with gain or loss of function and enhanced or delayed atrial myocyte repolarization. Addition genes include those encoding the sodium channel subunits that are associated with shorter wavelength of conduction impulses and more frequent wavelets in the atria resulting in hyperexcitability\cite{182}; genes of SERCA regulatory proteins\cite{183}; genes of the RAAS\cite{184}, and connexin 40\cite{185}. Another recent study has identified genetic variants in atrial natriuretic peptide (ANP)\cite{186}. AF patients with a frame shift mutation in this gene have high plasma ANP levels, implicating the ANP-cyclic guanosine monophosphate (cGMP) pathway in atrial electrical instability. In principle, these genetic studies might help to determine which patients’ could benefit most from ion channel blockers or other drug interventions.

With the current availability of genome-wide arrays capable of simultaneously assessing hundreds of thousands of single nucleotide polymorphisms (SNPs), dissection of the underlying genetic loci for AF is now easier and more comprehensive. Gudbjartsson et al\cite{187} performed a genome-wide association study in 550 patients with AF and 4,476 controls from Iceland to study potential SNPs conferring risk of AF. The study was further replicated in additional samples from European populations. Two SNPs on chromosome 4q25 were significantly associated with AF: rs2200733 and rs10033464. The risk of AF was increased by 1.72 and 1.39 per copy, respectively. The SNP with the strongest association, rs2200733, was replicated in a Chinese population, where the risk of AF was increased by 1.42 per copy. Intriguingly, this SNP has also been associated with risk of embolic stroke\cite{188}. As AF increases risk of stroke and morbidity, it is possible that the stroke risk associated with this SNP is actually due to increased risk of AF. Interestingly, both rs2200733 and rs10033464 have been shown to be associated with
early and late AF recurrence following catheter ablation\textsuperscript{189}.

There are no known genes present in the 4q25 locus containing these two variants. The nearest genes are PITX2 and ENPEP. PITX2 (paired-like home domain transcription factor-2) encodes a transcription factor important during cardiac development; this transcription factor is a primary determinant of asymmetric morphogenesis in the heart. The ENPEP gene encodes an aminopeptidase responsible for the degradation of Ang-II in the vascular system. Whether PITX2 or ENPEP is/are truly the relevant gene(s) at this locus is still an area of active investigation and needs to be fully evaluated, and the functional mechanisms underlying the significance of these SNPs warrants further study. Chung et al\textsuperscript{190} presented a study in AF patients with myocardial infarction identifying another SNP located in 4q25 locus (rs1461994) which is in perfect linkage disequilibrium with rs220733 SNP and which is associated with the expression of LRIT3 (Leucine-rich repeat, immunoglobulin-like domain and transmembrane domain-containing protein 3) and the C isoform of PITX2.

A meta-analysis of genome wide association studies was recently performed in lone AF cohort conducted using 1,335 individuals with lone AF and 12,844 controls\textsuperscript{191}. The study identified multiple SNPs associated with increased lone AF risk. Among those are the same SNPs located in the 4q25 locus previously shown by Gudbjartsson et al\textsuperscript{187}. The meta-analysis also identified several new SNPs, including one located on chromosome 1q21 (rs13376333). This SNP is intronic to KCNN3, a gene encoding a Ca\textsuperscript{2+}-activated potassium channel involved in atrial repolarization. This SNP was replicated in two independent cohorts with lone AF (combined odds ratio = 1.52).
The accumulation of genetic variants discovered thus far suggests that genetics may contribute to AF risk to a greater extent than originally imagined. These variants may regulate genes encoding hormonal factors or ionic channels and may identify high-risk patients who are susceptible to the potential side effects of ion channel-blockers, enzyme inhibitors, or receptor antagonists. For example, RAAS gene polymorphisms in patients with AF may determine their response to angiotensin-converting enzyme inhibitor therapy.

1.8.1. ET-1 and Genetic Polymorphisms

Polymorphic variants of the ET-1 gene may influence its gene expression, and post-transcriptional regulation may also be involved in the onset of AF and related cardiovascular diseases. Few studies have yet examined the relation between cardiac diseases and polymorphisms associated with genes in the ET-1 system\textsuperscript{192, 193}. One study reported that a G/T polymorphism associated with an amino acid substitution (Lys to Asn) at codon 198 in exon 5 of the ET-1 gene is associated with increased blood pressure (BP) in overweight people, suggesting that this polymorphism may strongly interact with BMI in the determination of BP levels, particularly in obese subjects\textsuperscript{194, 195}. Interestingly, the same SNP is also associated with the severity of obstructive sleep apnea in obese subjects\textsuperscript{196}, sleep apnea is associated with AF\textsuperscript{197}. ET-1 gene polymorphisms variants have also been associated with angina\textsuperscript{198} and HTN\textsuperscript{199}. Unlike HTN, studies in HF reported thus far have failed to link plasma ET-1 to SNPs located in the ET-1 gene\textsuperscript{200}, suggesting that changes in plasma ET-1 in HF are more related to acute changes and environmental factors related to the disease progression.
Intriguingly, in patients with severe ventricular dysfunction and malignant ventricular arrhythmias, the presence of polymorphisms in the ET-1 gene predicted increased risk of hemodynamic collapse during these arrhythmias\textsuperscript{201}. In AF, there is no evidence showing a relation of ET-1 polymorphism to AF persistence.

Endothelin-converting enzyme-I (ECE-1) is the main enzyme responsible for the final step of ET-1 processing and may contribute to pathology of cardiac disease. An association between an ECE-1 polymorphism (B C-338A) and BP levels has been reported in women but not in men. Females homozygous for the risk allele had significantly higher systolic and diastolic BP\textsuperscript{202}. This variant has also been shown to be associated with increased risk of CAD\textsuperscript{203}.

Interestingly, a SNP in exon 6 of the ETAR receptor gene had a marked influence on survival in patients with non-ischemic dilated cardiomyopathy\textsuperscript{204}. Carriers of the ETAR minor risk allele had a \textgreater5 fold increased risk of death within 2 years after diagnosis; this effect was independent from other predictors of survival in the same patients\textsuperscript{204}. Presence of this genetic variant might influence the response to ET-1 receptor antagonists if these compounds were to be used therapeutically in HF patients.

**1.9. Experimental and Clinical Trials of Endothelin Antagonists**

Accumulating evidence supporting the pathophysiological role of ET-1 led to interest in the development of ET-1 antagonists that could be used to treat cardiovascular diseases. In animal studies, use of ET-1 antagonists improved hemodynamic and structural remodeling in HF. For example, in a rat coronary artery ligation model of HF long-term use of bosentan, a non-selective endothelin receptor antagonist, improved survival\textsuperscript{205}. Improved survival was associated with a reduction in preload and afterload, increased
cardiac output, decreased left ventricular (LV) hypertrophy and dilatation, and decreased cardiac fibrosis. Similarly, in a canine model of HF induced by intra-coronary microembolization, chronic use (3 months, twice/day) of bosentan prevented the progression of LV dysfunction and ameliorated LV remodeling\textsuperscript{206}. There are no data available on the impact of endothelin antagonists on atrial remodeling.

In 2001, the FDA approved the administration of the ET-1 antagonist bosentan for the treatment of patients with pulmonary HT (PHTN)\textsuperscript{207}. Strong clinical and preclinical trial demonstrated the important role of ET-1 in the pathophysiology of PHTN\textsuperscript{207}. Patients with PHTN have increased plasma ET-1\textsuperscript{177, 208}. In addition, the pulmonary arteries of PHTN patients show increased ET-1 mRNA and protein abundance\textsuperscript{209}.

Following an initial study supporting the beneficial effects of bosentan on hemodynamics of PHTN patients\textsuperscript{210}, the efficacy of bosentan was further documented in another two multicenter trials\textsuperscript{211, 212}. Bosentan was associated with improved hemodynamics, reduced hospitalization, and reduced need for lung transplants. However, adverse effects associated with treatment included interactions with commonly used drugs (warfarin) and with cytochrome P450 (CYP450) liver enzymes. Thus, selective ETAR antagonists were developed to avoid such adverse effects; one of these (ambrisentan) is approved in the USA to treat patients with PHTN. Ambrisentan has shown no apparent pharmacokinetic interactions with common drugs or liver enzymes\textsuperscript{207}.

As described above, plasma ET-1 is increased in hypertensive patients\textsuperscript{57}. A randomized, double-blinded clinical trial in patients with HTN (n=379) documented a significant reduction in BP upon treatment with darusentan (an ETAR selective drug) in patients
who had not attained their BP treatment goals with three or more antihypertensive drugs\textsuperscript{213}.

Kiowski and coworkers were the first to publish that use of bosentan in HF patients reduced arterial and pulmonary artery pressures and increased cardiac index\textsuperscript{214}. In a larger trial, the research on endothelin antagonism in chronic HF study (REACH-1) sought to evaluate the efficacy of bosentan in HF\textsuperscript{214}. A total of 368 patients were randomized to either bosentan or placebo after 6 months of therapy. 50\% of patients who completed the study (113 bosentan, 58 placebo) tended to have favorable effect in term of death and hospitalization\textsuperscript{215}. Bosentan was also associated with improved hemodynamics\textsuperscript{216}.

In contrast, other HF clinical trials such as the “Endothelin Antagonist Bosentan for Lowering Cardiac Events in Heart Failure” (ENABLE I&II) and the “Enrasentan Clinical Outcomes Randomized trial of Endothelin antagonism,” (ENCORE) were not encouraging, as bosentan was not better than placebo but was associated with more fluid retention and more frequent adverse events\textsuperscript{217}.

Tezosentan is another dual ETAR and ETBR antagonist. Results of the randomized intravenous tezosentan (RITZ-2) program to assess the therapeutic potential of tezosentan in HF showed that tezosentan improved cardiac index, reduced capillary wedge pressure, improved hemodynamics, and improved dyspnea at doses of 25 and 50 mg/hr. However, the follow up RITZ 4 trial was neutral and did not show a significant difference in mortality between treatment and placebo\textsuperscript{218}. Similarly, another randomized clinical trial testing the therapeutic value of tezosentan did not show improved symptoms or clinical outcomes in patients with acute HF\textsuperscript{219}.
As use of ET-1 antagonists in HF clinical trials was either neutral or not beneficial, the drugs have not been approved by the FDA for treatment of HF. It is important to note that systemic blockade of both ET receptors may not be an optimal strategy. In healthy individuals, ETBR blockade is associated with peripheral vasoconstriction, reduced stroke volume and cardiac output\textsuperscript{220}. As ETBR mediates vasodilatation and is involved in ET-1 clearance, while ETAR is associated with vasoconstriction and increased Ca\textsuperscript{2+} overload\textsuperscript{10}, there is an impetus to test selective ETAR antagonists in HF. Use of non-selective ET-1 antagonists may mask the beneficial effects of antagonizing the ETAR subtype. Trials utilizing selective ETAR antagonists in HF are needed.

Although accumulating evidence suggests an important role of ET-1 in the pathology of HF, HT and PHTN, there is less evidence on the role of ET-1 in promoting AF. Thus, ET-1 antagonists have not yet been tested in AF patients. Whether there is data available on AF development/progression in patients treated with ET-1 antagonists in the described clinical trials is presently unknown, but would be of great interest.

1.10. Rationale and Hypothesis

Strong clinical and preclinical evidence suggest an important role for ET-1 in the setting of cardiovascular pathology. Observations of elevated plasma big ET-1 and ET-1 in patients with HF, HT, PHTN and cardiomyopathy\textsuperscript{57, 58, 59} suggest the possibility of ET-1 involvement in the etiology of these diseases. However, studies on AF are more limited, with some suggestions that AF patients with underlying HF disease have elevated plasma ET-1 levels\textsuperscript{61}. Thus, ET-1 may contribute to AF development in the setting of HF.
In vitro studies provide an intriguing possibility that ET-1 may have a causal role in AF pathogenesis by affecting atrial myocyte electrical properties and contractility\textsuperscript{63, 64}. However, in vivo studies in experimental models that seek to assess the impact of ET-1 on the atria are limited. Transgenic mice with cardiac overexpression of ET-1 have provided the strongest evidence that ET-1 promotes inflammation and fibrosis\textsuperscript{26}, pathways critical for the development of an AF substrate.

ET-1 is a strong mitogenic factor, promoting cardiac myocyte hypertrophy and fibroblast proliferation; however, it is unclear whether atrial ET-1 is increased in human AF in vivo and contributes to atrial fibrosis and AF persistence. We sought to test and translate in vitro findings in human patients with underlying cardiac disease and evaluate whether ET-1 is increased in human atria and contribute to AF persistence (Chapter 2).

Studies on human lone AF patients suggested that plasma ET-1 prior to pulmonary vein isolation (PVI) predicts AF recurrence following PVI\textsuperscript{60}. Similarly, genetic studies provided evidence of strong association of 4q25 SNP with AF risk and AF recurrence following catheter ablation\textsuperscript{189}. We sought to determine if plasma ET-1 are associated with the 4q25 SNP associated with AF risk in lone AF patients (Chapter 3).

The strong association of AF with HF suggests that ventricular dysfunction promotes atrial dysfunction and creates a substrate for AF. It is unclear if ventricular dysfunction increases atrial ET-1 by promoting atrial dysfunction. A study was also designed to test the effects of ventricular tachypacing induced HF on atrial ET-1 production and fibrosis (Chapter 4).

Many patients that undergo cardiac surgery develop POAF. We sought to test if ET-1 is increased following surgery and promotes post-operative AF. Use of omega-3 fatty acids
has been shown to reduce incidence of coronary artery disease and POAF\textsuperscript{110}. We tested the hypothesis that dietary omega-3 fatty acids would attenuate the development of an AF substrate and limit ET-1 expression in a canine POAF model (Chapter 5).

The literature shows that the ET-1 system modulates cardiac contractility and remodeling in multiple pathological conditions. Here, we test the hypothesis that ET-1 is increased in AF \textit{in vivo}, and that it contributes to the development of a substrate for AF and increases AF risk.
Chapter 2

Association of Left Atrial Endothelin-1 with Atrial Rhythm, Size and Fibrosis in Patients with Structural Heart Disease

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Abstract

Background: Atrial fibrillation (AF) promotes atrial remodeling and can develop secondary to heart failure (HF) or mitral valve disease. Cardiac endothelin-1 (ET-1) expression responds to wall stress, and can promote myocyte hypertrophy and interstitial fibrosis. We tested the hypothesis that atrial ET-1 is elevated in AF and is associated with AF persistence.

Methods and Results: Left atrial appendage (LAA) tissue was studied from coronary artery bypass graft, valve repair, and/or Maze procedure patients in sinus rhythm with no history of AF (SR, n=21), with history of AF but in SR at surgery (AF/SR, n=23), and in AF at surgery (AF/AF, n=32). The correlation of LA size with atrial protein and mRNA expression of ET-1 and ET-1 receptors (ETAR and ETBR) was evaluated. LAA ET-1 content was higher in AF/AF than in SR, but receptor levels were similar. Immunostaining revealed that ET-1 and its receptors were present both in atrial myocytes and in fibroblasts. ET-1 content was positively correlated with LA size, HF, AF persistence, and severity of mitral regurgitation (MR). Multivariate analysis confirmed associations of ET-1 with AF, hypertension (HTN) and LA size. LA size was associated with ET-1 and MR severity. ET-1 mRNA levels were correlated with genes involved in cardiac dilatation, hypertrophy and fibrosis.

Conclusion: Elevated atrial ET-1 content is associated with increased LA size, AF rhythm, HTN and HF. ET-1 is associated with atrial dilatation, fibrosis and hypertrophy, and likely contributes to AF persistence. Interventions that reduce atrial ET-1 levels and/or block its receptors may slow AF progression.
2.1. Introduction

Atrial fibrillation (AF) increases risk of stroke, morbidity and mortality. AF is present in 50% of patients undergoing mitral valve surgery and in 5-50% of patients with hypertension (HTN) and heart failure (HF). Atrial structural remodeling, encompassing myocyte hypertrophy, chamber dilatation and interstitial fibrosis, contributes to the development and progression of AF. Increased LA size reflects chronically increased atrial volume or pressure, and LA dilatation is associated with a poor clinical prognosis.

Stressors that promote atrial structural remodeling include HTN, mitral valve regurgitation (MR), HF, AF, and myocardial ischemia. Common to these pathologies is endothelin-1 (ET-1), an autocrine and paracrine mediator with mitogenic, inotropic and arrhythmogenic activity in cardiac muscle. ET-1 expression and release are promoted by a variety of stimuli, including hypoxia, ischemia, increased wall shear stress, stretch, and pressure overload, both in vitro and in vivo. Elevated ET-1 levels may promote atrial dilatation, hypertrophy and fibrosis. Increased atrial wall stress is associated with increased atrial collagen deposition, and ET-1 promotes fibroblast proliferation and extracellular matrix production. Changes in atrial geometry, conduction heterogeneity, and size may predispose the atria to persistent arrhythmia.

Plasma ET-1 levels are reported to be elevated in AF patients with underlying heart disease and in patients with HF and valvular heart disease. In HF patients, plasma ET-1 level was a strong and independent predictor of AF. Although elevated plasma ET-1 has been associated with increased risk of AF, local production and regulation of ET-1 in the atria has not yet been studied. Here we tested
the hypothesis that atrial ET-1 is increased in AF and is associated with LA enlargement, atrial fibrosis and AF persistence in patients with underlying cardiovascular disease.
2.2. Methods

2.2.1. Patient Selection
Left atrial appendage (LAA) specimens were obtained from patients referred to the Cleveland Clinic for cardiac surgical procedures in which the LAA was excised. Most patients had underlying structural heart disease. Clinical, laboratory and demographic data were obtained by query of the Cardiovascular Information Registry, a comprehensive surgical database. All patients provided informed consent; the protocol was approved by the Cleveland Clinic Institutional Review Board.
Patient samples were categorized into three groups based on arrhythmia history. Control patients were in sinus rhythm (SR), with no history of AF or atrial flutter, but had other cardiac conditions requiring surgery (e.g., coronary artery disease (CAD), mitral valve disease (MVD), HF). AF patients included both those presenting to surgery in AF (AF/AF) and in SR (AF/SR), as documented on the last electrocardiogram prior to surgery. Patients included in the SR, AF/AF and AF/SR groups were matched for age, gender, history of HF, HTN, and MR.

2.2.2. Echocardiographic Analysis of Left Atrial Dimensions
Echocardiographic studies were performed at our institution using digital imaging and archived for off-line analysis using Prosolv Cardiovascular software (Fuji Inc.). All studies were systematically reviewed and measured by a single experienced reader (AZ). LA dimensions were measured according to the recommendations of the American Society of Echocardiography\textsuperscript{229}. LA antero-posterior diameter (LAD) measurements were made in the parasternal long-axis view at end-systole. The largest antero-posterior
diameter is measured from the trailing edge of the posterior aortic wall to the leading edge of the posterior LA wall. LA volume (LAV) was calculated utilizing the biplane ellipsoid area-length method: Volume = \( \frac{8 \text{(A1)} \text{(A2)}}{3 \pi \text{(L)}} \) where A1 and A2 represent the maximal LA area acquired from the apical 4 and 2 chamber views, respectively. L is the shortest LA long-axis diameter, determined as the distance of the perpendicular line connecting the mitral annular plane and the superior aspect of the left atrium at either the 2 chamber or 4 chamber view. LA areas were measured both at end-systole and on the image immediately prior to mitral valve opening.

LA dimensions were indexed to body surface area (iLAD, iLAV), as previously described\textsuperscript{229}. The severity of mitral regurgitation is classified based on lesion severity (measured as effective regurgitant orifice area) and the yielding regurgitation volume and fraction overload, but it is also affected by left-ventricular systolic pressure and left-atrial compliance\textsuperscript{230}. MR severity was determined on scale of 0-4 of increasing severity; 0 represents no MR.

2.2.3. Enzyme Linked Immunoassay for Atrial ET-1

After transport from the operating room to the laboratory, separate sections of each LAA specimen were fixed in paraformaldehyde, or stored at -80°C for confocal and biochemical studies. Atrial ET-1 content was evaluated using an enzyme-linked immunoassay (Biomedica BI-20052, American Research Products, Inc, Belmont, MA). A transmural segment of the left atrial appendage (LAA, ~50mg) was homogenized for 20s in ice-cold mammalian protein extraction buffer (M-PER, Pierce, City) containing a protease-inhibitor mixture (Sigma). A Bradford assay (Bio-RAD Labs, Inc., Hercules, 46
CA) was used to measure protein concentrations. All samples were stored at -80°C until analysis. Samples were thawed at room temperature before the assay and diluted with 0.9% NaCl containing protease inhibitors at 0.8-µg/µl concentrations. A 50 µl aliquot (40 µg protein) was loaded into each well of a 96 well plate coated with a polyclonal rabbit anti-endothelin-1 antibody. Each sample was assessed in duplicate. The same (50 µL) aliquot volume was used for all tissue samples, controls and standards. A 200µl aliquot of the detection antibody (monoclonal mouse anti-endothelin-1 antibody) was then added to each well, and the plate was sealed to prevent evaporation. After overnight incubation, the liquid contents of the wells were discarded. Each well was washed five times (wash buffer), followed by addition of 200 µl of anti-mouse IgG antibody conjugated to horseradish peroxidase and incubation for 1 hr at RT. After washing again (5x), the substrate (TMB, 200 µl) was added into the wells and incubated for 30 min in the dark followed by 50µl of stopping buffer. The absorption of each well was immediately determined in an ELISA reader at 450nm, with a 690 nm reference. ET-1 quantity was proportional to the enzyme bound quantity, and values were interpolated from a calibration curve of standards.

2.2.4. Western Analysis

Western blot analysis was used to evaluate the expression of ETAR and ETBR proteins in LAA tissues. Tissues specimens (~50mg) were homogenized in M-PER buffer containing protease inhibitors, as described above. Proteins from the cell lysate were separated using SDS-PAGE (10% acrylamide gel), transferred to a nitrocellulose membrane, and probed with rabbit anti-ETAR or ETBR (1:200, Alomone labs). Blots were incubated with an IRDye 800 CW labeled donkey anti-rabbit secondary antibody,
washed, and imaged using a Li-Cor Odyssey multi-wavelength laser illumination scanner.

To correct for variations in protein loading, each blot was also probed with a mouse anti-GAPDH antibody (1:5000, Millipore). This antibody was detected using an IRDye 680 CW labeled anti-mouse secondary antibody. Dual-color scanned blots were analyzed on an identically sized region of protein in each lane. The cumulative density of the area within the box for each lane was used to normalize values of each receptor on the blot. The lane with the greatest protein density was set at unity. Loading variability was corrected by dividing the corresponding receptor intensity value in each lane by the relative abundance of GAPDH.

2.2.5. Microarray Analysis

Total RNA was isolated from each specimen (n=205) and mRNA levels were compared using Illumina microarray technology. Briefly, ~50 mg atrial tissue was homogenized in Trizol reagent (Invitrogen, CA) using a homogenizer with disposable generator probes. Following chloroform extraction and centrifugation, the aqueous phase was transferred to a new tube. Isopropyl alcohol was added and samples were stored at -80°C for overnight precipitation. After warming to room temperature, samples were centrifuged for 10 minutes. Ice cold 75% ethanol was added to the supernatant, and samples were again centrifuged for 10 minutes. Pellets were air dried for 5-10 minutes and RNA was dissolved in DEPC water. Total RNA was quantified by A_{260} measurement using a spectrophotometer.

RNA samples were processed by the Genomics Core at the Cleveland Clinic. Approximately 250 ng RNA was reverse transcribed into cRNA and biotin-UTP labeled
using the TotalPrep RNA Amplification Kit (Ambion, Austin, TX). cRNA was quantified using a nanodrop spectrophotometer and cRNA size distribution was assessed on a 1% agarose gel. cRNA was hybridized to Illumina Human HT12 Expression BeadChip arrays using standard protocols (Illumina, San Diego, CA). Arrays were scanned using a BeadArray reader. Expression data from all arrays were processed to remove batch variability prior to analysis.

2.2.6. Immunostaining and Confocal Microscopy

Immunostaining was used to evaluate the distribution of ET-1 and its receptors (ETAR and ETBR) in left atrial tissues. Transmural segments of freshly acquired LAA were placed in OCT medium, rapidly frozen and stored at -80°C. Frozen tissues were cryo-sectioned into 16-µm-thick sections. Sections were fixed in paraformaldehyde for 30 minutes at RT, blocked in 3%BSA (Sigma-Aldrich) for 1 hour, then incubated overnight with primary antibodies (rabbit anti-ETAR or anti-ETBR, 1:200, Alomone Labs, Jerusalem, or mouse anti-ET-1, 1:250, Abcam). To facilitate visualization of myocyte structure, sections were also incubated with either mouse monoclonal anti-α actinin (Sigma-Aldrich, 1:1000) or mouse anti-phalloidin labeled with Alexa fluor-647 (1:40, Molecular Probes). Sections were also stained with guinea pig anti-vimentin (1:800, ARP, Inc.) to visualize fibroblasts and other cells of mesodermal origin. Primary antibodies were visualized by use of appropriate Alexa-fluor conjugated secondary antibodies; DAPI was used to stain nuclei.

Negative controls included the omission of primary or secondary antibodies, and use of non-immune serums; these controls were included in all immunostaining experiments. Confocal immunofluorescence images of selected cardiac sections were obtained using a
Leica TCS SP AOBS spectral laser-scanning confocal microscope. Three-dimensional structures of each region were reconstructed from confocal immunofluorescence images recorded in z-series and analyzed using digital image-processing software (Volocity; Improvision, MA). Sections were evaluated at 63x magnification.

2.2.7. Statistical Analysis

Data are expressed as mean ± standard error, unless otherwise specified. Univariate and multivariate linear regression analyses were performed to evaluate the association of clinical and demographic variables with atrial ET-1 levels and LA size. Normally distributed variables are presented using bar plots and analyzed using ANOVA. Non-normally distributed variables are presented using box plots and analyzed using Mann-Whitney or Kruskal-Wallis tests. Spearman’s correlation was used to measure relation of ET-1 mRNA to variables that were not normally distributed. Univariate analyses were performed using GraphPad Prism 4; multivariate analyses were performed using JMP 7. Because atrial ET-1 levels were not normally distributed, a square root transformation of ET-1 levels was used in the multivariate linear regression model. The square root transformation of atrial ET-1 effectively normalized the dataset. Values of p<0.05 were considered statistically significant.
2.3. Results

2.3.1. Atrial Endothelin-1 and Endothelin-1 Receptor Studies

2.3.1.1. Patient Characteristics

LAA tissues from 76 patients were selected for biochemical and immunohistochemical analysis of atrial ET-1 and ET-1 receptor expression. Table 1 shows the three groups stratified based on atrial rhythm and prospectively matched by age, gender, and by cardiovascular comorbidities including HF, HTN and valvular disease: control patients SR (N=21), patients with a history of AF but in SR at time of surgery (AF/SR, N=23), and patients with a history of AF, presenting in AF at time of surgery (AF/AF, N=32). Surgical patients have complex cardiovascular problems, and many underwent treatment for more than one disorder (e.g. mitral valve repair, coronary artery bypass graft and Maze procedure, Table 2-1). These patients had significant cardiovascular disease; HTN, HF, CAD, and valvular diseases were present in 50%, 31%, 36%, and 78% of patients, respectively. LA dimensions were similar between rhythm groups; most patients had dilated left atria (median (IQR): 4.76 (4.11-5.13) cm).
Table 2-1: Patients characteristics (Tissue biochemistry, histology studies)

<table>
<thead>
<tr>
<th>Clinical Characteristics</th>
<th>SR (n=21)</th>
<th>AFSR (n=23)</th>
<th>AFAF (n=32)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>64 ± 2.9</td>
<td>64.1 ± 2.2</td>
<td>62.7 ± 1.9</td>
<td>0.55</td>
</tr>
<tr>
<td>Male sex, n (%)</td>
<td>11 (52.4)</td>
<td>15 (62.5)</td>
<td>23 (71.9)</td>
<td>0.35</td>
</tr>
<tr>
<td>Hypertension, n (%)</td>
<td>9 (42.9)</td>
<td>13 (54.2)</td>
<td>17 (53.1)</td>
<td>0.54</td>
</tr>
<tr>
<td>Body Mass Index</td>
<td>28.1 ± 1.3</td>
<td>27.7 ± 1.1</td>
<td>28.3 ± 1.1</td>
<td>0.89</td>
</tr>
<tr>
<td>CAD ≥50% stenosis, n (%)</td>
<td>9 (42.9)</td>
<td>7 (29.2)</td>
<td>12 (37.5)</td>
<td>0.37</td>
</tr>
<tr>
<td>Valvular disease, n (%)</td>
<td>17 (81.0)</td>
<td>20 (83.3)</td>
<td>23 (71.9)</td>
<td>0.38</td>
</tr>
<tr>
<td>Congestive Heart Failure, n (%)</td>
<td>5 (23.8)</td>
<td>8 (33.3)</td>
<td>11 (34.4)</td>
<td>0.44</td>
</tr>
<tr>
<td>Stroke history, n (%)</td>
<td>2 (9.5)</td>
<td>1 (4.2)</td>
<td>1 (9.4)</td>
<td>0.26</td>
</tr>
<tr>
<td>Diabetes mellitus, n (%)</td>
<td>3 (14.3)</td>
<td>2 (8.33)</td>
<td>4 (12.5)</td>
<td>0.90</td>
</tr>
<tr>
<td>Median AF duration, months (IQR)</td>
<td>0 (0-0)</td>
<td>48 (3.5-85.5)</td>
<td>60 (27-120)</td>
<td>0.28</td>
</tr>
<tr>
<td>Surgery Type</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mitral valve replacement/repair</td>
<td>14 (66.7)</td>
<td>13 (54.2)</td>
<td>17 (53.1)</td>
<td>0.90</td>
</tr>
<tr>
<td>Tricuspid valve replacement/repair</td>
<td>4 (19.0)</td>
<td>6 (25.0)</td>
<td>8 (25.0)</td>
<td>0.85</td>
</tr>
<tr>
<td>Coronary artery bypass graft</td>
<td>8 (38.1)</td>
<td>5 (20.8)</td>
<td>8 (25.0)</td>
<td>0.24</td>
</tr>
<tr>
<td>Maze procedure</td>
<td>2 (9.5)</td>
<td>21 (87.5)</td>
<td>31 (96.9)</td>
<td>1.1x10^-7</td>
</tr>
<tr>
<td>Echocardiographic Characteristics</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mitral valve stenosis, n (%)</td>
<td>2(9.5)</td>
<td>2(8.3)</td>
<td>4(12.5)</td>
<td>0.19</td>
</tr>
<tr>
<td>Mitral regurgitation, ≥2+, n (%)</td>
<td>16 (76.2)</td>
<td>13 (54.2)</td>
<td>19 (59.4)</td>
<td>0.23</td>
</tr>
<tr>
<td>Tricuspid regurgitation, ≥2+, n (%)</td>
<td>6 (28.6)</td>
<td>8(33.3)</td>
<td>14 (43.8)</td>
<td>0.53</td>
</tr>
<tr>
<td>Indexed LA diameter, cm/m^2</td>
<td>2.38 ± 0.08</td>
<td>2.39 ± 0.11</td>
<td>2.5 ± 0.10</td>
<td>0.62</td>
</tr>
<tr>
<td>Indexed LA volume, ml/m^2</td>
<td>47.1 ± 3.99</td>
<td>58.1 ± 6.79</td>
<td>59.5 ± 5.33</td>
<td>0.27</td>
</tr>
<tr>
<td>Indexed LA area (2 chamber, cm^2/m^2)</td>
<td>11.79 ±0.89</td>
<td>14.21 ± 1.06</td>
<td>14.05 ± 0.81</td>
<td>0.16</td>
</tr>
<tr>
<td>Indexed LA area (4 chamber, cm^2/m^2)</td>
<td>13.1 ± 0.86</td>
<td>14.2 ± 1.16</td>
<td>15.5 ± 0.98</td>
<td>0.27</td>
</tr>
<tr>
<td>Indexed Length (Apical shortest, cm^2)</td>
<td>2.81 ± 0.17</td>
<td>3.10 ± 0.16</td>
<td>3.16 ± 0.12</td>
<td>0.22</td>
</tr>
<tr>
<td># with known LA dimensions, n%</td>
<td>19 (90.5)</td>
<td>22 (91.7)</td>
<td>30 (93.8)</td>
<td>0.78</td>
</tr>
<tr>
<td>Left ventricular ejection Fraction%</td>
<td>52.7 ± 3.2</td>
<td>54.5 ± 2.1</td>
<td>50.5 ± 1.5</td>
<td>0.40</td>
</tr>
</tbody>
</table>

Use of medications

| ACE inhibitors, ARBs                                      | 8 (38.1)      | 14 (58.3)     | 14 (43.8)     | 0.36    |
| Other diuretics*                                          | 11 (52.4)     | 11 (45.8)     | 19 (59.4)     | 0.66    |
| Beta blockers                                             | 7 (33.3)      | 11 (45.8)     | 14 (43.8)     | 0.60    |
| Statins                                                   | 2 (9.5)       | 3 (12.5)      | 3 (9.3)       | 0.90    |

Values are mean ± SEM, unless indicated. AF: atrial fibrillation, CAD: coronary artery disease, LA: Left atria; ACE: angiotensin converting enzyme; ARBs: angiotensin-II receptor blockers. SR: sinus rhythm. ANOVA and Chi-square test was used to assess p value (SR vs. AF/AF) for continuous and categorical variables, respectively. * Other diuretics included thiazides and aldosterone antagonists, but not ACE inhibitors or ARBs.
2.3.1.2. ET-1 and AF Status

LAA ET-1 protein levels were evaluated in atrial homogenates. ET-1 content was greater in AF/AF patients than in SR patients (Median (IQR): 6.5 (4.3-10.4) vs. 4.1 (2.4-6.5) fmol/mg protein), p=0.0153/Kruskal-Wallis (Figure 2-1A). To further assess the relation of LAA ET-1 content to AF persistence, AF patients were grouped into three matched sets: permanent AF (Pm), persistent AF (Ps), and paroxysmal AF (Px), Table 2-2. Paroxysmal AF was defined as AF with spontaneous termination within 7 days. Persistent AF lasted more than 7 days and required cardioversion for restoration of sinus rhythm. Permanent AF was defined as long-lasting AF in which cardioversion was either contra-indicated or ineffective\textsuperscript{231}. Permanent AF patients had a greater LAA ET-1 content than paroxysmal AF patients (median (IQR): 7.0 (4.9-10.4) vs. 3.96 (2.7-8.15) fmol/mg protein, p=0.0286/ Kruskal-Wallis, Figure 2-1B).

2.3.1.3. Impact of Cardiovascular Disease on LAA ET-1 Content

LAA ET-1 content was evaluated as a function of comorbid cardiovascular conditions. LAA ET-1 content was higher in HF patients (median (IQR): 4.3 (2.9-6.5) vs. 7.2 (4.6-10.8) fmol/mg protein, p=0.0013/Mann-Whitney test (Figure 2-1C), and as a function of MR severity, p=0.0045 (Figure 2-1D). Atrial ET-1 was not correlated with left ventricular ejection fraction (Spearman’s r=-0.15, p=0.21).
Table 2-2: AF patient characteristics, tissue ET-1 studies

<table>
<thead>
<tr>
<th></th>
<th>Px AF (n = 16)</th>
<th>Ps AF (n = 10)</th>
<th>Pm AF (n = 25)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Clinical Characteristics</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age, years</td>
<td>61.6 ± 2.89</td>
<td>64.1 ± 3.12</td>
<td>63.3 ± 2.31</td>
<td>0.83</td>
</tr>
<tr>
<td>Male sex, n (%)</td>
<td>11 (68.7)</td>
<td>7 (70.0)</td>
<td>18 (72.0)</td>
<td>0.97</td>
</tr>
<tr>
<td>Hypertension, n (%)</td>
<td>8 (50.0)</td>
<td>5 (50.0)</td>
<td>13 (52.0)</td>
<td>0.98</td>
</tr>
<tr>
<td>Body Mass Index</td>
<td>28.6 ± 1.31</td>
<td>29.1 ± 1.98</td>
<td>27.5 ± 1.23</td>
<td>0.70</td>
</tr>
<tr>
<td>CAD ≥50% stenosis, n (%)</td>
<td>7 (43.8)</td>
<td>3 (30.0)</td>
<td>5 (20.0)</td>
<td>0.93</td>
</tr>
<tr>
<td>Valvular disease, n (%)</td>
<td>11 (68.75)</td>
<td>6 (60.0)</td>
<td>18 (72.0)</td>
<td>0.78</td>
</tr>
<tr>
<td>Congestive Heart Failure, n (%)</td>
<td>4 (25.0)</td>
<td>4 (40.0)</td>
<td>7 (28.0)</td>
<td>0.71</td>
</tr>
<tr>
<td>Stroke history, n (%)</td>
<td>1 (6.25)</td>
<td>1 (10.0)</td>
<td>0 (0.0)</td>
<td>0.32</td>
</tr>
<tr>
<td>Diabetes mellitus, n (%)</td>
<td>1 (6.25)</td>
<td>2 (20.0)</td>
<td>2 (8.00)</td>
<td>0.23</td>
</tr>
<tr>
<td>Median AF duration, months(IQR)</td>
<td>36 (5-79)</td>
<td>36 (16-78)</td>
<td>73 (32-153)</td>
<td>0.16</td>
</tr>
<tr>
<td><strong>Surgery Type</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mitral valve replacement/repair</td>
<td>10 (62.50)</td>
<td>6 (60.00)</td>
<td>14 (56.00)</td>
<td>0.35</td>
</tr>
<tr>
<td>Tricuspid valve replacement/repair</td>
<td>3 (18.75)</td>
<td>2 (20.0)</td>
<td>4 (16.0)</td>
<td>0.11</td>
</tr>
<tr>
<td>Coronary bypass graft</td>
<td>3 (18.75)</td>
<td>2 (20.0)</td>
<td>4 (16.0)</td>
<td></td>
</tr>
<tr>
<td>Maze procedure</td>
<td>16 (100)</td>
<td>10 (100)</td>
<td>25 (100)</td>
<td>1.00</td>
</tr>
<tr>
<td><strong>Echocardiographic Characteristics</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mitral regurgitation ≥2+, n (%)</td>
<td>7 (43.75)</td>
<td>7 (70.0)</td>
<td>14 (56.0)</td>
<td>0.35</td>
</tr>
<tr>
<td>Tricuspid regurgitation ≥2+, n (%)</td>
<td>4(25.0)</td>
<td>3(30.0)</td>
<td>11 (44.0)</td>
<td>0.35</td>
</tr>
<tr>
<td>Indexed LA diameter, cm/m^2</td>
<td>2.16 ± 0.12</td>
<td>2.83 ± 0.28</td>
<td>2.52 ± 0.10</td>
<td>0.05</td>
</tr>
<tr>
<td>Indexed LA volume, ml/m^2</td>
<td>51.7± 5.88</td>
<td>84.7 ± 13.10</td>
<td>60.46 ± 6.51</td>
<td>0.25</td>
</tr>
<tr>
<td>Indexed LA area (2 chamber, cm^2/m^2)</td>
<td>13.3 ± 1.16</td>
<td>17.7 ± 1.78</td>
<td>13.72 ± 0.92</td>
<td></td>
</tr>
<tr>
<td>Indexed LA area (4 chamber, cm^2/m^2)</td>
<td>13.5 ± 1.11</td>
<td>17.4 ± 2.47</td>
<td>15.9 ± 1.15</td>
<td>0.80</td>
</tr>
<tr>
<td>Indexed Length (Apical shortest, cm/m^2)</td>
<td>2.99 ± 0.19</td>
<td>3.45 ± 0.25</td>
<td>3.16 ± 0.14</td>
<td>0.20</td>
</tr>
<tr>
<td># with known LA dimensions, n (%)</td>
<td>16 (100)</td>
<td>8 (80.0)</td>
<td>21 (84.0)</td>
<td>0.20</td>
</tr>
<tr>
<td>Ejection Fraction,%</td>
<td>51.9 ± 2.89</td>
<td>54.7 ± 2.71</td>
<td>52.6± 1.17</td>
<td>0.75</td>
</tr>
<tr>
<td><strong>Use of medications</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACE inhibitors, ARBs, n (%)</td>
<td>6 (37.50)</td>
<td>6 (60.0)</td>
<td>12 (48.0)</td>
<td>0.53</td>
</tr>
<tr>
<td>Other diuretics, n (%)</td>
<td>7 (43.75)</td>
<td>4 (40.0)</td>
<td>16 (64.0)</td>
<td>0.136</td>
</tr>
<tr>
<td>Beta blockers, n (%)</td>
<td>4 (25.0)</td>
<td>6 (60.0)</td>
<td>13 (52.0)</td>
<td>0.29</td>
</tr>
<tr>
<td>Statins, n%</td>
<td>3 (18.75)</td>
<td>1 (10.0)</td>
<td>2 (8.0)</td>
<td>0.57</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM, unless otherwise indicated.
AF: atrial fibrillation; CAD: coronary artery disease; Px: paroxysmal; Ps: persistent; Pm: permanent; LA: Left atria; ACE: angiotensin converting enzyme; ARBs: angiotensin-II receptor blockers. P value (Pm vs. Px) was computed using ANOVA and Chi-square for continuous and categorical variables, respectively.
Figure 2-1: Left atrial ET-1 content in patients with structural cardiovascular diseases.

Box plots show median, 25\textsuperscript{th} and 75\textsuperscript{th} percentile values; whiskers indicate minimum and maximum values for LA ET-1 in SR, AF/SR or AF/AF patients at surgery (A); in Px (paroxysmal), Ps (persistent), Pm (permanent) AF (B, Kruskal-Wallis); and in heart failure (HF) or no HF (C, Mann-Whitney). Panel D is a box plot showing median values of LA ET-1 content in patients with mitral regurgitation (MR) of increased severity (0-4, Kruskal-Wallis). “r” is Spearman’s correlation coefficient.
By univariate analysis, LAA ET-1 levels were similar between hypertensive and non-hypertensive patients (Median (IQR): 4.93 (3.79-9.7) vs. 5.1 (3.3-10.5) fmol/mg protein, p=0.35/Mann-Whitney test). By multivariate analysis, MR severity, HTN and HF were associated with the square root of atrial ET-1. However, as MR severity and HF are also linearly correlated with iLAD (MR, r=0.60, p= 1.02x10^{-7}; HF, r=0.294, p=0.0129), these parameters were not included in the final multivariate model. By step-wise analysis, CAD, diabetes, gender, BMI, use of beta-blockers and ACE inhibitors were not correlated with atrial ET-1, and thus were excluded from the final model. Multivariate analysis showed that, in addition to AF status, age, HTN and LA size were associated with square root of atrial ET-1 content (Table 2-3, R^2=0.49, P=3.95x10^{-9}, DF=65). Interestingly, this analysis revealed that statin-treated patients had lower LA ET-1 content than non-statin treated patients (p=0.05).
Table 2-3: Multivariate factors associated with square root of left atrial ET-1 content

Response = square root of ET-1 fmol/mg total protein
Unadjusted $R^2 = 0.5346$, $p=3.947 \times 10^{-9}$
Adjusted $R^2 = 0.4910$
# of observations= 71, DF=65

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<td>Hypertension</td>
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<td>0.0614</td>
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<td>Indexed LA diameter, cm/m$^2$</td>
<td>0.7503</td>
<td>0.1434</td>
<td>1.98x10$^{-6}$</td>
<td>5.23</td>
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<td>Use of statins</td>
<td>-0.2103</td>
<td>0.1057</td>
<td>0.051</td>
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AF status: (coded as SR=1, AF/SR=2, AF/AF=3), LA: Left atria. $R^2$ is coefficient of determination. $\beta$ is the standardized coefficient (slope/std error).
2.3.1.4. Determinants of LA Size

Echocardiographic data available from 71 patients was used to assess the relationships between atrial rhythm, LA size and LA ET-1 content. Atrial ET-1 content was positively correlated with LA size; patients with higher ET-1 levels had larger iLADs (Spearman’s \( r=0.61, p=1.08 \times 10^{-7} \), Figure 2-2A) and iLAVs (Figure 2.2B, \( r=0.5, p=1.2 \times 10^{-4} \)). This relationship was further evaluated among rhythm groups. Patients in AF at surgery (AF/AF) showed a stronger correlation of atrial ET-1 content with iLAD (\( r=0.78, p=2.9 \times 10^{-5} \)) than the AF/SR group (\( r=0.62, p=0.0002 \)). Intriguingly, no correlation was observed between atrial ET-1 level and iLAD among SR patients (\( r=0.24, p=0.39 \)). As the groups were matched for other cardiovascular conditions, these data suggest that atrial rhythm modulates the relationship between LA ET-1 content and LA size. Because reentrant electrical activity depends on tissue mass, it is not surprising that AF persistence is also associated with iLAD (Figure 2-2C). Together, these data show that ET-1 is associated with the factors that underlie AF persistence.

Severity of MR was also strongly associated with increased iLAD (\( p=3.8 \times 10^{-5} \), Kruskal-Wallis, Figure 2-2D) and iLAV (\( p=0.00016 \), Kruskal-Wallis).

Multivariate analysis identified severity of MR and square root of atrial ET-1 as the strongest predictors of LA enlargement, as assessed by both iLAD (\( R^2=0.51, p=4.67 \times 10^{-10} \), DF=66, Table 2-4) and iLAV (\( R^2=0.40, p=5.95 \times 10^{-6} \), DF=62). Interestingly, female gender was positively correlated with iLAD (Table 2-4); however, the effect size was small. In our dataset, neither AF status nor history of HTN were significant univariate predictors of iLAD and were not included in multivariate regression models for iLAD.
LA enlargement predicts mortality for HF patients. HF was a univariate predictor of both iLAD and atrial ET-1, and a predictor of iLAD by stepwise analysis; however, presence of ET-1 in the model masked this relation. Although MR severity and atrial ET-1 are related, both were strong predictors of iLAD in our multivariate model (Table 2-4).
Figure 2-2: Correlation of left atrial ET-1 with left atrial size in AF patients

A, B) Scatter plots showing the relationship of LA ET-1 to indexed LA diameter (A) and to indexed LA volume (B); “r” is Spearman’s correlation coefficient. C) Relation of atrial ET-1 to severity of AF among patients with AF history. Px: paroxysmal, Ps: persistent, Pm: permanent AF (ANOVA). D) Box plots showing relation of mitral regurgitation (MR) severity to indexed LA diameter (Kruskal-Wallis).
### Table 2-4: Multivariate predictors of left atrial size

Response = Indexed LA diameter (cm/m²)
Undejusted $R^2 = 0.5452$, $p = 4.67 \times 10^{-10}$
Adjusted $R^2 = 0.5102$
# of observations = 71, DF = 65

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<td>Female sex</td>
<td>0.1027</td>
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<td>MR severity</td>
<td>0.1244</td>
<td>0.0312</td>
<td>1.72 \times 10^{-4}</td>
<td>3.99</td>
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<tr>
<td>Square root ET-1, fmol/mg protein</td>
<td>0.2374</td>
<td>0.0642</td>
<td>4.47 \times 10^{-4}</td>
<td>3.70</td>
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<tr>
<td>Heart Failure</td>
<td>0.0759</td>
<td>0.0514</td>
<td>0.145</td>
<td>1.48</td>
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</tbody>
</table>

MR: Mitral regurgitation (coded as a continuous variable, 0-4); LA: Left atria, $R^2$ is the coefficient of determination. Beta ($\beta$) is the standardized coefficient (slope/std error).
2.3.1.5. Sources of Atrial ET-1

Although ET-1 is prominently produced by vascular endothelial and smooth muscle cells\textsuperscript{10}, ET-1 has also been reported to be produced by cardiac myocytes\textsuperscript{232} and fibroblasts\textsuperscript{233}. Confocal microscopy (Figure 2-3) confirmed that atrial ET-1 staining (red) is detectable in both cardiac myocytes and in fibroblasts (green). Consistent with our biochemical results, the intensity of ET-1 staining was associated with atrial rhythm. ET-1 staining was heterogeneously distributed.
Figure 2-3: Distribution of ET-1 in atrial myocytes and fibroblasts of AF patients

Representative confocal immunofluorescence images of LA sections from 3 patients: one in sinus rhythm (SR), one with AF history but in SR at surgery (AF/SR), and a patient in AF at surgery (AF/AF). Sections A were stained with ET-1 antibody (red) and phalloidin antibody to stain myocytes (green). Sections B were stained with the same ET-1 antibody (red) and with vimentin antibody to stain fibroblasts (green) (B). Each panel shows images representing ET-1 staining alone (red) or an overlay of ET-1 with phalloidin or vimentin (green).
**2.3.1.6 ET-1 Receptor Distribution and Density in Human AF**

ET-1 receptor activity modulates intracellular signaling pathways that affect contractility, excitability and transcription. In a subset of 45 patients (9 SR, 14 AF/SR, and 21 AF/AF), western blot analysis was used to assess ET-1 receptor density (ETAR and ETBR) relative to GAPDH. ETAR and ETBR density did not differ between study groups (Kruskal-Wallis test). This finding was confirmed by multivariate analysis, adjusting for possible confounders (e.g. HF, HTN, MR, age, sex, LA size, CAD) (Figure 2-4 A-D). LAA ET-1 content was weakly correlated with ETAR expression (Figure 2-4E), but not with ETBR expression, suggesting that ET-1 may modulate ETAR protein expression. Interestingly, ETAR protein expression was significantly correlated with ETBR protein expression (Figure 2-4F), suggesting that ET-1 independent mechanisms also modulate receptor expression.

Receptor distribution was also evaluated using immunohistochemistry. Confocal imaging showed no differences in ETAR distribution among the groups (Figure 2-5). ETAR (green) was abundantly expressed in cardiac myocytes (red) and in fibroblasts (purple), with no obvious differences in receptor localization between groups. ETAR expression in cardiac myocytes was most apparent in the cell membrane and intercalated disk regions, with less cytosolic staining, and minimal localization in the nucleus (blue). In fibroblasts, ETAR was expressed in the cytosol and the nucleus (Figure 2-5).

In contrast to ETAR, distribution of ETBR (green) in cardiac myocytes was more cytosolic, and ETBR was more abundant in the nucleus. In fibroblasts, ETBR was expressed in the cytosol and the nucleus (Figure 2-6).
Figure 2-4: Endothelin receptor type A and B density among AF patients

A and C show representative western blots of ETAR and ETBR proteins from left atrial appendage of patients with sinus rhythm (SR, n=9), with AF but SR at surgery (AF/SR, n=14) and in AF (AF/AF=22) at surgery. Panels B, D show box plots of receptor intensity relative to GAPDH of the blots in A and C by atrial rhythm (Kruskal-Wallis). Panels E, F are scatter plots showing relation of ETAR protein density with ET-1 protein and ETBR protein density, respectively. “r” is Spearman’s correlation coefficient.
Figure 2-5: ETAR protein distribution among AF patients

Representative confocal immunofluorescent images of LA sections stained for ETAR protein in patients with sinus rhythm (SR), with AF but in SR at surgery (AF/SR), and in AF at surgery (AF/AF). For each patient, 3 images document ETAR staining (green), A; an overlay of ETAR (green), vimentin to stain fibroblasts (purple), and DAPI to stain nuclei (blue), B; and an overlay of ETAR (green), α-actinin to stain myocytes (red), and DAPI to stain nuclei (blue), C. Note the predominance of ETAR in the myocyte membranes, intercalated discs, and in the cytosol. Vacuolization of atrial myocytes in AF/AF patient (less packed or empty spaces inside myocytes) is indicative of myocyte degeneration. Scale bar: 50 µm.
Figure 2-6: ETBR protein distribution among AF patients

Representative confocal immunofluorescent images of LA sections stained for ETBR protein in patients with sinus rhythm (SR), with AF but in SR at surgery (AF/SR), and in AF at surgery (AF/AF). For each patient, 3 images document ETBR staining (green), A; an overlay of ETBR (green), vimentin to stain fibroblasts (purple), and DAPI to stain nuclei (blue), B; and an overlay of ETBR (green), α-actinin to stain myocytes (red), and DAPI to stain nuclei (blue), C. Note the predominance of ETBR in the myocyte membranes, cell cytosol, and the nucleus. Scale bar: 50 µm
2.3.2. Microarray Results

Microarrays facilitate simultaneous comparisons of mRNA levels for many different genes. Illumina HT-12 expression arrays were used to characterize mRNA expression profiles in LAA specimens from 205 patients, 66 of whom overlapped with those in Table 2.1, above. Table 2-5 summarizes clinical and demographic characteristics of these patients.

Atrial ET-1 protein content and mRNA levels (EDN1) were correlated (Spearman’s r=0.32, p=0.0108, Figure 2-7A), suggesting that the ET-1 protein detected has an atrial origin. As reported in the subset of patients with LAA ET-1 measurements (Figure 2-2A), EDN1 expression levels were also correlated, though more weakly, with iLAD (r=0.22, p=0.01). Interestingly, Figures 2-7 B,C show that mRNA expression of endothelin converting enzyme (ECE2) was associated both with AF and with AF persistence, suggesting that ET-1 processing is activated during AF.

To gain more insight into the pathways related to ET-1 signaling, we performed an empirical Bayes regression analysis of 7099 probes that were detected in at least 75% of the samples at a p<0.01 threshold level and showed signs of variability across samples and which were consistently above background. Sample probe expression was correlated with ET-1 mRNA (EDN1) expression using a regression procedure. Microarray regression analysis was adjusted for sex, age, history of AF, HF, HTN and MVD. The analysis identified more than 1500 genes that were associated with EDN1 expression. We then focused our analysis on probes that were decreased or increased by at least 10% for a 20% increment in EDN1 expression. This analysis identified 129 genes that were associated with ET-1 expression. Table 2-6 shows nested group of genes that are
associated with ET-1 mRNA under the described criteria (unpublished data). This analysis confirms that many of the genes involved in cardiac hypertrophy and fibrosis are associated with ET-1 mRNA expression.

Plasma BNP has been identified as a predictor of AF. In vitro, ET-1 has been reported to regulate atrial BNP expression. To assess whether this occurs in human atria, we evaluated the relationship between atrial ET-1 protein and mRNA (EDN1) with BNP mRNA (NPPB). EDN1 (Figure 2-7D) and ET-1 protein expression were both strongly correlated with NPPB expression. Atrial NPPB abundance was also associated with AF and HF (data not shown).
Table 2-5: Patient population characteristics, mRNA expression array studies

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<td>Body Mass Index</td>
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<td>History of AF</td>
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<td>CAD ≥50% stenosis, n (%)</td>
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<td>Valvular disease, n (%)</td>
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<td>Congestive Heart Failure, n (%)</td>
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<td>Stroke history, n (%)</td>
<td>17 (8.3)</td>
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<tr>
<td>Diabetes mellitus</td>
<td>34 (16.6)</td>
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**Surgery Type**

- Mitral valve replacement/repair 71 (34.8)
- Tricuspid valve replacement/repair 22 (10.7)

**Echocardiographic Characteristics**

- Mitral regurgitation, ≥2+, n (%) 135 (66.2)
- Tricuspid regurgitation, >2+, n (%) 104 (51.0)
- Indexed LA diameter, cm/m² 2.37± 0.04
- Left ventricular ejection Fraction (%) 50.9±1

Values are expressed as mean ± SEM, unless otherwise indicated.

AF: atrial fibrillation; CAD: coronary artery disease; LA: Left atria.
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List of top genes associated with 10% increase or decrease (-) with a 20% increase in ET-1 mRNA expression. FC: fold change; FC20%: is the fold change associated with a 20% increase in ET-1 mRNA expression.
Figure 2-7: ET-1 gene expression and processing during AF. A Scatter plot shows the relation of LA ET-1 mRNA (EDN1) to ET-1 protein; (A). Box plots show median, 25th and 75th percentile values; whiskers indicate minimum and maximum values for relation of LA endothelin converting enzyme 2 mRNA (ECE2) to rhythm (AF/AF vs. SR patients, B) and AF persistence (Pm>Ps>Px) (C); Kruskal-Wallis test. Panel D shows the relation of LA EDN1 to mRNA level of brain natriuretic peptide, NPPB. “r” is Spearman’s correlation coefficient.
In addition to promoting myocyte hypertrophy, ET-1 can stimulate fibroblast proliferation and extracellular matrix deposition. Interstitial fibrosis (primarily composed of collagen) can be a critical element of the substrate for AF. We assessed the relationships between atrial ET-1 and collagen expression. The mRNA levels of collagen isoform 1 (Fig 2-8A), as well as collagen isoforms 3 and 4 were each positively correlated with atrial EDN1 (COL3A1: Spearman’s $r=0.39$, $p=6.8 \times 10^{-6}$; COL4A1: $r=0.42$, $p=0.0023$, respectively). In a subset of samples, quantitative image analysis of Masson’s trichrome stained sections documented a similar relationship between atrial ET-1 protein and collagen deposition ($r=0.39$, $p=0.065$, Figure 2-8B), suggesting a functional relationship between atrial ET-1 protein and development of atrial fibrosis. ET-1 may have both paracrine and autocrine activities that promote atrial fibroblasts proliferation.

Platelet derived growth factor (PDGF) signaling is implicated as a modulator of fibroblast proliferation. CTGF also promotes cardiac fibrosis, and is modulated by ET-1. Our microarray analysis suggests a coordinate regulation of both PDGF and CTGF expression by ET-1 (Figures 2-8 C, D).
Figure 2-8: ET-1 mRNA is associated with genes involved in cardiac hypertrophy and fibrosis. Panel A shows the relation of EDN1 to collagen isoform 1 mRNA expression (COL1A2). Panel B shows the relation of ET-1 protein to extent of fibrosis (as determined by quantitative analysis of Masson's Trichrome stained LAA sections). Panels C, D show the relation of EDN1 mRNA abundance to platelet derived factor D (PDGFD) and CTGF mRNA. "r" is Spearman's correlation.
2.4. Discussion

Here we evaluated the hypothesis that atrial ET-1 is increased in AF and is associated with LA enlargement, atrial fibrosis and AF persistence in patients with structural heart disease.

Consistent with earlier plasma studies\textsuperscript{61}, we also found that LAA ET-1 protein levels were elevated in AF patients with HTN, HF, and MR – all conditions associated with increased hemodynamic stress. Myocyte hypertrophy and altered expression of several cardiac-specific genes are adaptive responses to hemodynamic stress. In experimental studies, ET-1 has been shown to modulate intracellular Ca\textsuperscript{2+} release\textsuperscript{234}, induce cardiac myocyte hypertrophy\textsuperscript{10} and modulate fibroblast proliferation and extracellular matrix deposition\textsuperscript{134}.

2.4.1. Local Activation of Atrial ET-1 in AF Patients with Structural Heart Disease

Plasma ET-1 levels are reported to be elevated in patients with diastolic dysfunction and elevated atrial pressures\textsuperscript{235}. Although circulating levels of ET-1 were reported to be elevated in AF patients with underlying cardiac disease\textsuperscript{61}, little is known about atrial ET-1 content or its relevance to atrial pathophysiology. In a small study, pro-ET-1 mRNA and protein expression were evaluated in right atrial samples from patients with and without valvular disease, in sinus rhythm or in AF\textsuperscript{179}. Atrial ET-1 was increased with AF only in the subset with underlying valve disease. In patients with mitral valve disease, LA enlargement is profound, and LA dilatation is associated with poor clinical prognosis. No studies have assessed the impact of AF or structural heart disease on LA ET-1 content or receptor expression.
Here we report that LA ET-1 protein is elevated in AF patients with underlying cardiac disease, and that elevated ET-1 levels are associated with AF rhythm and persistence. Atrial rhythm was an important determinant of LA ET-1 content, and we documented an increased abundance of both ET-1 protein and mRNA in the fibrillating LA. This may be due to the elevated wall stress associated with AF and atrial dilatation. Although there was a trend for a reduction of LAA ETAR protein in AF patients (Figure 2-4A), no significant differences in ET-1 receptor expression were detected between rhythm groups. Although modulation of ETAR by ET-1 was not strong, ETAR expression was significantly correlated with ETBR protein expression. ETAR and ETBR have opposing effects on blood pressure; ET-1 binding to ETAR promotes vasoconstriction while binding to ETBR promotes vasodilatation. The nuclear localization of ETBR may suggest a greater impact on transcriptional regulation. These data suggest a complex relationship between agonist concentration and receptor subtype distribution; ET-1 receptor expression may be regulated in an ET-1 independent manner.

The LA is frequently dilated in HF patients, even among those with preserved LV ejection fraction. Development of AF in patients with MR is independently associated with HF and death. HTN is also an important risk factor for AF. Here we report that increased LAA ET-1 is associated with each of these conditions in AF patients, and that this relationship is modulated by atrial rhythm. Using confocal microscopy, we show that ET-1 is present in both atrial myocytes and fibroblasts. By modulating blood pressure, left atrial myocyte Ca\textsuperscript{2+} handling, fibroblast proliferation, cardiac hypertrophy and the activation of cardiac specific gene programs, ET-1 may exacerbate cardiac dysfunction and accelerate the development or progression of HF and AF.
2.4.2. Atrial ET-1 Content and Left Atrial Geometry

Atrial structural remodeling is observed in clinical AF and in experimental AF models\textsuperscript{134}. LA enlargement is a marker of diastolic dysfunction\textsuperscript{238} and an independent predictor for development of post-operative AF\textsuperscript{223}, stroke and morbidity\textsuperscript{224}. In a murine study, cardiac-specific overexpression of ET-1 promoted atrial and ventricular hypertrophy, dysfunction, dilatation, inflammation and fibrosis\textsuperscript{26}, leading to dilated cardiomyopathy and impaired survival.

LA size increases in response to both increased left ventricular filling pressure and mitral regurgitation\textsuperscript{237}. Patients with MVD have an increased risk of developing AF. Independent risk factors for AF in MVD were an age $>$65 years and a baseline LA size greater than 5 cm\textsuperscript{237}. Here, we demonstrate that atrial ET-1 levels are strongly correlated with LA size, MR severity and age. AF risk increases with advancing age\textsuperscript{239} and ET-1 expression is increased in aged aortas\textsuperscript{240}.

2.4.3. ET-1 and Atrial Fibrosis

The PDGF\textsuperscript{134} and CTGF\textsuperscript{142} signaling pathways are prominent modulators of cardiac fibrosis, and fibrosis is an important determinant of AF persistence\textsuperscript{32}. Atrial fibroblasts are reported to be more sensitive than ventricular fibroblasts to a variety of pro-fibrotic stimuli, including PDGF and ET-1\textsuperscript{134}. ET-1 mRNA levels were strongly associated with expression of PDGFD and its B type receptor mRNA, and with CTGF. These associations are likely functionally significant, as both ET1 protein and mRNA levels were associated with increased expression of the major cardiac collagen isoforms, and
with atrial fibrosis. Here, patients with permanent AF had higher atrial ET-1 levels and larger LA than those with paroxysmal AF, suggesting that AF persistence may be modulated by ET-1 production. By accelerating the development of atrial fibrosis and dilatation, ET-1 may contribute to the structural remodeling that is characteristic of persistent and permanent AF\textsuperscript{241}.

ET-1 expression is modulated at both transcriptional and post-transcriptional levels. In AF patients, both mRNA and protein levels of ET-1 were elevated. Increased endothelin converting enzyme mRNA expression suggests that processing of atrial ET-1 may be enhanced in AF.

In recent studies, plasma levels of brain natriuretic peptide (BNP) have also been shown to predict the development of AF\textsuperscript{242} and postoperative AF\textsuperscript{243}. Both BNP and ET-1 expression are enhanced by hemodynamic stress\textsuperscript{17,244}. It is intriguing that atrial, but not ventricular, BNP expression is modulated by ET-1\textsuperscript{244}. Thus, whether BNP is a marker or a mediator of AF risk is unclear. Our data show that atrial expression of BNP mRNA was strongly associated with ET-1 mRNA and protein levels. Although speculative, the prognostic value of plasma BNP as a predictor of AF may depend on this relationship.

**2.5. Study Limitations**

A primary limitation of our study is the limited number of subjects. Ascertainment of rhythm history is challenging in surgical patients; misclassification of patients (with respect to rhythm history) would tend to minimize differences between groups. Although this study demonstrates an association of LAA ET-1 content with LA size in AF patients with underlying cardiovascular diseases, it cannot prove a causal role of ET-1 as a mediator of LA hypertrophy, dilatation, fibrosis or AF persistence.
2.6. Conclusions

LA dilatation is enhanced by AF in subjects with concomitant structural heart disease. Increased LAA ET-1 is associated with and may contribute to increased AF persistence and LA dilatation. Our data suggest that both ET-1 gene expression and processing are activated during AF. ET-1, in turn, enhances expression of genes involved in cardiac dilatation, hypertrophy, and fibrosis. The combined influence of these factors may contribute to the impact of ET-1 on AF persistence. Our study suggests that increased expression of atrial ET-1 is associated with the progression of atrial dysfunction. Thus, interventions that decrease atrial ET-1 expression (e.g., statins, etc.) or block its receptors might be useful in slowing the progression of AF.

Pharmacologic strategies for treating AF are frequently directed at rhythm control and are mostly ineffective. Numerous studies have focused on the role of angiotensin-II as a modulator of atrial electrical and structural remodeling. In experimental studies, angiotensin receptor blockade is insufficient to prevent the development atrial fibrosis in the setting of ventricular dysfunction. Endothelin-1 signaling may be a target for AF-focused therapeutic interventions.
Chapter 3

Plasma Endothelin-1 Levels are Associated with a Common Chromosome 4q25 Genetic variant Linked with Atrial Fibrillation

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Running title: Association of plasma endothelin-1 with AF risk SNP

Key words: Endothelin-1, Lone atrial fibrillation, SNP, risk allele, Pulmonary hypertension.
Abstract

Background: Atrial fibrillation (AF) has a hereditable component. Recent studies have identified single nucleotide polymorphisms (SNPs) on chromosome 4q25 that are strongly associated with AF and stroke risk. Although the biological significance of this locus remains unclear, the genes nearest the 4q25 SNPs are PITX2 and ENPEP, both of which have plausible links to endothelin-1 (ET-1) signaling. ET-1 is implicated in stroke and modulates atrial electrical activity and structure.

Methods and Results: We evaluated the relationship of the 4q25 SNP most strongly associated with AF (rs2200733) with plasma ET-1 (pET-1) levels in a series of lone AF patients. pET-1 was assessed by ELISA in samples from 119 individuals (30 T/T, homozygous for the risk allele; 43 C/T heterozygous, and 46 C/C homozygous for the major allele). Groups were matched for age, gender and history of hypertension.

pET-1 was signficantly higher in lone AF patients homozygous for the risk allele (T/T, \(p=0.0004\)) than in the C/T or C/C patients. pET-1 was also positively correlated with age (\(r=0.299, p=0.0008\)), pulmonary hypertension (PHTN, \(p=0.0029\)), indexed left atrial (LA) area (\(r=0.26, p=0.008\)), and systolic blood pressure (\(r=0.17, p=0.07\)), but not with diastolic blood pressure or BMI. Multivariate analysis confirmed the influence of genotype, PHTN, LA area and age on pET-1.

Conclusion: The 4q25 SNP rs2200733 AF risk allele is associated with higher pET-1 in lone AF patients. pET-1 is also positively associated with age, PHTN and LA size—all of which are common risk factors for AF. ET-1 may contribute to AF in a genotype-dependent manner in individuals with this risk allele.
3.1. Introduction

Atrial fibrillation (AF) is the most frequent sustained cardiac arrhythmia encountered in clinical practice. Although AF frequently occurs in the context of structural heart disease (valvular disease, coronary artery disease, heart failure), AF can also occur in individuals without evidence of structural heart disease (lone AF). AF has been associated with increased risk of stroke, ventricular dysfunction and death.

Recent studies provide strong evidence of a genetic contribution to AF and genome wide association studies have identified variants conferring risk for AF in different populations. The single nucleotide polymorphism (SNP) rs2200733, located on chromosome 4q25, had the strongest association with AF, both in patients with lone AF and in those with AF in the context of structural heart disease. With a minor allele frequency of ~14% in subjects with European ancestry, rs2200733 was associated with a ~72% increased risk of AF per copy. The mechanisms and the functional significance of this variant are not yet known.

The gene nearest rs2200733 (~150 kilobases away) is the paired-like homeodomain transcription factor 2 (PITX2), known to have a critical function in the development of left–right asymmetry in the heart. In a mouse model, PITX2 deletion suppressed a default pathway for sinoatrial node formation in the left atrium (LA) and was associated with lower endothelin-1 (ET-1) expression. ET-1 signaling is critical for the development of the cardiac neural crest, aortic arch vessels and atrioventricular cushions. Intriguingly, ET-1 knockout mice have a cardiac phenotype that is similar to that of PITX2 knockout mice. ET-1 activates the G-protein coupled receptors...
ETAR and ETBR. ETBR receptors mediate vasodilatation through enhanced nitric oxide production and are involved in ET-1 clearance, while ETAR is associated with vasoconstriction and mobilization of intracellular Ca\(^{2+}\). ETAR is a G\(_q\)-coupled receptor that activates phospholipase C, promoting formation of diacylglycerol and inositol trisphosphate (IP3). ET-1 is a potent vasoconstrictor peptide with mitogenic properties. ET-1 exerts both direct and indirect effects on cardiac myocytes through modulation of intracellular Ca\(^{2+}\) homeostasis and protein kinase C (PKC)/mitogen activated protein kinase (MAPK) pathways. ET-1 is implicated in the initiation and progression of cardiac dysfunction and vascular disease via modulation of vasoconstrictor, inflammatory, and growth factor pathways.

The gene next nearest rs2200733 (~230 kilobases) is ENPEP, encoding aminopeptidase-A, a protease responsible for the degradation of angiotensin II (Ang-II). Angiotensin-II can induce ET-1 expression via the extracellular signal regulated kinase (ERK) pathway. Angiotensin-II and ET-1 are parallel and interacting pathways that modulate blood pressure, atrial Ca\(^{2+}\) cycling and contractility, and interstitial fibrosis. Based on its relation to PITX2, angiotensin-II, calcium cycling, and profibrotic signaling effects, ET-1 seems a plausible candidate linking 4q25 with risk of AF. Here, we tested the hypothesis that plasma ET-1 levels are associated with rs2200733 genotype, and that lone AF patients carrying the rs2200733 AF-risk allele will have elevated plasma ET-1 levels.
3.2. Methods

3.2.1. Patient Selection

This study was performed by analyzing blood samples from lone AF patients referred to the Cleveland Clinic and recruited into the Cleveland Clinic Lone AF Genebank, a biorepository of DNA, plasma, and clinical data. All patients provided informed consent, and studies were approved by the Institutional Review Board of the Cleveland Clinic. Inclusion criteria included age $\geq$ 18 years old, history of recurring or current lone AF, left ventricular ejection fraction (LVEF) $\geq$ 50%, with no significant structural heart disease. Exclusion criteria included history of significant valvular heart disease (>2+ valve regurgitation or any valve stenosis), significant CAD (>50% coronary artery stenosis), prior percutaneous coronary intervention or coronary artery bypass surgery, and history of congenital heart disease, (except isolated patent foramen ovale). Hypertensive subjects were not excluded. Clinical, laboratory and demographic data were prospectively collected into the Lone AF Genebank data registry.

AF burden was characterized by maximal AF categorization according to current AHA/ACC/HRS clinical guidelines\textsuperscript{257}. Paroxysmal AF (Px) was defined as AF that terminated within 7 days. Persistent AF (Ps) was defined as AF lasting more than 7 days or requiring electrical or pharmacological cardioversion for restoration of sinus rhythm. Permanent AF (Pm) was defined as long-lasting AF in which cardioversion failed or was not attempted.
Patient groups were stratified by rs2200733 genotype. We selected all available patients with the homozygous T/T genotype, and then matched the C/T and C/C groups for gender, age, body mass index, and history of hypertension.

3.2.2. Blood Collection

Blood was collected by venipuncture in K$_2$EDTA tubes. After centrifugation, DNA was collected from the buffy coat, and frozen plasma samples were stored at -80°C.

3.2.3. DNA Isolation

DNA was isolated from the buffy coat using MasterPure$^\text{TM}$ DNA purification kits (EPICENTRE Biotechnologies, Madison/WI), per manufacturer’s instructions. After thawing, the buffy coat was treated with red cell lysis solution. After centrifugation, the white cell pellet was suspended in cell lysis solution and treated with the MPC protein precipitation reagent. Samples were vortexed and centrifuged. The supernatant was mixed with isopropanol and a DNA pellet was obtained by centrifugation. After rinsing with ethanol, the DNA was resuspended in 200 µl TE buffer at room temperature and quantified by spectrophotometer at 260 nm.

3.2.4. RS2200733 SNP Genotyping

Genotyping was carried out using Illumina Human Hap610 and Human Hap550 Bead chips (Illumina, SanDiego, CA, USA), as previously described$^{258}$. These arrays contain more than 550,000 haplotype tagged SNPs derived from phases I and II of the International HapMap project. Briefly, each sample was whole-genome amplified, fragmented, precipitated and resuspended in appropriate hybridization buffer. Denatured samples were hybridized on the BeadChips. After hybridization, the BeadChips were
processed for the single-base extension reaction, stained, and imaged on an Illumina Bead Array Reader. Normalized bead intensity data obtained for each sample were loaded into the Illumina Bead Studio software, which converts fluorescence intensities into SNP genotypes. Samples with call rates less than 97% were excluded.

### 3.2.5. Plasma ET-1 Measurements

Plasma ET-1 concentrations were determined using enzyme-linked immunoassays (Biomedica BI-20052, distributed by American Research Products, Inc.). EDTA-plasma was stored at -80°C until analysis. Plasma samples (25 µL) were diluted 1:1 with 0.9% NaCl solution. Human plasma samples, controls and standards (all 50 µL) were pipetted into microtiter wells coated with polyclonal rabbit anti-endothelin-1 antibody. Samples were assayed in duplicate. A 200µl aliquot of the detection antibody (monoclonal mouse anti-ET-1) was added to each well, and the plate was sealed to avoid evaporation. After overnight incubation, the contents of the wells were discarded and washed five times with washing buffer, followed by addition of 200 µl of anti-mouse IgG antibody conjugated to horseradish peroxidase and incubation for 1 hour at room temperature. After incubation, the contents were again discarded, and the plate was re-washed five times. A 200 µl aliquot of the substrate was added into the wells and incubated for 30 min in the dark, followed by 50µl of stopping buffer. Absorption was immediately determined in a plate reader at 450nm against 690 as reference. The quantity of ET-1 in each sample was proportional to the enzyme bound quantity and was interpolated from a calibration curve of standards. The assay was quite reproducible, with an average intra-assay coefficient of variation of <4%, and inter-assay coefficient of variation <10%.
3.2.6. Statistical Analysis

Baseline demographic and laboratory data are expressed as mean ± standard error for continuous variables and percentages for discrete variables. Univariate and multivariate regression analyses were performed to evaluate the ability of clinical and demographic variables to predict plasma ET-1 levels. To test if values were normally distributed, the D’Agostino and Pearson Omnibus normality test was used. Plasma ET-1 levels were not normally distributed, therefore ET-1 levels were log_{10} transformed for both univariate and multivariate analyses. Linear regression analysis was used to assess differences across genotype groups, while Student’s unpaired t-test was used to assess differences between two groups. Univariate analysis was performed using Graph Pad Prism 4 software. Multivariate analyses were performed using JMP 7 software. Graphics were prepared with Origin 8.0. Values of p<0.05 were considered statistically significant. Since the data were log_{10} transformed, fold change responses for univariate and multivariate predictors of plasma ET-1 were computed as (\exp (\ln(10)\text{slope})-1)*100.
3.3. Results

3.3.1. Patient Characteristics

Plasma ET-1 levels were ascertained in 119 lone AF patients. Samples were stratified based on rs2200733 SNP genotype into three matched groups: 30 patients homozygous for the minor risk allele (T/T), 43 patients heterozygous for the minor risk allele (C/T), and 46 patients homozygous for the major allele (C/C). Table 3-1 summarizes baseline demographic and clinical characteristics of each group. Although patients had no structural heart disease, 54% and 26% of patients had a history of hypertension (HTN) and pulmonary HTN (PHTN), respectively. PHTN was documented as an increase of right ventricular pressure (a precursor of pulmonary hypertension) >30mmHg. As noted in the Methods, the distribution of hypertension was matched between genotype groups. In this study, 27.7% of patients had paroxysmal AF, 63% had persistent AF, and 9.3% had permanent AF.
Table 3-1: Patients characteristics

<table>
<thead>
<tr>
<th>Genotype</th>
<th>T/T n = 30</th>
<th>C/T n = 43</th>
<th>C/C n = 46</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Clinical Characteristics</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age, years</td>
<td>55.0±1.9</td>
<td>57.7±1.3</td>
<td>54.6±1.7</td>
<td>0.33</td>
</tr>
<tr>
<td>Male, n (%)</td>
<td>27 (90)</td>
<td>26 (60)</td>
<td>23 (50)</td>
<td>0.37</td>
</tr>
<tr>
<td>Body Mass Index, kg/m²</td>
<td>29.8 ± 1</td>
<td>29.9±0.9</td>
<td>31.1±0.9</td>
<td>0.31</td>
</tr>
<tr>
<td>Paroxysmal AF, n (%)</td>
<td>7 (23)</td>
<td>10 (23)</td>
<td>16 (34)</td>
<td>0.59</td>
</tr>
<tr>
<td>Persistent AF, n (%)</td>
<td>18 (60)</td>
<td>30 (69)</td>
<td>27 (59)</td>
<td>0.37</td>
</tr>
<tr>
<td>Permanent AF, n (%)</td>
<td>5 (16)</td>
<td>3 (7)</td>
<td>3 (6)</td>
<td>0.18</td>
</tr>
<tr>
<td>Hypertension, n (%)</td>
<td>12/27 (44)</td>
<td>22/38 (58)</td>
<td>25/43 (58)</td>
<td>0.3</td>
</tr>
<tr>
<td>Systolic blood pressure, mmHg</td>
<td>128 ± 3.5</td>
<td>130±2.8</td>
<td>129±2.6</td>
<td>0.9</td>
</tr>
<tr>
<td>Diastolic blood pressure, mmHg</td>
<td>79.0 ± 2</td>
<td>82.7±2.29</td>
<td>77.3±1.65</td>
<td>0.4</td>
</tr>
<tr>
<td>Pulmonary hypertension, n (%)</td>
<td>5 (28)</td>
<td>12 (33)</td>
<td>8 (19)</td>
<td>0.29</td>
</tr>
<tr>
<td>Prior stroke, n (%)</td>
<td>1 (3)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0.9</td>
</tr>
<tr>
<td>Diabetes mellitus, n (%)</td>
<td>1 (3.6)</td>
<td>3 (7)</td>
<td>3 (7)</td>
<td>0.58</td>
</tr>
<tr>
<td><strong>LA Size</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Indexed LA diameter, cm/m²</td>
<td>n=28 (93%)</td>
<td>n=38 (88%)</td>
<td>n=39 (85%)</td>
<td></td>
</tr>
<tr>
<td>Indexed LA area, cm²/m²</td>
<td>1.94±0.07</td>
<td>1.94±0.05</td>
<td>1.84±0.04</td>
<td>0.17</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM, unless otherwise indicated. T/T: homozygous for the minor risk allele; C/T: heterozygous for the minor risk allele; C/C: homozygous for the major allele; AF: atrial fibrillation; LA: Left atria. P values determined by ANOVA.
3.3.2. Univariate Predictors of Plasma ET-1 in Lone AF

Plasma ET-1 levels were not normally distributed. A log_{10} transformation effectively normalized the dataset. Univariate analysis showed that the log of plasma ET-1 was associated with rs2200733 genotype (Figure 3-1). The log of plasma ET-1 levels increased with increased copy number of the AF risk allele (T) (r^2 = 0.1, p=0.0004, additive model, linear regression analysis).

Table 3-2 summarizes the univariate predictors of plasma ET-1 in our study population. Age is commonly accepted as the strongest predictor of AF risk. Interestingly, age was a significant univariate predictor of plasma ET-1 (p=0.0008, r=0.3, Figure 3-2).

In contrast, AF persistence, body mass index and gender were not associated with plasma ET-1 levels. Plasma ET-1 was significantly associated with PHTN (p=0.002, Figure 3-3A). Although univariate analysis showed no relation of plasma ET-1 to HTN, plasma ET-1 was weakly associated with systolic but not diastolic blood pressure (r=0.17, p=0.07, Figure 3-3B).

Since hypertension increases preload and promotes left atrial enlargement, we evaluated the relationship between plasma ET-1 and the diameter or area of the left atrium (LA), indexed to body surface area. Figure 3.3C-D shows that plasma ET-1 was associated with both indexed LA diameter (r=0.23, p=0.02) and indexed LA area (r=0.26, p=0.008).
Table 3-2: Univariate predictors of plasma ET-1

Response = $\log_{10}$ [plasma ET-1], fmol/ml

# of observations: 119; 30 TT, 43 CT, 46 CC

<table>
<thead>
<tr>
<th>Predictor</th>
<th>Estimate (slope, B)</th>
<th>Std error</th>
<th>P value</th>
<th>Standardized coefficient ($\beta$)</th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs2200733 SNP genotype</td>
<td>0.176</td>
<td>0.0487</td>
<td>0.0004</td>
<td>3.61</td>
<td>50%</td>
</tr>
<tr>
<td>Age, per year</td>
<td>0.0126</td>
<td>0.00367</td>
<td>0.0008</td>
<td>3.43</td>
<td>2.9%</td>
</tr>
<tr>
<td>Hx of Pulmonary HTN</td>
<td>0.29</td>
<td>0.095</td>
<td>0.0029</td>
<td>3.04</td>
<td>94%</td>
</tr>
<tr>
<td>Female, sex</td>
<td>-0.00158</td>
<td>0.0856</td>
<td>0.98</td>
<td>-0.02</td>
<td>-0.34%</td>
</tr>
<tr>
<td>Body mass Index (Kg/m²)</td>
<td>0.00358</td>
<td>0.00672</td>
<td>0.59</td>
<td>0.53</td>
<td>0.82%</td>
</tr>
<tr>
<td>Systolic BP, mm Hg</td>
<td>0.0041</td>
<td>0.0022</td>
<td>0.07</td>
<td>1.83</td>
<td>0.53%</td>
</tr>
</tbody>
</table>

Echocardiographic variables,
(n=100)

<table>
<thead>
<tr>
<th>Predictor</th>
<th>Estimate (slope, B)</th>
<th>Std error</th>
<th>P value</th>
<th>Standardized coefficient ($\beta$)</th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indexed left atrial area (cm²/m²)</td>
<td>0.0465</td>
<td>0.0174</td>
<td>0.0087</td>
<td>2.67</td>
<td>11%</td>
</tr>
<tr>
<td>Indexed left atrial diameter</td>
<td>0.293</td>
<td>0.124</td>
<td>0.02</td>
<td>2.36</td>
<td>96%</td>
</tr>
</tbody>
</table>

HTN: hypertension; BP: blood pressure. R² is the coefficient of determination; $\beta$ is the standardized coefficient (slope/std error); fold change is % change of non-log transformed plasma ET-1 per unit increase of the predictor.
Figure 3-1: Plasma ET-1 is associated with the rs2200733 SNP in lone AF patients
Bar plot shows values for $\log_{10} \text{[plasma ET-1]}$ as a function of rs2200733 genotype in lone AF patients homozygous for the minor risk allele of rs2200733 SNP (T/T, n=30), heterozygous of the minor risk allele (C/T, n=43), and homozygous for the major allele (C/C, 46). (p-value by regression analysis).
Figure 3-2: Plasma ET-1 is associated with age in lone AF.
A scatter plot shows the relationship of log₁₀ [plasma ET-1] with age. “r” is Pearson’s correlation coefficient.
Figure 3-3: Univariate predictors of plasma ET-1 in lone AF.

A) Box plots show 5th, 25th, median, 75th and 95th percentile values for log10 [plasma ET-1] in patients with (PHTN) and without (no PHTN) pulmonary hypertension (p-value by two-tailed T-test). B-D) Scatter plots show the relationship of log10 [plasma ET-1] to systolic blood pressure (B); indexed left atrial area, cm²/m² (C), and indexed LA diameter, cm/m² (D). In each plot, “r” is Pearson’s correlation coefficient and p is the significance of this correlation.
3.3.3. Multivariate Predictors of Plasma ET-1 in Lone AF

To determine the independent predictors of plasma ET-1 in lone AF patients, a multiple regression analysis was performed in 100 patients with available echocardiographic measurements of left atrial diameter and left atrial size (Table 3-3). By step-wise analysis, AF persistence (Pm vs. Ps vs. Px) was not associated with plasma ET-1 and was not included in the model. Multiple regression analysis revealed that the minor risk allele (T) of SNP rs2200733 (p=0.0015), age (p=0.015), and history of PHTN (0.039) were significant independent predictors of plasma ET-1 in lone AF patients. Although not reaching statistical significance, indexed LA area (p=0.087) also tended to predict plasma ET-1 levels.

The rs2200733 SNP was the strongest independent predictor of plasma ET-1 (β=3.27) and was associated with 47% and 116% increase in plasma ET-1 for one and two copies of the minor risk allele T, respectively.
Table 3-3: Multivariate predictors of plasma ET-1

Response: $\log_{10}$ [plasma ET-1], fmol/ml
Whole model $R^2 = 0.283$, $P<0.0001$
Adjusted $R^2 = 0.229$
# of observations: 100; 27 TT, 36 CT, 37 CC

<table>
<thead>
<tr>
<th>Predictor Parameter of ET-1</th>
<th>Estimate (slope, B)</th>
<th>Std error</th>
<th>P value</th>
<th>Standardized coefficient ($\beta$)</th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs2200733 SNP genotype</td>
<td>0.1667</td>
<td>0.0516</td>
<td>0.0015</td>
<td>3.27</td>
<td>46.8%</td>
</tr>
<tr>
<td>Age, per year</td>
<td>0.0092</td>
<td>0.00374</td>
<td>0.01549</td>
<td>2.47</td>
<td>2.14%</td>
</tr>
<tr>
<td>Pulmonary HTN</td>
<td>0.214</td>
<td>0.1007</td>
<td>0.0394</td>
<td>2.13</td>
<td>63.7%</td>
</tr>
<tr>
<td>Indexed left atrial area (cm$^2$)</td>
<td>0.0284</td>
<td>0.0164</td>
<td>0.087</td>
<td>1.73</td>
<td>6.7%</td>
</tr>
<tr>
<td>Female, sex</td>
<td>-0.065</td>
<td>0.086</td>
<td>0.45</td>
<td>-0.75</td>
<td>13.9%</td>
</tr>
<tr>
<td>Systolic BP</td>
<td>0.00229</td>
<td>0.0024</td>
<td>0.34</td>
<td>0.95</td>
<td>0.669%</td>
</tr>
<tr>
<td>Body mass Index</td>
<td>0.0026</td>
<td>0.0067</td>
<td>0.7</td>
<td>0.39</td>
<td>0.6%</td>
</tr>
</tbody>
</table>

HTN: hypertension; BP: blood pressure. $R^2$ is coefficient of determination; $\beta$ is the standardized coefficient (slope/std error); fold change is the % change of non-log transformed plasma ET-1 by unit increase of the predictor.
3.4. Discussion

Genome wide association studies have identified multiple genetic variants that are associated with AF risk\textsuperscript{187, 247, 248,191}. Among the SNPs with the strongest association to AF is rs2200733, located in a non-coding region on chromosome 4q25\textsuperscript{5}. The biological significance of this SNP has not yet been established.

A number of studies have focused on PITX2, the gene nearest this locus. Based on its role in cardiac development and expression in the atria and pulmonary veins, PITX2 seems a plausible candidate gene\textsuperscript{259}. ET-1 is also important in cardiac development, and both PITX2 and ET-1 are regulated by the Wnt/\(\beta\)-catenin pathway. Interestingly, a mouse PITX2 knockout model has lower cardiac expression of ET-1\textsuperscript{10}. It has also been suggested the ET-1 receptor, ETAR, is downstream of PITX2\textsuperscript{260}. Perhaps due to its critical role during development, there is thus far little evidence that the 4q25 locus is associated with PITX2 abundance in adult heart\textsuperscript{5}. ENPEP (encoding aminopeptidase-A) is the next nearest gene encoded in this region of chromosome 4q25. Changes in the expression or activity of aminopeptidase-A would affect angiotensin-II degradation, and might indirectly affect ET-1 homeostasis.

In the present study, we evaluated the hypothesis that plasma ET-1 levels are elevated in lone AF patients with the minor (AF risk) allele of rs2200733. Our study provides novel insight regarding the pathophysiological significance of this variant in AF. Here, we show that in individuals with lone AF, having one copy of the rs2200733 AF risk allele is associated with a 47% increase in plasma ET-1 levels, while individuals homozygous for the risk allele had more than twice the plasma ET-1 levels of subjects without the T
allele. Importantly, plasma ET-1 is also associated with age, PHTN, and indexed LA area - all known risk factors of AF.

Common risk factors for AF include advanced age, history of hypertension, valvular heart disease, HF and diabetes. AF in the presence of multiple risk factors is associated with increased risk of stroke and death. Lone AF occurs in 1.6 to 11.4% of all cases of AF. Increasing left atrial size, age, family history and development of HTN may also increase the risk of cardiovascular events in lone AF patients.

ET-1 levels in plasma and atrial tissue are increased in AF patients with structural heart disease. ET-1 affects the electrical and contractile activity of cardiac myocytes and stimulates myocyte hypertrophy and myofibrillogenesis. ET-A receptor mediated diacylglycerol and IP3 production modulates PKC/MAPK signaling and IP3-mediated intracellular calcium release. These pathways likely contribute to the effects of ET-1 on the atria. Enhanced reactive oxygen species production (via activation of NADPH oxidase and modulation of mitochondrial respiration) may also be significant. Oxidant stress in AF can lead to heterogeneous conduction, via changes in connexin trafficking. ET-1 also promotes atrial fibrosis via direct actions on atrial fibroblasts; this activity contributes to arrhythmia persistence. In sum, ET-1 might contribute to both the triggers that initiate AF, and to the progression of AF via changes in atrial conduction and structure. ET-1 has biologic plausibility as a modifier of AF risk.

The relation of ET-1 to SNPs associated with AF risk has not previously been evaluated. Our finding of an association of plasma ET-1 with rs2200733 is intriguing, based on the impact of ET-1 on cardiac structure and function. It has been shown that plasma ET-1 levels prior to pulmonary vein isolation (PVI) predict AF recurrence 3 to 6 months after
PVI\textsuperscript{60}. It is intriguing that the presence of polymorphisms on chromosome 4q25 (rs220073 and rs10033464) predict early (7 days) and late (3-6 months) AF recurrence following catheter ablation. These data strongly support our finding and suggest that the 4q25 locus modulates plasma ET-1\textsuperscript{189}. However, the mechanism by which the chromosome 4q25 locus regulates plasma ET-1 is still unknown. In addition to the possible link via PITX2, ET-1 and angiotensin-II signaling are highly interrelated. This locus may modulate ET-1 levels via PITX2 or Ang-II dependent pathways, or may regulate microRNAs modulating ET-1 expression or processing.

Elevated plasma ET-1 levels have been associated with HTN\textsuperscript{266}, PHTN\textsuperscript{59} and HF\textsuperscript{58}. ET-1 gene expression is enhanced by pathological pressure overload, as in that associated with pulmonary hypertension\textsuperscript{59}. Here, we document elevated plasma ET-1 in patients with lone AF and evidence of PHTN. That both HTN and PHTN are associated with increased risk of AF is well-described\textsuperscript{19-21}. Whether PHTN was present at the time of initial AF diagnosis and contributed to the etiology of AF, or if it became manifest with time in predisposed AF patients, cannot be deduced from this study.

ET-1 synthesis is increased in response to ischemia and hemodynamic stress\textsuperscript{18}, thus plasma ET-1 levels are often elevated in patients with heart failure\textsuperscript{22} and other stroke risk factors\textsuperscript{267}. ET-1 infusion has been used experimentally to cause stroke\textsuperscript{268}. As rs2200733 has been associated with both AF\textsuperscript{187} and ischemic stroke\textsuperscript{188}, the common link to this SNP may suggest the presence of undiagnosed AF. Alternatively, one might speculate that ET-1 contributes to both conditions, and underlies the genetic association with this locus.

LA size is an important marker of cardiac dysfunction\textsuperscript{269}. LA enlargement is an independent predictor for development of post-operative AF\textsuperscript{223}, stroke and morbidity\textsuperscript{224}. 
We have recently shown that LA ET-1 content is associated with AF persistence and LA enlargement\textsuperscript{62}. Concordant with that study, here we report that there was a trend for plasma ET-1 levels to be correlated with LA area (p=0.087 by multivariate analysis), suggesting that plasma ET-1 may have a cardiac origin, and/or that plasma ET-1 contributes to atrial hypertrophy and dilatation.

AF risk increases with advancing age\textsuperscript{239}. The mechanisms by which aging influences AF risk are complex and multifactorial; they may be related to functional or structural changes in the vascular system or the myocardium, and to changes in the neurohumoral environment\textsuperscript{237}. In this study, plasma ET-1 was significantly associated with age (Figure 2). Interestingly, a recent study\textsuperscript{240} suggested that release of ET-1 is dramatically increased in aged endothelium. It seems plausible that age-dependent increases in ET-1 contribute to the age-related risk of AF.

### 3.5. Limitations

This study was limited to a cohort of lone AF patients; in future studies it will be of interest to replicate our findings in different populations (e.g., healthy controls, and in AF patients with underlying cardiac disease). The fraction of plasma ET-1 that is produced in the myocardium is uncertain and probably variable. Additional studies are warranted to better characterize the source(s) of ET-1 in individuals with this genetic variant. It would also be of interest to evaluate the relationship between plasma ET-1 levels and atrial ET-1 content in the same individuals, in order to identify the source(s) of ET-1 production.
3.6. Significance and Conclusions

To achieve the goal of personalized medicine in the field of AF, we must first understand the biological significance of the polymorphisms associated with AF risk. These efforts may identify pathways for intervention that could guide the development of individualized AF treatments. Previous studies have shown that the rs2200733 SNP T allele is associated with increased risk of AF and stroke; however, the mechanism(s) are not known. This is the first study to identify a potential biological mediator that could link this SNP with these pathologic conditions.

The rs2200733 SNP risk allele is associated with higher plasma ET-1 levels in lone AF patients and is correlated with PHTN, LA area and age -- important risk factors of AF. ET-1 is a potent vasoconstrictor associated with hypertension and stroke. The association of rs2200733 with plasma ET-1 levels may underlie the increased risk of AF in patients in a genotype dependent manner. In future studies, it will be of interest to determine whether targeted interventions that reduce ET-1 production or block its receptors (e.g. ET-1 antagonists) can limit AF vulnerability and related morbidity in patients with the rs2200733 AF risk allele.
Chapter 4

Endothelin-1 Expression and Fibrosis are Greater in the Atria than in the Ventricles During the Onset of Heart Failure Induced by Ventricular Tachypacing

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Running title: Atrial versus ventricular endothelin-1

Key Words: Endothelin-1, atrial fibrillation, Heart Failure, Inositol Trisphosphate Receptor.
Abstract

**Background:** Heart failure (HF) is associated with arrhythmogenic remodeling of the atria (A) and ventricles (V). Expression of endothelin-1 (ET-1), a vasoconstrictor and mitogen, increases with wall stress and promotes electrical/structural remodeling by coupling to PLC/IP3/MAPK pathways. Here we evaluated ET-1 protein expression in the atria and ventricles during the development of HF.

**Methods and Results:** ET-1 levels were evaluated by ELISA in plasma, A and V tissues from a canine HF model induced by ventricular tachypacing (VTP). Right atrial (RA) and left ventricular (LV) samples were analyzed from animals sacrificed after 12 hr, 24 hr, 1, 2, and 5 weeks (W) VTP and compared to tissues from non-operated control dogs (CTR). At 2W VTP, tissue ET-1 levels were evaluated in LA, RA, pulmonary veins (PV), LV and RV. Fibrosis was assessed with Masson’s trichrome staining; cellular distribution of ET-1 protein was assessed with immunostaining. IP3R-I was evaluated by western blot. RA ET-1 increased at 24 hr and remained elevated up to 5W. LV ET-1 changes were remarkably lower and slower. No significant changes in plasma ET-1 were detected. Regional and temporal changes in IP3R-I abundance paralleled changes in ET-1. At 2W VTP, fibrosis, ET-1 and IP3R-I proteins were increased in LA, RA, and PV. Atrial changes were 2-5x greater than in the ventricles. ET-1 and IP3R-I proteins were expressed in both A and V myocytes, but IP3R-I was more abundant in intercalated disc region and nuclei of A myocytes.

**Conclusions:** Atria and PVs have a more robust elevation of ET-1 expression than the ventricles; increased fibrosis and IP3R-I followed changes in ET-1. VTP-stimulated ET-1 production likely promotes atrial electrical and structural remodeling and contributes to
atrial arrhythmogenesis. ET-1 signaling may be a novel target for AF therapy.
4.1. Introduction

Heart failure (HF) is associated with a complex series of changes at several levels, starting from the cellular to the whole organ level. Changes include functional and structural alterations that can culminate in the death of myocytes, hemodynamic and/or neurohormonal alterations, and changes in the composition of the extracellular matrix composition and extent of fibrosis. HF promotes electrical and structural remodeling in both the atria and ventricles. Cardiac tissues from human HF and animal HF models reveal greater structural remodeling of the atria than is observed in the ventricles. Atrial interstitial fibrosis typically exceeds ventricular fibrosis, and both the inducibility and duration of AF episodes increase with duration of ventricular tachypacing, suggesting that HF promotes atrial fibrosis and creates a substrate for AF. However, the mechanisms underlying development of atrial fibrosis in HF are not yet well understood.

Angiotensin-II (Ang-II), a critical myocardial vasoconstrictor and growth factor, is more abundant in the atria than in the ventricles. However, use of Ang-II antagonists only partially attenuate atrial structural and electrical remodeling, suggesting that other critical factors also promote electrical and structural remodeling in the atria.

Endothelin-1 (ET-1) is a potent vasoactive and mitogenic factor that acts on fibroblasts, smooth muscle cells and cardiac myocytes. ET-1 modulates the contractile properties of cardiac myocytes and stimulates myocyte growth and myofibrillogenesis. Through two G protein coupled receptors (GPCR), the ETAR and ETBR, ET-1 activates Gq-PLC with subsequent production of diacylglycerol (DAG) and inositol trisphosphate (IP3). Binding of IP3 to its receptors (IP3R) promotes release of intracellular Ca stores and
contributes to ET-1 signaling\textsuperscript{47}. The role of IP3Rs in cardiac myocytes is unclear, but is of interest\textsuperscript{45,46,81}.

In cardiac hypertrophy and HF, plasma and ventricular ET-1 levels and IP3R expression are upregulated\textsuperscript{56}. Elevated plasma ET-1 levels have been shown to predict AF recurrence after pulmonary vein isolation\textsuperscript{60}. Plasma ET-1 levels are elevated in AF patients with concomitant structural heart disease\textsuperscript{61}, however, studies measuring ET-1 expression locally in failing or fibrillating atria are lacking. We have recently shown that atrial ET-1 is increased in AF patients with underlying cardiac disease and is associated with atrial rhythm, size and fibrosis\textsuperscript{62}. AF is often comorbid with HF; HF creates a substrate for AF development and progression\textsuperscript{121}. However, differences between atrial and ventricular ET-1 content have not yet been characterized during the development of HF.

The present study was designed to compare temporal and regional changes of ET-1 content, IP3R-I expression and fibrosis in left and right atria (LA, RA) and ventricles (LV, RV) during the early stages of HF due to ventricular tachypacing in a canine model. To gain a better understanding of cardiac regional differences, the pulmonary veins (PVs), a common site of origin of ectopic triggers\textsuperscript{28}, were also evaluated for the extent of fibrosis, and ET-1 and IP3R-I protein expression.
4.2. Methods

4.2.1. Canine HF Model

Dogs (20 to 35 kg) were implanted with a pacemaker capable of high rate (240 bpm) pacing, with a lead positioned in the right ventricular apex. Ventricular pacing was delivered for 12 hrs, 24 hrs, 1 week, 2 week or 5 weeks at 220-240-bpm to induce HF (VTP, n=5-11 per pacing period) as previously described\textsuperscript{121}. Control dogs were not pacemaker implanted (CTR; n=10). PVs, atrial and ventricular tissues from CTR and VTP dogs were snap-frozen and stored at −80°C for biochemistry and histology analysis.

4.2.2. Enzyme Linked Immunoassay for Plasma and Tissue ET-1 Measurements

ET-1 content was evaluated using an enzyme-linked immunoassay (Biomedica BI-20052, American Research Products, Inc., Belmont, MA). Freshly drawn EDTA-blood was put immediately on ice and centrifuged within one hour. Tissue sample (LA, RA, LV, RV, or PV tissues, ~50mg each) were homogenized in M-PER buffer containing protease inhibitor mixture (Sigma). All samples were stored at -80°C until analysis. Samples were thawed on ice and assayed at room temperature. Tissue samples were diluted using 0.9% NaCl containing protease inhibitors at 1.5-µg/µl concentration, and a total of 50 µl was loaded into each well (75µg total protein). Plasma samples were diluted at 1:1 ratio. Samples, controls and standards (all 50 µL) were pipetted into microtiter wells coated with polyclonal rabbit anti-endothelin-1 antibody. Samples were assayed in duplicate. A 200µl aliquot of the detection antibody (monoclonal mouse anti-ET-1) was added to each well, and the plate was sealed to avoid evaporation. After overnight incubation, the
The contents of the wells were discarded and washed five times with washing buffer, followed by addition of 200 µl of anti-mouse IgG antibody conjugated to horseradish peroxidase and incubation for 1 hour at room temperature. After incubation, the contents were again discarded, and the plate was re-washed five times. A 200 µl aliquot of the substrate was added into the wells and incubated for 30 min in the dark, followed by 50µl of stopping buffer. Absorption was immediately determined in a plate reader at 450nm. The quantity of ET-1 in each sample was proportional to the enzyme bound quantity and was interpolated from a calibration curve of standards.

4.2.3. Western Analysis

Western blot analysis was used to evaluate the expression of IP3R type 1 (IP3R-I) protein in cardiac tissues. Tissues specimens (~50mg) were homogenized in M-PER buffer containing protease inhibitors (Sigma). For each sample, the same tissue homogenate was used for ET-1 and IP3R-I protein analysis. Proteins from the cell lysate were separated using SDS-PAGE (8% polyacrylamide gels), transferred to a nitrocellulose membrane, and probed with goat anti-IP3R-I antibody (1:150, Santa Cruz). Blots were incubated with donkey anti-goat secondary antibody, washed, and imaged using a Li-Cor Odyssey multi-wavelength laser illumination scanner.

To correct for variations in protein loading, each blot was also probed with a mouse anti-GAPDH antibody (1:5000, Millipore). Dual-color scanned blots were used to analyze the protein density in each lane. The integrated intensity of the area within the box for each lane was used to measure the protein density. The lane with the greatest protein density was set at unity. Loading variability was corrected by dividing the corresponding receptor intensity value in each lane by the relative abundance of GAPDH. Mean
GAPDH protein expression was unchanged between groups (control LV: 0.822±0.035; VTP LV: 0.77±0.019; control RA: 0.825±0.034; VTP RA: 0.73±0.022, $P=\text{NS}$).

### 4.2.4. Immunostaining and Confocal Microscopy

Immunostaining was used to evaluate the distribution of ET-1 and IP3R-I in cardiac sections from LA, RA, LV, RV and PVs at 2W VTP. Fresh tissue specimens were placed in OCT medium and rapidly frozen and stored at -80°C. Frozen tissues were cryo-sectioned into 16-µm-thick sections. Sections were fixed for 30 min in paraformaldehyde at RT, blocked in 3%BSA (Sigma-Aldrich) for 1 hour, and incubated with primary antibodies (rabbit anti IP3R-I, 1:50 Santa Cruz, and mouse anti-ET-1, 1:200, Abcam) overnight at 4°C. Sections were then incubated with Alexa fluor secondary antibodies at room temperature for 2 hours. To visualize myocyte structure, sections were also probed with mouse anti-phalloidin (Molecular Probes, 1:40), followed by mounting on glass slides. DAPI was used to visualize nuclei. Negative controls included omission of primary or secondary antibodies and were included in all immunostaining experiments.

Confocal immunofluorescence images of selected cardiac sections were obtained using a Leica TCS SP AOB5 spectral laser-scanning confocal microscope. Three-dimensional structures of each region were reconstructed from confocal immunofluorescence images recorded in z-series and analyzed using digital image-processing software (Volocity; Improvision, MA).

Stained slides were visually examined and imaged using a 63x objective. Maximum intensity projections (MIP) of confocal slices (0.5 µm separation) were used for image presentations.
4.2.5. Masson’s-Trichome and Histologic Analysis

In the subset of animals with frozen tissue blocks (OCT), the extent of tissue fibrosis and collagen deposition in both HF (2W VTP) and control animals was assessed and compared between atria, ventricles, and PVs. Fibrosis was evaluated using Masson’s-Trichrome staining.

OCT sections were washed with PBS (3X) and incubated with Bouin’s Fluid overnight. Slides were rinsed with de-ionized water and stained with Masson’s Trichrome. Following gentle water washing, slides were dehydrated using graded alcohols (75, 95, 100%), cleared in xylene, and cover-slipped. Photomicrographs were taken using high resolution-transmitted light bright-field-mosaic microscopy with a 5x objective. A series of tiles representing the total specimen area for an image was recorded for each specimen and used for quantitative assessment of % area of fibrosis (including both epicardial and interstitial fibrosis). Separate images were also obtained at 20x magnification for quantitative assessment of interstitial fibrosis using bright field light microscope (Leica DMR Upright Microscope, Leica Microsystems, Heidelberg, Germany).

Digital images were evaluated using a custom-programmed automated computerized image analysis algorithm (Image Pro 0.6.1, Media Cybernetics). Area of fibrosis (blue) was expressed as % area of the total specimen area.

4.2.6. Statistical Analysis

2-way ANOVA was used to assess differences and interactions across multiple groups, followed by Bonferroni post-tests for individual pair comparisons. Non-normally distributed variables were analyzed using Kruskal-Wallis tests. Two-sample analysis of normally distributed variables was assessed using a t-test. Spearman’s correlation was
used to assess the relationship between ET-1, IP3R-I protein and % area of fibrosis. Values of p<0.05 were considered statistically significant. Values are expressed as mean±SEM.
4.3. Results

4.3.1. Regional Differences in Cardiac Fibrosis

Colleagues at Montreal Heart Institute have previously showed that atrial fibrosis exceeds ventricular fibrosis, and is increased in a time dependent manner by ventricular tachypacing (for 12 hrs to 5 weeks)\textsuperscript{121}. Here, we assessed regional differences in myocardial fibrosis in LA, RA, LV, RV and PV at 2 weeks of VTP, a time at which the cardiac dysfunction is moderate.

Figure 4-1 shows representative bright field montage images assembled from 5x images in each cardiac section from CTR and HF dogs. The corresponding quantitative analysis of % area of total cardiac fibrosis (epicardial and myocardial fibrosis) is summarized in panel C, below. In CTR hearts, both the LA (10.96±2.6%) and RA (10.2±1.6%) were more fibrotic than the ventricles (LV: 1.64±0.27%, RV: 4.69±1.18%) and the extent of total cardiac fibrosis was increased in the atria (LA: 21.3±1.79%, RA: 20.9±1.99%, p<0.05, ANOVA) but not in the ventricles at 2W VTP (LV: 2±0.57% LV, RV: 2.9±0.73%; p>0.05). Interestingly, the PV from CTR hearts showed more extensive fibrosis in both the epicardium and the myocardium (28.99±7.14%), with a further, marked increase in fibrosis at 2 weeks of VTP (46.5±5.9%, p<0.01). A statistically significant interaction was observed between tissue origin and condition (P=0.0043), suggesting that the extent of cardiac fibrosis is influenced both by the cardiac chamber and by the presence of HF at 2W VTP.

A similar analysis of cardiac sections at higher power (to assess interstitial fibrosis) revealed that interstitial fibrosis in the atria and the PVs greatly exceeded that in the
ventricles, Figure 4-2. Following 2W VTP, atrial interstitial fibrosis increased from 3.2±0.5% to 16.9±2.5%, whereas the ventricles showed an increase from 2.04±0.42% to 3.45±0.63%. Intriguingly, PVs from control hearts had 12.8±3.7% baseline interstitial fibrosis, and this increased to 34±1.38% after 2 weeks VTP (Figure 4-2C). A statistically significant interaction was observed (p<0.0001), suggesting that interstitial fibrosis is similarly dependent on tissue origin and condition.

At 2W VTP, qualitative assessment suggests that reparative fibrosis is present more than reactive fibrosis, particularly in the region of the PVs. Reparative fibrosis, with transversely running zones of fibrosis, is expected to interrupt longitudinal electrical conduction more than reactive fibrosis which is typically localized along lateral myocyte boundaries (Figure 4-2B).
Figure 4-1: Masson’s-Trichome analysis of regional cardiac fibrosis in HF

Representative bright field images taken at 5x magnification in left atria (LA), right atria (RA), left ventricle (LV), right ventricle (RV) and pulmonary vein (PV) from control (CTR, A) and HF dogs (2W VTP, B). Sections were stained with Masson’s-Trichome to image fibrosis (blue) and cardiac myocytes (red), Scale bar: 50 µm. C) Quantitative analysis of total % area of fibrosis at 5x magnification, n= 4-6 per observation, p<0.05; # CTR versus 2W VTP.
Figure 4-2: Masson’s-Trichrome analysis of regional interstitial fibrosis in HF
Representative bright field images taken at 20x magnification in left atria (LA), right atria (RA), left ventricle (LV), right ventricle (RV) and pulmonary vein (PV) from control (CTR, A) and HF dogs (2W VTP, B). Sections were stained with Masson’s-Trichrome to image fibrosis (blue) and cardiac myocytes (red), Scale bar: 50 µm. C) Quantitative analysis of % area of interstitial fibrosis at 20x magnification, n= 3-5 per observation, p<0.001; # CTR versus 2W VTP, ANOVA.
Temporal and Regional Changes of ET-1 Content in HF

Plasma ET-1 was measured in samples from CTR dogs and in dogs following 24hr, 2W, and >3W VTP. Plasma ET-1 was quite variable between study groups, and particularly in the CTR dogs. Although there was a trend for plasma ET-1 to increase following tachypacing from 24 hr to >3W, no statistical difference was observed in the VTP animals relative to the CTR animals (Figure 4-3A).

To assess time course of changes in ET-1 content, the RA and the LV were analyzed at 12 hr, 24 hr, 1W, 2W, and 5W VTP (Figure 4-3B). Basal ET-1 content was markedly higher in CTR RA than in LV (mean±SEM; 2.9±0.29 vs 0.83±0.13 fmol/mg protein, p<0.0001). Relative to CTR, RA ET-1 significantly increased following 24hr VTP (1.56-fold, p<0.05), peaking at 2 weeks (2.69-fold, p<0.001), and remained elevated up to 5 weeks of VTP (1.52-fold, p<0.001). Changes in the LV were markedly slower and ET-1 levels were lower in the LV than in the RA. A highly significant interaction was observed between tissue origin and duration of VTP (p<0.0001). These data suggest that changes in atrial ET-1 occur earlier and have a greater magnitude than changes in the ventricles during the development of HF.

Since 2 weeks VTP is associated with moderate HF, we sought to further evaluate the regional differences in ET-1 contents at that time in the PVs, LA, RA, LV, and RV (Figure 4-3C). Both atria and the PVs showed marked increases of ET-1 protein. Intriguingly, CTR PV and RA showed higher ET-1 levels than CTR LA, with 2.67 and 2.69 fold increases in HF, respectively. At 2W VTP, the LA showed a 4.4 fold increase in ET-1 protein relative to CTR (5.6±0.734 vs 1.27±0.127 fmol/mg protein). The LV and RV showed markedly less ET-1 protein expression compared to the atria in both CTR
and HF dogs. ET-1 content of the atria and PVs were 3-5 fold greater than in the ventricles, Figure 4-3C. A statistically significant interaction was observed (p<0.0001) suggesting that ET-1 protein content is influenced by condition (HF) and tissue origin. These data suggest that the atria and the PVs are intrinsically different from the ventricles, and that they are more sensitive to the hemodynamic stress associated with ventricular tachypacing.

Changes in ET-1 protein and fibrosis were similar and parallel across the myocardium. Correlation analysis revealed that regional ET-1 content is associated with regional % area of total cardiac fibrosis (5x magnification) at 2W VTP (Spearman’s r= 0.66, p<0.0001) as well as with interstitial fibrosis obtained at 20x magnification (Spearman’s r= 0.73, p<0.0001), Figure 4-3D. Note that increased ET-1 protein was associated with increased fibrosis in the atria and PVs, consistent with the hypothesis that ET-1 contributes to atrial remodeling.
Figure 4-3: Temporal and regional changes of ET-1 contents in HF

A) Box plot shows median, 25th and 75th percentile values; whiskers indicate minimum and maximum values for plasma ET-1 in control (CTR) and VTP dogs (VTP: ventricular tachypacing for 24hr, 2 week, and >3 week), n= 4-10 per group, Kruskal Wallis. B) Column plot shows temporal changes of ET-1 in the right atria (RA) and left ventricle (LV) following tachypacing for 12 hr, 24 hr, 1 week (1W), 2W and 5W relative to CTR. N= 5-11 each observation, P<0.05; # VTP RA vs. CTR RA. * P<0.001; LV vs. RA, ANOVA.

C) Column plot shows regional changes of ET-1 in the left atria (LA), RA, LV, and RV at 2W VTP, # p<0.001; CTR vs VTP, ANOVA. D) Scatter plot shows the relationship of cardiac ET-1 content (panel C) with the corresponding interstitial fibrosis in Fig 4-2C. A: atria, V: ventricle, PV: pulmonary vein. r is Spearman’s correlation coefficient.
4.3.3. Temporal and Regional Changes of IP3R-I Protein Expression in HF

ET-1 can causes increases in intracellular Ca$^{2+}$ by modulating IP3R activity$^{10}$. Although IP3R-I is the predominant isoform in human atria$^{49}$, little is known about its role or abundance in failing atria and ventricles. We measured IP3R-I protein expression in PVs, atria, and ventricles and compared temporal changes following 12 hrs to 5W VTP relative to CTR.

Figure 4.4A shows representative western blots of IP3R-I protein in both RA and LV following VTP (12hr-5 weeks) with corresponding quantitative analysis of the protein density relative to GAPDH. Relative to CTR RA, atrial IP3R-I protein started to increase at 24hr VTP, markedly increased at 1W VTP, and remained elevated up to 5W VTP, p<0.05. The LV showed a transient but mild increase at 24 hr (p=0.2), followed by slow changes from 1W to 5W VTP. At 1W, right atrial IP3R-I density was 5-fold higher than in the left ventricles (P<0.01). A statistically significant interaction was observed (p=0.03), suggesting that IP3R-I density is influenced by the duration of tachypacing and by tissue origin.

In an additional set of samples (some of which overlapped with those in Figure 4-4A), tissue homogenates from LA, RA, LV, RV and PV obtained at 2W VTP were homogenized and run on western blots to assess the expression of IP3R-I protein. Figure 4-4B shows representative blots of IP3R-I protein expression at 2W VTP, with corresponding quantitative analysis of the protein density relative to GAPDH. Similar to ET-1 content, IP3R-I protein abundance increased markedly in the LA and RA (p<0.001) following 2W VTP. In the ventricles, changes in IP3R-I protein were more variable. In the LV, IP3R-I protein was not changed. Although IP3R-I protein was increased in the
PV and RV (t-test, p=0.001 PV, p=0.02 RV), the difference was not statistically significant by 2-way ANOVA analysis. Atrial IP3R-I protein abundance was 2-5-fold greater than in the ventricles. A statistically significant interaction was observed between tissue origin and condition (p=0.027), suggesting that IP3R-I protein abundance is also influenced by condition and tissue origin at 2 weeks VTP.

Atrial IP3R-I protein changes paralleled the changes in ET-1. Correlation analysis revealed that the temporal changes of ET-1 protein were correlated with the corresponding IP3R-I protein relative density in the RA (p=0.04, Spearman’s r= 0.36, Figure 4-4C) but not in the LV (p=0.18, Spearman’s r= -0.26), suggesting that IP3R-I protein expression may be partially modulated by atrial ET-1 signaling. Hemodynamic stress has also been shown to modulate IP3R expression\textsuperscript{49} and may further contribute to the observed atrial changes in IP3R-I expression.
Figure 4-4: Temporal and regional changes of IP3R-I protein in HF

A) Representative western blots of IP3R-I and GAPDH from RA and LV following 12 hr, 24 hr, 1W, 2W, and 5W VTP with corresponding quantitative analysis relative to GAPDH, n=5-9 each observation. P<0.05; # VTP RA vs. CTR RA, * p<0.01 LV vs. RA, ANOVA. B) Representative western blots of IP3R-I and GAPDH from LA, RA, LV, RV, PV at 2W VTP with corresponding quantitative analysis relative to GAPDH, n=5-8 each observation. P<0.001; # VTP vs. CTR, ANOVA. C) A scatter plot showing relationship between temporal changes of ET-1 in the RA and corresponding IP3R-I protein density, r is Spearman’s correlation coefficient.
4.3.4. Localization and Distribution of ET-1 and IP3R-I Proteins

Immunostaining and confocal microscopy were used to evaluate the source of cardiac ET-1 and the distribution of IP3R-I in both atria and ventricles. Figure 4-5 shows representative confocal images of ET-1 in HF (2W VTP) and CTR dogs. It is apparent that ET-1 (red) is present in cardiac myocytes, as it is co-localized with phalloidin (purple, a cardiac myocyte marker). Immunostaining confirmed the changes of ET-1 in cardiac tissues that were evaluated by ELISA. In CTR dogs, ET-1 protein staining was higher in RA, PV>LA>>LV, RV. Following 2W VTP; LA, RA, and PV ET-1 content were significantly increased, and ET-1 was detectable in RV>LV.

Figure 4-6A shows representative confocal images of IP3R-I in HF (2W VTP) and CTR dogs. Immunostaining confirmed regional differences of IP3R-I in failing atria and ventricles. Relative to CTR dog, IP3R-I protein expression in cardiac myocytes was increased following 2W VTP in LA, RA>> PV>RV. In the LV, IP3R-I protein was unchanged.

Interestingly, atrial myocytes from failing atria and PVs (2W VTP) showed differential localization of IP3R-I relative to ventricular myocytes. Unlike ventricular myocytes which showed predominantly a cytosolic distribution of IP3R-I, IP3R-I protein in failing atrial myocytes was also localized in the intercalated discs (arrows) and nuclei (arrow heads), Figure 4-6B.

These data suggests that differential localization of IP3R-I may also promote differential atrial responses, and might promote enhanced atrial ET-1/IP3 signaling.
Figure 4-5: ET-1 protein distribution in cardiac myocytes

Representative confocal immunofluorescence images (63x objective) from left and right atria (LA, RA), ventricles (LV, RV), and pulmonary vein (PV) of CTR and HF dog (2W VTP). Sections were stained with ET-1 antibody (red) and phalloidin antibody to stain for cardiac myocytes (purple). Scale bar: 50 µm.
Figure 4-6: Distribution of IP3R-I protein in cardiac myocytes
A) Representative confocal immunofluorescence images (63x objective) of left and right atria (LA, RA), ventricles (LV, RV), and pulmonary veins (PV) from CTR and HF dogs (2W VTP). Sections on Figure 4.5 were also stained with IP3R-I antibody (green), and DAPI (blue) to stain nuclei. B) Representative confocal immunofluorescence images from the RA at 2W VTP (63x objective). Sections were stained with IP3R-I antibody (green), phalloidin to stain myocytes (purple), and dapi to stain nuclei (blue). Images were zoomed in 2 and 4 times (2x Z, 4x Z). IP3R-I protein is distributed in intercalated discs (♠) and nuclei (♣) of atrial myocytes. Scale bar: 50 µm.
4.4. Discussion

Here we describe in detail the regional and temporal changes of cardiac ET-1 and IP3R-I proteins during development of HF. We examined the PVs, atria and ventricles as a function of the duration of ventricular tachypacing. The study revealed consistent differences and responses between PVs, atria, and ventricles. Atrial changes in ET-1 and IP3R-I were faster, larger and more sustained than in the ventricles. Tissue interstitial fibrosis was much more intense in atrial chambers, and especially in the left atrial tissues near the PVs.

Fibrosis increases mechanical stiffness, disrupts conduction and impairs myocardial oxygen supply. Atrial remodeling associated with the onset of HF creates a substrate for the development of AF. Atrial remodeling and fibrosis are frequently observed in patients with AF and HF. Although atrial fibrosis forms a substrate for atrial arrhythmia, the kinetics and mechanism(s) underlying the development of atrial fibrosis during HF are not yet well understood.

The renin-angiotensin system (RAAS) is prominently associated with cardiac fibrosis in HT, myocardial infarction, and HF. Although angiotensin-II (Ang-II) activates mitogen activated protein kinases (MAPK/ERK) and promotes fibrosis, in experimental studies, Ang-II antagonists were insufficient to fully prevent atrial fibrosis.

ET-1, acting through PKC, also activates the ERK proteins. In vascular smooth muscle cells, ET-1 promotes hypertrophy, proliferation, and synthesis of extracellular matrix proteins. It was previously shown that ET-1 promotes proliferation of atrial but not
ventricular fibroblasts in both CTR and HF tissues\textsuperscript{134}. Here, we show that ET-1 is increased earlier and to a greater extent in the atria and the PVs than in the ventricles. In the atria, ET-1 started to increase before onset of HF (at 24hr VTP), and remained elevated until after development of HF (5W VTP). The increase of atrial ET-1 was associated with increased interstitial fibrosis in the atria and the PVs, with much less in the ventricles. These data suggest that ET-1 may promote atrial structural remodeling and create a substrate for AF. Our data also reveal regional differences in ET-1 protein in normal and failing atria and ventricles; ET-1 content in normal RA, PV is >LA>> LV, RV. Following VTP, the increase of atrial ET-1 protein was earlier and markedly higher than in the ventricles, suggesting that there are intrinsic differences in cardiac chambers and their responsiveness to stresses. In a study of porcine congestive HF model induced by VTP\textsuperscript{272}, an increase in myocardial ET-1 mRNA was reported with a distribution in which RA>LA>RV and LV, suggesting that regional changes in ET-1 protein levels may begin with regulation at the transcriptional level. Unlike reports in human and experimental HF\textsuperscript{58,272}, we did not observe consistent differences in plasma ET-1 between the groups. This may reflect the rather large variability within the CTR group, as well as the limitation of a small sample size. The duration of ventricular tachypacing used in the current study ranged from 12 hr to 5 weeks; this duration may not be sufficient to promote profound heart failure or changes in vascular ET-1 expression. This further suggests that myocardial changes in ET-1 expression may occur earlier than those in the vascular system. In addition, myocardial ET-1 changes may not be large sufficient to modulate circulating ET-1 levels. Studies in a porcine HF model (RV tachypacing, 3 weeks) suggest that pulmonary release of ET-1 contributes to increased plasma ET1\textsuperscript{272},
In our study, rapid pacing for 3 weeks was insufficient to induce congestive HF in all animals; this may explain some of the variation in plasma ET-1 that we observed. Expression and release of ET-1 is enhanced by several stimuli, including hypoxia\(^\text{16}\), increased wall shear stress, and pressure overload\(^\text{18}\). It has previously been reported that ventricular tachypacing is associated with increased atrial pressure\(^\text{111}\), suggesting that differential hemodynamic wall stresses may promote greater ET-1 expression in the atria than in the ventricles. As the VTP rate was sometimes slowed between 3 and 5 weeks, the decline in tissue ET-1 content between these time points may reflect the altered hemodynamics associated with slowing the rate of VTP, rather than an adaptive response of the atria.

Cardiac overexpression of ET-1 promotes inflammatory cell infiltration and cytokine production, cardiac hypertrophy, increased interstitial collagen/fibrosis, and early-onset HF\(^\text{26}\). We have recently shown that ET-1 protein abundance in the left atrial appendage is increased in AF patients with underlying cardiac disease, and that ET-1 was associated with AF persistence\(^\text{62}\). Interestingly, ET-1 was associated with LA enlargement as well as with increased mRNA expression of platelet derived factor (PDGF) and connective tissue growth factor (CTGF)\(^\text{62}\). In the canine VTP model of HF, microarray analysis of gene expression revealed a differential expression of PDGFs and their receptors on canine atrial fibroblasts compared to those from the ventricles\(^\text{134}\). ET-1 promotes atrial but not ventricular fibroblasts proliferation\(^\text{134}\). Here too, ET-1 may be a mediator of selective expression of atrial PDGF and fibrosis in HF. Interestingly, atrial cells from IP3R-II deficient mice are less prone to develop pro-arrhythmic disturbances in Ca\(^{2+}\) signaling upon exposure to exogenous ET-1\(^\text{47}\),
suggesting that the acute arrhythmogenic effects of ET-1 are mediated by intracellular Ca\(^{2+}\) release by IP3Rs. IP3R-I is the predominant isoform in human atrial and rat Purkinje myocytes, whereas atrial and ventricular myocytes from most other animal species express predominantly IP3R-II, and to lesser extent, IP3R-III isoforms. Here we show that ET-1 is increased in the atria earlier and to a greater extent than in the ventricles. This increase is associated with a parallel increase in IP3R-I protein in the atria but less in the ventricles, clearly suggesting that ET-1 signaling is enhanced in the atria and may promote arrhythmic disturbances via IP3Rs. It is intriguing that the inotropic response to ET-1 is much greater in the failing atria (+75%) than in the failing ventricles (+10%), and may be mediated by differential IP3R expression.

Our data suggest the presence of adaptive mechanisms in both atria and ventricles in response to an increment in IP3R-I protein expression. Atrial IP3R-I increased following 24hr of VTP, peaking at 1W, and started to decline after that, although it still remained higher than in the control animals. In contrast, in the ventricles, the increase of IP3R-I seemed to occur transiently at 12-24 hours and declined rapidly afterward.

Although IP3 can cause an increase of cytosolic intracellular Ca\(^{2+}\) and may acutely modulate inotropy and arrhythmia, intracellular Ca\(^{2+}\) may also activate calcineurin phosphatase activity, also promoting fibrosis and hypertrophy. Calcineurin activation leads to changes in gene expression by facilitating translocation of transcription factors from the nuclear factor of activated T cells (NFAT) family and contribute to hypertrophic pathways that facilitate transcriptional changes. ET-1 has also been shown to activate nuclear IP3 production and Ca\(^{2+}\) release via nuclear IP3Rs. This can activate histone deacetylase nuclear export, thereby regulating cardiac myocyte transcription and...
hypertrophy\textsuperscript{81}. Here, we also demonstrate that IP3R-I protein is markedly expressed and co-localized with the nuclei of atrial myocytes (to a greater extent than observed in ventricular myocytes), and may also promote nuclear Ca\textsuperscript{2+} release. Further, we show that IP3R-I is distributed in atrial gap junctions and intercalated discs, areas that are critical for modulation of excitation-contraction coupling. This suggests that IP3R-I signaling may mediate/modulate atrial electrical abnormalities.

The PVs are an important source of ectopic beats that frequently initiate paroxysms of AF\textsuperscript{28}. PV foci are commonly targeted for treatment with radio-frequency ablation\textsuperscript{28} of the regions around the entry of the PVs into the left atrium (eg., pulmonary vein atrum isolation). However, the mechanisms underlying the arrhythmogenicity of PVs are still poorly defined. Our canine study reveals that the atrial areas located close to the PVs show extensive interstitial fibrosis, which normally occupies 12\% of tissue area, increasing to 34\% in the early stages of HF. These data suggest that fibrosis in the PV region may lead to a greater susceptibility to develop abnormal conduction or multiple wavelets/reentries than other cardiac regions. Importantly, ET-1 and IP3R-I proteins are also increased in PV tissues from HF dogs. This further suggests that ET-1 signaling pathways are enhanced in the PVs and may promote electrical and structural remodeling resulting in arrhythmogenic effects.

4.5. Study Limitations

This study was limited to evaluate temporal and regional changes of ET-1 and IP3R-I proteins in HF. In future studies, it will be of interest to evaluate temporal changes of IP3R-II during the onset of HF. Regional comparisons were performed following 2 weeks of ventricular tachypacing. At this time, the cardiac dysfunction was associated
with only moderate evidence of HF. A greater degree of ventricular dysfunction would seem likely to be accompanied by a greater increase in ventricular ET-1 abundance and fibrosis. Plasma ET-1 was variable between animals. Future studies with a larger sample size are warranted to evaluate temporal changes of plasma ET-1 associated with VTP. The findings of this study relate to a potential role of ET-1/IP3 signaling on AF substrate, and do not yet prove a causal relationship.

4.6. Significance and Conclusions

Prevention or attenuation of atrial structural and electrical remodeling is a critical goal of AF management, given the inadequacy of currently available atrial antiarrhythmic drugs. Although use of Ang-II converting enzyme inhibitors (ACE-inhibitors) and Ang-II receptor antagonists reduced experimental HF-related ventricular structural remodeling and AF development, these drugs are insufficient to prevent electrical remodeling and can only partially attenuate structural remodeling. This suggests that additional critical pathways are activated in the atria that also promote AF and HF related remodeling. The present study demonstrates for the first time that ET-1 is enhanced in the atria to greater extent and earlier than the ventricles. This is associated, perhaps causally, with increased expression of IP3R-I in atrial myocytes. Importantly, ET-1, IP3R-I protein, and extent of fibrosis are abundant in the PVs. ET-1 is both an important vasoconstrictor and pro-fibrotic factor. IP3R activation following the stimulation of ET-1 receptors may be a primary mediator of electrical and structural remodeling resulting in atrial failure before the ventricles. ET-1 receptor blockade prevented ventricular fibrosis in a HF model induced by intra coronary micro-embolization\textsuperscript{206}. Atrial ET-1 markedly exceeds ventricular ET-1 protein. Efforts to
target cardiac ET-1 using ET-1 selective receptor antagonists, or approaches that blunt ET-1 synthesis, may limit the prominent atrial electrical and structural remodeling in HF, attenuating the development and progression of AF.
Chapter 5

Impact of dietary fish oil on the substrate for post-operative atrial fibrillation in a canine cardiac surgery model

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Abstract
Aims: Pre-treatment with ω3 fatty acids has been reported to reduce the incidence of atrial fibrillation (AF) following cardiac surgery. In a canine cardiac surgery model, we tested the effects of dietary ω3 fatty acids on atrial electrophysiologic properties, inflammatory markers, and endothelin-1 (ET-1) system.

Methods and Results: Adult mongrel dogs (15-25 kg) received either control chow (SC, n =11), or chow supplemented with fish oil (FO, containing EPA/DHA, 0.6g /kg/day, n=9) for 3 weeks before surgery. A left thoracotomy was performed, the pericardium was opened, and the left atrial appendage (LAA) excised. Atrial pacing/recording wires were placed, and the pericardium/chest was closed. AF (>30 s) was inducible on post operative day (POD) 2 in 4/6 SC, but no FO animals. AERP was prolonged, heart rate (HR) was slowed, and HR variability was enhanced in FO dogs. The ratio of ω6/ω3 lipids decreased from ~15-20 in SC to to 2-3 in FO dogs. FO treatment lowered pre-surgical and stabilized post-surgical atrial arachidonate levels. Plasma CRP levels were not different between groups. Peak neutrophil/lymphocyte ratio was lower and decayed faster in FO-treated animals. Extensive inflammatory cell infiltration (myeloperoxidase staining) was present in SC atria and was reduced in FO-treated dogs. Treatment also decreased iNOS protein, plasma ET-1, ET-1 in the LAA, and IP3R-II expression in terminal left atria.

Conclusions: Dietary FO may attenuate AF following cardiac surgery by modulating autonomic tone and reducing inflammation, iNOS and ET-1 expression.
5.1. Introduction

Post-operative atrial fibrillation (POAF) following cardiac surgery affects 20-50% of patients, depending primarily on age and type of surgery. POAF is associated with increased risk of morbidity and mortality. Efforts to prevent POAF have been largely unsuccessful, perhaps due to the complex etiology of the disorder and fragile state of the patients. Factors thought to contribute to POAF include: systemic inflammatory activation\(^8^4\); elevated leukocyte count\(^8^3\), atrial fibrosis\(^2^7^4\), inflammatory tachycardia and changes in atrial action potential morphology\(^2^7^5\); and oxidant stress resulting from increased NADPH oxidase activity\(^9^1\). Inflammatory and oxidant mechanisms likely contribute to AF that occurs both in the post-operative setting, and more broadly, in the context of age-related degenerative changes\(^2^6^4\).

Preclinical models help to elucidate the mechanisms underlying post-operative atrial arrhythmogenesis. These include the canine sterile pericarditis model, in which talc is used as an inflammatory stimulus\(^2^7^6\), and an atrial incision model\(^2^7^7\). Altered conduction patterns, due in part to altered expression and distribution of connexins as a result of inflammatory cell infiltration, were found to contribute to arrhythmogenesis in the post-operative state\(^2^7^7, 2^7^8\). In both models, prednisone decreased neutrophil infiltration and reduced the duration of AF episodes induced with burst pacing\(^2^7^7, 2^7^9\). A randomized, blinded clinical trial similarly showed that steroids can reduce the incidence of AF following cardiac surgery\(^2^8^0\), but concerns about the impact of steroids on wound healing and glucose homeostasis following surgery have limited enthusiasm for this approach.
5.1.1 Omega-3 Fatty Acids for Prevention of Post-operative AF

The highly unsaturated fatty acid lipid pool (HUFA, composed of polyunsaturated fatty acids of 20-22 carbon length) includes both \( \omega_6 \)-fatty acids (arachidonic acid, AA, etc.) and \( \omega_3 \)-fatty acids (eicosapentaenoic acid, EPA; docosahexaenoic acid, DHA). As \( \omega_3 \) and \( \omega_6 \) fatty acids are structurally similar, the enzymes that metabolize \( \omega_6 \) polyunsaturated fatty acids (\( \omega_6 \)-PUFA) also metabolize \( \omega_3 \)-PUFA. While \( \omega_6 \) metabolites tend to promote inflammation and vasospasm, \( \omega_3 \) metabolites typically have less inflammatory activity\(^1\). The ratio of \( \omega_6 \) to \( \omega_3 \) PUFA in tissue HUFA varies as a function of diet. Diets containing abundant vegetable oils and chicken have higher \( \omega_6/\omega_3 \) ratios than diets with a greater abundance of fish. This ratio varies regionally. The American diet maintains \( \sim 15\% \) \( \omega_3 \) in tissue HUFA, while diets in Japan or Iceland maintain the \( \omega_3 \) fraction above 50\%\(^2\). Populations with the lowest fraction of \( \omega_3 \) in the HUFA tend to have the highest incidence of cardiovascular mortality\(^2\).

In 2005, Calò and colleagues published a clinical trial in which bypass graft surgery patients were treated with a supplement containing 2g \( \omega_3 \)-PUFA (derived from fish oil, FO) for a minimum of 5 days prior to surgery, with treatment continued following surgery. Treated patients had a lower incidence of POAF (15.2\%) than untreated control patients (33\%, \( p=0.013 \)). As side effects associated with dietary FO are minimal, this study generated significant clinical interest. Recent efforts to replicate this study using a similar dose with oral delivery have been negative\(^{113, 282} \). Although baseline dietary differences may contribute, reasons for the lack of consistent results cannot be easily determined from clinical trials. More broadly, the impact of FO and \( \omega_3 \)-PUFA on atrial physiology and pathophysiology in the context of cardiac surgery is poorly understood.
To evaluate the mechanisms whereby supplemental FO modulates the development of POAF, we designed a preclinical study in which plasma markers and atrial specimens could be obtained at defined times prior to and following cardiac surgery. We sought to evaluate the impact of FO treatment on pathways that contribute to POAF. We assessed the impact of a dietary FO supplement on plasma and atrial lipid composition, inflammatory cells and mediators, atrial ectopy, heart rate and AF inducibility, and the expression of endothelin-1 and relevant related proteins.
5.2. Methods

5.2.1. Experimental Diets

Adult mongrel dogs of either sex (15-25 kg) were randomly assigned to one of two groups: 1) control chow surgical group (SC) was fed a standard lab chow (Teklad #8653, containing 10% fat with negligible ω3 content, n=11); or 2) a fish oil (FO) group, n=9, that was fed the Teklad #8653 chow supplemented with fish oil (Virginia Prime Platinum, Omega Protein, Inc.), containing 0.6g/Kg/day EPA/DHA representing 20% of their daily caloric intake, for 3 weeks prior to an initial cardiac surgery. A similar FO diet decreased infarct size and reduced ventricular arrhythmias in dogs with experimental myocardial infarction\textsuperscript{283}.

FO was stored frozen in daily aliquots. Individual aliquots were thawed and added to chow just prior to feeding each morning.

5.2.2. Surgical Model

5.2.2.1. Initial Survival Surgery

Under surgical anesthesia, a left thoracotomy was performed, the pericardium was opened and the left atrial appendage (LAA) was excised using a surgical cutting stapler (Ethicon EZ 45). The excised LAA tissue (POD0) served as an internal control for histologic and biochemical studies. Pacing and recording wires were placed in the left atrial epicardium, externalized through the wound and the pericardium/chest was closed. Animals received antibiotics and analgesics (buprenorphine) and were allowed to recover until terminal study at POD2 (n=9) or POD4 (n=11). Animals in the FO group and control chow surgical group (SC) received the same diet after surgery as before.
5.2.2.2. Terminal Surgery

For the acute experiments, animals were placed under general anesthesia (using thiopental and isoflurane) and a median sternotomy surgery was performed to expose the heart. Euthanasia was performed by inducing ventricular fibrillation. The heart was removed, flushed with cooled cardioplegia solution, and atrial tissue specimens were rapidly collected. Some tissues were snap-frozen in liquid nitrogen for biochemical and gene expression studies. Additional specimens were frozen in OCT media for immunostaining and confocal microscopy, and adjacent sections were fixed in formalin for conventional histology. All study protocols were performed in accord with NIH guidelines and approved by the Cleveland Clinic IACUC.

5.2.3. Blood Draws

For FO animals, the first blood draw was obtained prior to initiation of the FO diet. For SC and FO animals, a blood sample was obtained on the day of the initial surgery, prior to anesthesia (POD 0). Tubes were centrifuged (1000g, 15 min) to separate plasma and serum from the cellular components. Plasma and serum was stored in 2 mL cryotubes at -80°C until analysis (for endothelin-1, CRP, and lipid composition). Similar blood draws were performed daily each morning thereafter until the terminal surgery (on post-operative day 2 or post-operative day 4).
5.2.4. Inflammatory Cells Analysis

White blood cell count (WBC) was evaluated prior to treatment and daily from POD 0 to POD 4 using a clinical analyzer (Advia 120) programmed with a canine specific parameter set. The analyzer provides an estimate of monocyte and neutrophil count using myeloperoxidase detection and cell size analysis. Other cell types are distinguished based on cell size. A CBC/differential (complete blood cell count with differential) was obtained.

5.2.5. Holter Recording

In 13 animals, Holter recordings were obtained using a 3-lead digital Holter recorder (IQMark Advanced Holter, Midmark Diagnostics). ECG electrodes (DryContact, Orbital Research) were connected to Holter leads. The chest and leads were covered with a surgical sock. The Holter recorder was then placed in the side pocket of a jacket following the initial cardiac surgery. Holter recordings were downloaded onto a laboratory computer daily and analyzed in batch mode at the end of the study to obtain measures of atrial ectopy, heart rate (HR), and HR variability. Data analysis was performed using the IQMark Holter Analysis suite. Ectopic activity, R-R intervals and data fidelity were manually reviewed.

5.2.6. Conscious Testing

The atrial effective refractory period (AERP) and AF inducibility were determined daily in the conscious state from POD 1 to POD 4 using a Bloom stimulator to perform the extra-stimulus and burst pacing protocols. AERP was determined at a basic cycle length of 400 ms. Arrhythmia inducibility was assessed using a burst pacing protocol (20 Hz
stimulation for 5s or 30s) at 0.1 ms duration, 2x threshold output. Inducibility (defined as AF >30s) was evaluated in duplicate at each stimulus duration.

5.2.7. Lipid Analysis

For analysis of plasma and atrial lipid composition, frozen plasma and atrial tissue samples were transferred on dry ice to Lipid Technologies, Austin, MN. Fatty acid composition was assessed using capillary gas chromatography. Lipids were extracted from the plasma using chloroform/methanol (2:1 vol) according to the method of Folch et al. Phospholipids were separated from neutral lipids by thin layer chromatography. Fatty acid methyl esters (FAME) were formed through transesterification with boron trifluoride (12%) in excess methanol. Methyl ester samples were evaporated under nitrogen and resuspended in heptane containing methyl-tridecanoic acid (NuChek Prep, Elysian, MN) as an internal standard. FAME were separated using a capillary gas chromatograph with a bonded phase, fused silica capillary column. Identities of sample methyl ester peaks were determined by comparison of authentic FAME (NuChek Prep).

5.2.8. Myeloperoxidase (MPO) Staining

Freshly collected sections were fixed in 10% formalin, dehydrated and embedded in paraffin blocks. Tissue sections (~5 µm thickness) were deparaffinized, hydrated, and washed in PBS. Hydrogen peroxide (3%) was used to remove endogenous peroxidases. Following blocking, sections were sequentially incubated in primary rabbit anti-MPO antibody (1:500, Dako). Visualization was accomplished with a daiminobenzidine (DAB substrate) solution until the desired staining intensity was obtained. The sections were then counterstained with hematoxylin, cleared, and mounted. Nuclei appear in blue color while MPO staining appears in brown/black color. Images were obtained at 5, 10, and
20x magnification for qualitative assessment using bright field light microscope (Leica DMR Upright Microscope, Leica Microsystems, Heidelberg, Germany).

**5.2.9. Western Analysis**

Western blot analysis was used to evaluate the level of protein expression of inducible nitric oxide synthase (iNOS) and inositol triphosphate receptor type 1 (IP3R-1) in LAA, LA, and RA tissues. Tissues (~50mg) specimens were homogenized for 20s in ice-cold M-PER mammalian protein extraction buffer (Pierce) containing a protease-inhibitor mixture (Sigma). Bradford assay was used to measure protein concentration. Proteins from the cell lysate were separated using SDS-PAGE (8% acrylamide gel), transferred to a nitrocellulose membrane, and probed with goat anti-IP3R type1 (1:100, Santa Cruz) and rabbit anti-iNOS (1:500, Abcam).

Blots were incubated with appropriate secondary antibodies, washed, and scanned (at 700 and 800 nm) with the Li-Cor Odyssey multi-laser illumination scanner. The integrated intensity of the area within the box for each lane was used for analysis. To correct for variations in protein loading, protein density of iNOS and IP3R-I was normalized to GAPDH (1:5000, Millipore). The lane with the greatest protein density was set at unity. Loading variability is corrected by dividing the corresponding protein value in each lane by the relative abundance of GAPDH.

**5.2.10. C-reactive Protein Plasma Measurements (CRP)**

Plasma CRP was assessed using a canine-specific CRP ELISA (KT-093, Kamiya Biomedical Company, Seattle, WA). Freshly collected EDTA-plasma was put immediately on ice and centrifuged within one hour. Samples were stored at -80°C until
analysis. Quantity of CRP was proportional to enzyme bound quantity and was interpolated from a calibration curve of standards.

5.2.11. ET-1 Measurements in Canine Plasma and Atrial Tissues

Plasma and atrial ET-1 contents were determined by ELISA (Biomedica Bl-20052, American Research Products, Inc). Freshly collected EDTA-plasma was put immediately on ice and centrifuged within one hour. For tissue samples, LAA, LA, RA, and PV tissue (~50mg) was homogenized on M-PER buffer containing protease inhibitor mixture as described before (see western analysis). Samples were stored at -80°C until analysis. Samples were thawed on ice and moved to room temperature before the assay. Tissue samples were diluted using 0.9% NaCl containing protease inhibitors at 1.5-µg/µl concentration, and a total of 50 µl was loaded into each well (75µg total protein). Plasma samples were diluted at 1:1 ratio using 0.9% NaCl solution. Canine plasma or tissue samples, controls and standards (50 µl) were applied in microtiter wells coated with polyclonal rabbit anti-endothelin-1 antibody. Samples were assayed in duplicate. Quantity of ET-1 was proportional to enzyme bound quantity and was interpolated from a calibration curve of standards.

5.2.12. Immunostaining and Confocal Microscopy

Immunostaining was used to evaluate the distribution and protein intensity of IP3R type I and II, ETAR and ETBR from LAA, LA, RA, and PV sections. Alpha-actinin antibody or phalloidin were used to image cardiac myocytes. The distribution of ET-1 protein in cardiac sections was also assessed.
Atrial tissue specimens were immersed in OCT medium, rapidly frozen in liquid N\textsubscript{2}, and stored at -80\textdegree C. Frozen tissues were cryo-sectioned into 16-\(\mu\)m-thick preparations. Sections were fixed for 30 min in paraformaldehyde at RT, blocked in 3\%BSA (Sigma-Aldrich) for 1 h, and incubated with primary antibodies (rabbit anti ETAR or ETBR, 1:200, Alomone labs; rabbit anti IP3R1 or goat anti IP3RII antibody, 1:50, Santa Cruz biotechnology, Inc; mouse anti-ET-1, 1:250, Abcam) at 4\textdegree C overnight. To visualize myocytes structure, tissues were also incubated with mouse monoclonal anti-\(\alpha\) actinin (Sigma-Aldrich, 1:1000) or mouse anti-phalloidin (Molecular probes, 1:40). In addition, sections were stained with guinea pig anti vimentin to visualize fibroblast structure. Sections were then treated with Alexa fluor secondary antibodies at RT for 2 h followed by mounting on glass slides with dapi to stain nuclei. Omission of primary or secondary antibodies as negative controls was included in all immunostaining experiments.

Confocal immunofluorescence images of selected cardiac sections were obtained by using a Leica TCS SP AOBS spectral laser-scanning confocal microscope at 63x magnification. Three-dimensional structures of each region were reconstructed from confocal immunofluorescence images recorded in z-series and analyzed using digital image-processing software (Volocity; Improvision, MA).

5.2.13. Histologic Assessment of Receptor Staining

Stained slides were visually examined at 63x magnification. For quantitative assessment of the extent of atrial receptor staining, maximum intensity projections (MIP) of receptor-labeled, confocal slices (0.5 mm separation, 6-8 slices with equal # for each protein) and corresponding \(\alpha\)-actinin images were batch processed for quantitative analysis of receptor staining using customized macros and algorithms written for Image Pro Plus
v6.2 (Media Cybernetics, Silver Springs, MD). In order to evaluate receptor expression in cardiac myocytes only, receptor area and intensity was measured within α-actinin area.

Briefly, each set of α-actinin and corresponding receptor images were imported consecutively. Since α-actinin has a striated staining pattern that does not necessarily coincide with receptor staining, a number of filters were applied to cluster these striations for a true representation of myocyte tissue area and boundaries. To generate a mask representing receptor staining, predefined size and intensity threshold were applied to filtered receptor MIP images. In order to analyze receptor presence within myocytes only, the binary receptor and myocyte masks were “multiplied” together. This resulting myocyte-only receptor mask was in turn multiplied with the original, unprocessed receptor MIP image. Summing all non-zero intensity values within this image produced a myocyte-localized; receptor integrated optical density (IOD) value. Likewise, summing all binary pixels in the myocyte-only receptor mask and in the myocyte mask produced myocyte-localized receptor area and total myocyte area values respectively. Finally, to determine average receptor intensity normalized to myocyte area, the receptor IOD was divided by α-actinin area.

Computerized image analysis was performed in 6 animals of each study group (3 pairs each at POD2 and POD4).
5.2.14. Statistical Analysis

Two-group analysis of normally distributed variables was assessed using t-test. Paired t-test was used to assess differences between matched cardiac chambers in the same animal, while unpaired t test was used to asses differences between two independent groups. ANOVA was used to assess differences across multiple groups followed by Bonferroni post test for individual pair comparisons. Values of $p<0.05$ were considered statistically significant. All values are expressed as mean±SEM.
5.3. Results

5.3.1. Fish oil / Plasma / Atrial Lipid Content

To increase atrial ω3 content, we used a diet highly enriched in ω3 PUFA. Figure 5-1B shows that the atrial ω3 % in HUFA increased from 7.5% to 35%, and plasma ω3 % in HUFA increased to ~60% (Figure 5-1A). Plasma ω3 % in the FO group fell towards the tissue levels following surgery. Plasma and atrial levels were comparable in the SC group, suggesting that lipid changes in the FO group had not reached steady state. As ω3-PUFA content increased, the arachidonic acid (AA)% in HUFA decreased in plasma from 78% to 38% (Figure 5-1C). In the LAA on POD 0, the AA % was 78% in SC compared to 58% in the FO group (Figure 5-1D). The POD0 ω6/ω3 ratio dropped from 18 to 1.3 in plasma, and from 21 to 3 in LAA (Figure 5-2A, B). Leukotriene B4 (LTB₄) is an arachidonic acid (20:4 ω6) metabolite that acts as a chemokine to attract neutrophils into injured tissues. LTB₅, the homologous ω3 metabolite, has ~1/1000x the chemotactic activity of LTB₄. LTB₄ formation is attenuated when the abundance of EPA (20:5 ω3) is increased; Figure 5-2D shows that FO treatment reduced the LAA 20:4/20:5 ratio from 134:1 to 4:1.
Figure 5-1: Plasma and tissue ω3 and arachidonic acid

Scatter plots shows plasma (A) and atrial ω 3% (B), arachidonic acid (AA) % (C, D) in the highly unsaturated fatty acid lipid pool (HUFA) of surgical control (SC) and fish oil treated (FO) animals at baseline (BL, 3weeks prior to surgery), at post operative day 0 (initial surgery) and post operative day 1-4 following surgery.
Figure 5-2: Plasma and tissue $\omega_6/\omega_3$ and AA/EPA ratio

Scatter plots shows plasma (A) and atrial (B) $\omega_6/\omega_3$ ratio, and 20:4/20:5 (AA/EPA) ratio (C, D) in the highly unsaturated fatty acid lipid pool (HUFA) of surgical control (SC) and fish oil treated (FO) animals at baseline (BL, 3weeks prior to surgery), at post operative day 0 (initial surgery) and post operative day 1-4 following surgery.
5.3.2. C-reactive Protein / Inflammatory Cells

Figure 5-3A shows that CRP levels peaked on POD1 in both the surgical control (SC) and fish oil (FO) treated dogs. FO treatment had no impact on plasma CRP levels. Surgery also evokes a cellular inflammatory response, dominated by an increase in neutrophil count. Figure 5-3B shows that although the neutrophil/lymphocyte ratio (NLR) peaked at the same time in control and FO treated animals, the peak NLR was lower and recovered more quickly in the FO treated group.

5.3.3. Heart Rate / Heart Rate Variability (HRV) / Arrhythmia Inducibility

Cardiac surgery evokes a profound inflammatory response, often accompanied by inflammatory tachycardia. Holter and arrhythmia inducibility data was obtained from 6 control and 7 FO treated animals following the initial cardiac surgery. Figure 5-3C shows that post-operative heart rate was more elevated in the SC animals following surgery than in those receiving a FO supplemented chow. The treatment groups were significantly different (p=0.0003), with FO associated with a lower heart rate following surgery. In addition to differences in mean heart rate, Figure 5-3D shows that RMSSD and SDANN, heart rate variability parameters associated with vagal tone, were enhanced by fish oil treatment. In the SC animals, atrial effective refractory period (AERP) was 70±22.8 ms, significantly shorter than the AERP in the FO group (122±34.5 ms, p=0.009). In cardiac surgery patients, AF is most common on POD2. AF inducibility was evaluated in 6 CTR and in 7 FO treated dogs on POD2. Atrial arrhythmias (>30s duration) were induced by burst pacing in 4/6 control, but in none (0/7) of the FO treated animals tested (p=0.02).
**Figure 5-3: Biomarkers, heart rate variability (HRV) and atrial arrhythmia inducibility**

A) Scatter plot shows plasma CRP at baseline (BL, 3 weeks before FO treatment), at post operative day 0 (day of surgery and prior to LAA excision), POD 1, 2, and 4 in both SC and FO treated animal. (n=8-9 per group)

B) Scatter plot shows neutrophil to lymphocyte ratio at BL, and POD 0-4.

C) Scatter plot shows heart rate following surgery (POD 0-4).

D) Column plot showing mean±SEM of the root mean square of the successive normal sinus RR interval differences (RMSSD) and the standard deviation of the averaged normal sinus RR intervals for all 5-minute segments (SDANN). For SC, n=6; FO n=7. * p<0.05 for FO vs. SC
5.3.4. Myeloperoxidase Staining

Myeloperoxidase (MPO) is abundantly expressed in neutrophils and macrophages. Tissues from left atrial appendage (LAA, POD0), and left and right atrium (LA, RA, POD2, 4) were evaluated for evidence of injury, and MPO staining (as a measure of macrophage/neutrophil infiltration). Figure 5-4 shows tissue sections from representative SC and FO treated animals that had terminal surgery on POD 4. Extensive inflammatory cell infiltration (and MPO staining, brown/black) was evident in the LA near the wound of the control animal, but staining was also apparent in the LA away from the wound and in the RA. In contrast, in FO animals, MPO staining was markedly lower, particularly away from the region of the wound and in the RA.
Figure 5-4: Myeloperoxidase (MPO) staining

Bright field images show MPO staining (brown/black) recorded at 5x magnification from left atrial appendage obtained at time of surgery (LAA), terminal left atria near the wound (LA) and remote from wound, and terminal right atria (RA) at POD4 from (A) a single representative surgical control (SC), and (B) a fish oil (FO) treated animal (B).
5.3.5. iNOS Protein Expression

Figure 5-5A shows representative western blots of iNOS protein expression in atrial tissues. FO treatment decreased iNOS protein abundance in POD0 LAA (p=0.046), Figure 5-5B. Relative to baseline LAA, iNOS protein tended to be higher following cardiac surgery in the LA of SC animals at POD2 (p=0.09), Figure 5-5C. FO treatment significantly decreased iNOS in terminal LA and to a lesser extent in the RA, Figure 5-5C-D.

5.3.6. Plasma and Atrial ET-1 Measurements

Plasma ET-1 was evaluated at baseline prior to FO treatment (BL), prior to surgery (POD0), and daily thereafter (POD1, POD2 and POD4) (Figure 5-6A). The FO diet significantly lowered plasma ET-1 levels measured on POD0 and each day thereafter. Although plasma ET-1 tended to increase after cardiac surgery at POD4, the change was not significant in either group.

ET-1 levels were measured in LAA tissues (initial surgery), and from terminal LA and RA tissues on POD2 and POD4, Figure 5-6B-D. Analogous to the plasma studies, the FO diet significantly lowered LAA ET-1 levels at the initial surgery (mean±SEM, fmol/mg total protein: 1.23±0.07 vs. 0.72±0.10, SC vs. FO, p=0.002), Figure 5-6B. Relative to POD0 ET-1 expression in the LAA, ET-1 protein expression was higher in the LA in both study groups (POD2, mean±SEM, fmol/mg protein: 2.40±0.49 vs. 1.75±0.44; POD4: 1.83±0.19 vs. 2.00±0.61; p<0.05, SC vs. FO groups, respectively). ET-1 content of terminal RA tissues was twice that of the LA (POD2, mean±SEM, fmol/mg protein = 4.69±0.98 vs. 3.08±0.45, SC vs. FO, p=0.03, 0.07, respectively). To determine whether
the greater abundance of ET-1 in the RA was due to cardiac surgery or to regional variation, LA and RA specimens were evaluated from control chow non-surgical dogs (NSC, n=7). In all groups, RA ET-1 content was higher than in the LA, suggesting that there are intrinsic regional differences in ET-1 expression (Figure 5-6D).

Although FO tended to lower RA and LA ET-1 expression at POD2, this difference was not statistically significant (Figure 5-6C).

Immunostaining confirmed that ET-1 (red) is higher in the LA than in the LAA and is present in cardiac myocytes (phalloidin, purple), Figure 5-7. FO treatment lowered ET-1 protein expression in LA cardiac myocytes, but had less effect in the RA.
Figure 5-5: iNOS protein expression

Representative Western blots of iNOS protein expression and GAPDH from left atrial appendage (LAA) at initial surgery (POD0), terminal left atria (LA), terminal right atria (RA) after surgery at post operative day 2 (POD2) or POD4 in SC and FO groups. B-D) Bar plots showing Mean±SEM of iNOS protein band relative density relative to GAPDH. N=4-7 per group. *p<0.05.
Figure 5-6: Plasma and cardiac endothelin-1 (ET-1) measurements

A) Scatter plot shows plasma ET-1 at baseline (BL, 3 weeks before FO treatment), POD 0 (day of surgery and prior to LAA excision), POD 1, 2, and 4 in both SC and FO groups. * p<0.05 for FO vs. SC. # p<0.05 for BL vs. POD 0 (FO group). N=9 per group.

B) Column plot shows mean±SEM of ET-1 protein in left atrial appendage (LAA) obtained at time of surgery.

C-D) Column plots show mean±SEM of ET-1 expression in POD0 LAA, terminal left atria (LA), and terminal right atria (RA) on POD2 (C) and POD4 (D) in non surgical control (NSC), SC and FO animals.
Figure 5-7: Endothelin 1 distribution in cardiac myocytes

Representative confocal images of ET-1 staining (red) from LAA (initial surgery) and terminal LA and RA in SC and FO groups at POD2. Overlay images are stained with phalloidin (purple, myocytes) and DAPI (blue, nuclei). Scale bar is 50µm.
5.3.7. Endothelin Receptor Intensity and Distribution

ET-1 acts on two receptors, type A (ETAR) and B (ETBR). Immunostaining revealed that ETAR and ETBR expression was abundant in atrial myocytes (α-actinin, red). No marked differences in receptor localization were observed between study groups (Figures 5-8, 9).

Relative to POD0 LAA, ETAR expression was lower in SC terminal LA at POD2 (P<0.05), but not at POD4. In FO treated group, ETAR was lower in LA at POD2, but the difference was not significant. At POD4, ETAR expression in FO treated LA was higher (P<0.05, Figure 5-8). FO treatment had no impact on ETAR expression at POD2, but showed more abundant ETAR expression at POD4 in terminal LA (P=0.018). These data suggest that ETAR is reduced after cardiac surgery (mainly at POD2). This effect was reversed by FO treatment at POD4. ETAR expression in the RA tended to be similar to POD0 LAA expression, suggesting that injury in the LA is associated with changes of ETAR expression in the LA more than in the RA.

ETBR expression was similar between FO and SC groups at POD2 and POD4, (Figure 5-9). Relative to POD0 LAA, ETBR expression in terminal LA was lower at POD2 and POD4 in SC and FO treated groups.
Figure 5-8: ETAR protein expression following surgery

Representative confocal images of ETAR staining (green) in SC (A) and FO (B) groups at post operative day POD 2 & 4 with corresponding overlay images with α-actinin (red) to stain cardiac myocytes, vimentin (a marker of cells of mesodermal origin) to stain fibroblasts (purple) and DAPI to stain nuclei (blue). LAA: left atrial appendage at initial surgery (POD0); LA: terminal left atria; RA: terminal right atria. Note that ETAR is mostly localized in the plasma membrane of atrial myocytes with less expression in the cytosol. ETAR is also colocalized in fibroblasts (purple). C-D) bar plots showing Mean±SEM ETAR integrated optical density (IOD) normalized to α-actinin area in atrial myocytes at post operative day 2 (POD2, C) and POD4 (D). N=6 per group. * p<0.05
Figure 5-9: ETBR protein expression following surgery

Representative confocal images of ETBR staining (green) in SC (A) and FO (B) groups at post-operative day (POD) 2 with corresponding overlay images with α-actinin (red) to stain cardiac myocytes, vimentin (a marker of cells of mesodermal origin) to stain fibroblasts (purple) and DAPI to stain nuclei (blue). LAA: left atrial appendage at initial surgery (POD0); LA: terminal left atria; RA: terminal right atria. Note that ETBR is mainly expressed in the cytosol of cardiac myocytes with less expression in the plasma membrane. ETBR staining is also present in fibroblasts (purple). C-D) bar plots showing Mean±SEM ETBR integrated optical density (IOD) normalized to α-actinin area in atrial myocytes at post operative day 2 (POD2, C) and POD4 (D). * p<0.05, n=6 per group.
5.3.8. IP3 Receptor Expression and Distribution

ET-1 can modulate intracellular Ca\(^{2+}\) via generation of inositol trisphosphate (IP3) that interacts with IP3 receptors type I and II. Like ET-1 receptors, IP3Rs were distributed in atrial myocytes (Figure 5-10, 11). No changes in receptor localization were observed between study groups.

Image analysis revealed no differences in IP3R-I expression between FO and SC dogs. Relative to POD0 LAA, IP3R-I expression was higher in the LA (Figure 5-10) at both POD2 and POD4. Western blot analysis confirmed the changes observed by immunostaining (Figure 5-10).

Unlike IP3R-I, FO treatment significantly reduced IP3R-II expression in terminal LA at both POD2 and POD4 (Figure 5-11). Relative to POD0 LAA, IP3R-II expression was higher in terminal LA of SC but not FO treated animals. In both study groups, RA IP3R expression was similar to that in the LAA.
Figure 5-10: IP3R-I protein expression following surgery

Representative confocal images of IP3R-I staining (green) in SC (A) and FO (B) groups at post operative day (POD) 2 with corresponding overlay images with α-actinin (red) to stain cardiac myocytes, vimentin to stain fibroblasts (purple) and Dapi to stain nuclei (blue). LAA: left atrial appendage at initial surgery (POD0); LA: terminal left atria; RA: terminal right atria. Note that IP3R-I expression in atrial myocytes is mainly cytosolic and co-localized with α-actinin. IP3R-I is also expressed in fibroblast. C-D) bar plots showing Mean±SEM IP3R-I integrated optical density (IOD) normalized to α-actinin area in atrial myocytes at post operative day 2 (POD2, C) and POD4 (D). * <0.05, n=6 per group. E is representative western blot of IP3R-I protein expression and GAPDH from left atrial appendage (LAA, POD0), terminal left atria (LA), terminal right atria (RA) after surgery at POD4 in SC and FO groups.
Figure 5-11: IP3R-II protein expression following surgery

Representative confocal images of IP3R-II staining (green) in SC (A) and FO (B) groups at POD2, with corresponding overlay images with α-actinin (red) to stain cardiac myocytes, vimentin to stain fibroblasts (purple) and DAPI to stain nuclei (blue). LAA: left atrial appendage at initial surgery (POD0); LA: terminal left atria; RA: terminal right atria. Note that IP3R-II is highly colocalized with α-actinin, with a striated distribution pattern.

C-D) Column plots show mean±SEM IP3R-II integrated optical density (IOD) normalized to α-actinin area in atrial myocytes on POD2 (C) and POD4 (D). * p<0.05, n=6 per group.
5.3.9. *Endothelin-1/IP3 Signaling in the Pulmonary Vein*

The pulmonary veins (PVs) are an important source of atrial ectopic beats that can trigger AF. We measured ET-1 protein expression in the PVs and compared changes between study groups. ET-1 content in terminal PV (SC, n=3) tended to be higher than non-surgical ET-1 content (NSC, n=7) and FO treated PVs (n=3) (Mean±SEM, fmol/mg total protein = 4.8±0.64, 3.18±0.24, 3.28±0.95; p=0.09, for SC, NSC, and FO groups, respectively), Figure 5-12A. Immunostaining revealed the presence of ET-1 staining in atrial myocytes of the PV and documented that FO reduced ET-1 in the PV, Figure 5-12B.

Atrial myocytes (actinin, red) of the PV showed abundant expression of endothelin receptors (green, Figure 5-12C) and IP3Rs (green, Figure 5-12D), suggesting the presence of downstream component of ET-1 signaling pathway in the PVs.
Figure 5-12: ET-1 expression in the pulmonary veins:

A) Total ET-1 contents in LA, RA, and pulmonary veins (PV) from all SC, FO animals (n=9-10 LA&RA, n=3 PV; POD2 and 4) and normal non-surgicated hearts (NSC, n=7 LA, RA &PV). B) Representative confocal images of ET-1 staining (red) in SC and FO groups at POD 2 and corresponding overlay images with phalloidin (purule) and Dapi (blue). C-D) representative confocal images of ET receptors (ETAR, ETBR; green) and IP3Rs (IP3R-I&II; green) in atrial myocytes (α-actinin, red) of the pulmonary vein (PV).
5.4. Discussion

POAF increases length of stay and hospital costs, and is a predictor of increased morbidity and mortality. Perhaps because the mechanisms underlying POAF are so poorly understood, available prophylactic interventions are generally ineffective.

A clinical study suggested that a dietary fish oil supplement (2g/day, beginning 5-7 days before surgery) was effective in decreasing the incidence of POAF\(^{286}\). Clinically, the impact of modifying atrial vs. plasma \(\omega3\)-content has not been carefully evaluated, with respect to determining the degree of change needed to impact arrhythmogenesis following cardiac surgery. Changes in plasma and atrial lipid composition in response to dietary supplement are time-and dose-dependent. Although plasma levels can change quickly, steady state tissue changes require about 3 weeks\(^{287}\).

The impact of a given dose of \(\omega3\) PUFA also depends on the level of competing \(\omega6\) fatty acids in the diet, and in tissue lipid stores. The composition of tissue lipid stores varies globally by region, reflecting variations in dietary preference and availability. Canine atrial tissues have a very low fraction of \(\omega3\) PUFA, with an \(\omega6/\omega3\) ratio of 21. The plasma and atrial \% \(\omega3\) in the SC HUFA pool is 8\%, similar to that of the American population. In contrast, individuals consuming a Mediterranean diet have \(\sim35\%\) \(\omega3\) in the HUFA, and those consuming a traditional Japanese diet have more than 50\% \(\omega3\) in HUFA\(^{281}\). Our experimental diet shifted the \(\omega3\)-HUFA content of the atria from a level similar to that of individuals regularly consuming an American diet to one closer to that of individuals consuming a Mediterranean diet. This change lowered post-operative heart
rate, improved heart rate variability, decreased inflammatory cell infiltration, and attenuated the expression of iNOS and elements of the endothelin-1 system.

5.4.1. Impact of Fish Oil on Post-operative Tachycardia and Heart Rate Variability

FO treatment slowed heart rate and enhanced heart rate variability after surgery. Tachycardia following cardiac surgery is common, and is associated with increased risk of POAF\textsuperscript{275}. Tachycardia might result from enhanced sympathetic tone, loss of vagal tone, or modulation of pacemaker activity. HR variability analysis suggests that vagal tone was preserved and/or enhanced by FO treatment. Vagal stimulation is associated with anti-inflammatory activity\textsuperscript{288}. As arachidonic acid (AA) metabolites PGF\textsubscript{2a} and TxA\textsubscript{2} are implicated in the genesis of inflammatory tachycardia\textsuperscript{289}, decreased AA abundance in the FO-treated animals may contribute to heart rate slowing. Dietary ω3-PUFA can also suppress pacemaker channels in the SAN node (I\textsubscript{f})\textsuperscript{290}.

5.4.2. Impact of Fish Oil on Post-operative CRP and Inflammatory Cells

Previous studies in canine surgical models suggest a critical role for inflammatory cell infiltration as a mediator of post-operative AF\textsuperscript{277,279}, and shown that interventions which limit this infiltration can limit conduction heterogeneity, AF inducibility, and AF episode duration. With excision of the LAA during initial surgery as the primary trauma (rather than application of talc and gauze to the epicardium, or creation of a right atrial incision), the surgical model used in this study is somewhat more clinically relevant than those reported previously. Here, the post-surgical systemic inflammatory response (as reflected by plasma CRP levels) was not affected by FO treatment, and was not different between animals in which AF was vs. was not inducible. The systemic inflammatory response may primarily reflect the trauma associated with opening the chest and pericardium, a
feature common to all protocols. That the CRP response did not vary by treatment group suggests that CRP is not strongly modulated by dietary lipids, and may not be causally linked to the arrhythmogenic substrate for POAF. In contrast, atrial MPO staining was strongly modulated by FO treatment. Atrial MPO is implicated in atrial fibroblast proliferation and the development of interstitial fibrosis. FO treatment attenuated atrial MPO staining, particularly in the areas remote from the surgical wound, likely as a result of decreased LTB₄ production.

5.4.3. Impact of Fish Oil on Atrial Endothelin-1 System

ET-1 acts through ETAR and ETBR receptors and activates PKC/IP3/NADPH oxidase pathways. We recently reported that atrial ET-1 abundance is increased in AF patients with underlying cardiac disease, and that atrial ET-1 was associated with atrial rhythm, size and fibrosis. Here we show that atrial, but not plasma ET-1 was higher following surgery in both study groups. FO strongly reduced plasma ET-1. Plasma ET-1 prior to pulmonary vein isolation (PVI) is reported to predict AF recurrence following PVI; ET-1 may also promote POAF. In the atria, FO reduced initial ET-1 expression by 42%. Following surgery, the changes were less significant. Intriguingly, RA ET-1 levels were higher than in the LA, suggesting that there are regional differences in this system. Right atrial mRNA expression of both ETAR and ETBR was lower at time of surgery in patients that experienced POAF than in those that did not. Both ETAR and ETBR expression were lower in the LA following surgery in SC animals. The biological significance of a downregulation of ET-1 receptors is unclear. A recent study suggested that both ET receptors are regulated by jun activation domain-binding protein (Jab1)
which promote ubiquitination and degradation of the receptors following long term ET-1 stimulation.293

By increasing IP3 production, elevated ET-1 can enhance atrial contractility, and perhaps arrhythmia inducibility. After binding to its receptors, IP3 modulates calcium via release from intracellular stores. In cardiac diseases including AF and HF, expression of IP3Rs and membrane receptors that couple to PLC/IP3 signaling is increased, suggesting a role for IP3 signaling in the etiology of these diseases.49 Both IP3R-I and IP3R-II were higher following surgery in SC animals. While FO had no impact on ETBR or IP3R-I expression, it markedly reduced IP3R-II expression in the post-surgical LA. In most species, IP3R-II is the predominant atrial isoform.49 FO reduced atrial ET-1 and IP3R-II expression and may attenuate the likelihood of arrhythmogenic disturbances associated with enhanced IP3 signaling or intracellular Ca2+ release.

Interestingly, ET-1 content in SC PV tended to be higher than non-surgical control PV content. Arrhythmogenic ectopic triggers originate in the PVs, and FO reduced ET-1 content in the PVs. This effect may contribute to its ability to limit AF inducibility. The finding that endothelin and IP3 receptors are also expressed in atrial myocytes of the PVs indicates that components of IP3 signaling pathway exist in atrial myocytes of the PV and may be activated with enhanced ET-1 production.

In vitro studies have shown that treatment of cultured myocytes with EPA (ω3-PUFA) prevented ET-1 mediated induction of iNOS expression and cardiac myocyte hypertrophy.97 Here, we observed that FO attenuated both ET-1 production and iNOS expression. ET-1 receptor activation also stimulates NADPH oxidase activity.264 As
both iNOS and ET-1 levels were reduced by FO treatment, it seems reasonable to hypothesize that atrial and systemic oxidant stress is attenuated by FO treatment.

5.5. Limitations

Due to technical limitations, baseline heart rate / HRV data was not obtained prior to surgery. Electrophysiologic testing was performed in only a subset of the animals, and AF was only inducible in 4/6 control animals. The small sample size limits the ability to define the factors associated with AF inducibility. To achieve the desired shift in atrial lipid content within 3 weeks required a dose larger than that used in any of the clinical trials using dietary PUFA (0.6 g/kg/day). Studies in a canine ventricular tachypacing model demonstrated that a two week pretreatment with ~0.2g/kg/day ω3 PUFA was also effective in minimizing hemodynamic changes, atrial fibrosis and AF inducibility. Additional studies are needed define the threshold ω6/ω3 ratio needed to impact atrial arrhythmogenesis.

5.6. Conclusions

This study demonstrates that cardiac surgery provokes profound changes in plasma and atrial inflammatory markers. Use of dietary fish oil at levels that shifted atrial ω3-content to levels similar to those of individuals consuming a Mediterranean diet decreased the post-operative neutrophil to lymphocyte ratio and evidence of atrial neutrophil infiltration. Fish oil slowed post-operative heart rate and enhanced heart rate variability, in a manner consistent with improved vagal tone. Fish oil also reduced the plasma and atrial ET-1 levels, and the atrial expression of iNOS, IP3R-II. These elements have a critical role in calcium cycling and atrial conduction. In animals with substantially
elevated atrial and plasma \( \omega_3 \) levels, the substrate for post-operative arrhythmogenesis is favorably altered.
Summary and Future Directions

6.1. Summary and Conclusions

In the paragraphs below, I summarize the primary findings of my studies:

1. Left atrial size is increased in patients as a function of age, degree of mitral valve regurgitation, ventricular dysfunction and atrial fibrillation. Others have shown that left atrial enlargement is associated with poor clinical outcomes. In the excised left atrial appendage of human patients, I have found that ET-1 protein (but not its receptors) is notably increased in the patients who are in AF at the time of cardiac surgery. This suggests that expression of the ET-1 ligand, rather than the receptor, is dynamically modulated by atrial rhythm in patients with underlying cardiac disease. ET-1 levels were also associated with AF persistence, suggesting that enhanced ET-1 expression may promote atrial dysfunction and increase the persistence of AF.

2. Among surgical patients with a history of AF, LAA ET-1 elevation correlates with increased left atrial size. This association suggests that ET-1 activation in AF promotes LA remodeling.

3. Microarray expression analysis revealed that ET-1 mRNA was less elevated in AF than ET-1 protein. However, the mRNA expression of ECE-2 (endothelin-converting enzyme, isoform 2) was also upregulated in AF patients and was associated with AF persistence. These data suggest that AF-related changes in ET-1 expression begin at the transcriptional level, but are amplified by enhanced processing of the precursor protein,
big ET-1.

4. ET-1 mRNA levels were positively associated with the mRNA expression of profibrotic molecules including PDGF and the CTGF, suggesting that, in human atria, ET-1 modulates atrial remodeling via activation of these signaling pathways.

5. In both canine and human studies, I have documented the presence of ET-1 protein in atrial myocytes and fibroblasts. This suggests that ET-1 may have both paracrine and autocrine activities that promote atrial electrical and structural remodeling.

6. In lone AF patients, plasma ET-1 was associated with age, PHTN, and atrial size; all are common risk factors of AF.

7. The single nucleotide polymorphism (SNP) rs2200733 on chromosome 4q25 is one of the SNPs most strongly associated with AF risk; however, the mechanisms linking the SNP with AF are still unknown. In a study of plasma ET-1 levels in lone AF patients, plasma ET-1 was significantly higher in lone AF patients homozygous for the rs2200733 risk allele than in patients with the major allele, suggesting that ET-1 may contribute to increased AF risk in individuals with this genotype.

8. In a canine heart failure (HF) model induced by ventricular tachypacing (VTP), ET-1 levels increased in response to VTP within 24 hours in the right atria and sustained through 5 weeks of VTP. The increase in atrial ET-1 levels occurred earlier and was markedly higher than in the left ventricles. Following 2W VTP, ET-1 was increased in both left and right atria and PVs but markedly less in both ventricles. Regional and temporal changes of of IP3R-I protein paralleled changes in ET-1 protein. IP3R-I was upregulated in the atria but markedly less in the ventricles, suggesting the presence of an enhanced ET-1/IP3 signaling pathway in the atria.
9. Atrial fibrosis was markedly increased in the canine VTP HF model. Fibrosis was selectively increased in the atria and the PVs, much more than in the ventricles. This resembles the selective human atrial fibrosis observed clinically in HF patients\textsuperscript{118}. As changes in fibrosis and IP3R-I paralleled but lagged the changes in ET-1, these data are consistent with the hypothesis that ET-1 signaling underlies these changes and acts as an important modulator of atrial electrical/structural remodeling and arrhythmogenesis.

10. Cardiac surgery creates an inflammatory/autonomic environment that creates a substrate for AF. In a canine cardiac surgery model, surgery provoked profound changes in heart rate, and in plasma and atrial inflammatory markers. Left atrial but not plasma ET-1 levels were increased following cardiac surgery. Increased atrial ET-1 was temporally associated with increased neutrophil/lymphocyte ratio, atrial MPO staining, atrial iNOS protein expression and AF inducibility.

11. In the canine cardiac surgery model, increased LA ET-1 levels were accompanied by a down regulation of ET-1 receptors, but an upregulation of IP3Rs. Enhanced ET-1 production and upregulation of IP3Rs may promote changes in intracellular Ca\textsuperscript{2+} leading to changes in transcriptional regulation, and/or in atrial arrhythmia.

12. Both canine studies revealed regional differences in ET-1 protein in the myocardium. In control animals, ET-1 content in the RA and PVs was approximately twice the level present in the LA. This suggests that there are intrinsic differences in cardiac chambers that affect ET-1 protein expression. Regional differences in gene expression may also influence cardiac responses to various stressors.

13. Pulmonary vein ectopy is implicated as an important cause of AF\textsuperscript{28}. Our data support a possible role of ET-1 in abnormal PV physiology. Increased ET-1 expression here in
response to stress (wall stress or ischemia) may contribute to increased PV fibrosis and IP3R-I expression. Thus, ET-1 may contribute to abnormal conduction (local re-entry) and the origin of ectopic activity.

14. The heart consumes lipids as a primary source of energy. Dietary fatty acids have an impact on cardiac metabolism, autonomic activity and inflammation. As a consequence, the balance of \( \omega 6/\omega 3 \) polyunsaturated fatty acids (PUFA) in the diet can modulate heart rate, and the response to tissue injury. Both atrial and plasma \( \omega 3 \)-PUFA levels may impact arrhythmogenesis following cardiac surgery. In our cardiac surgery model, we evaluated the impact of an experimental diet that contained fish oil (a rich source of \( \omega 3 \)-PUFA) at levels sufficient to shift the atrial \( \omega 3 \)-content from levels similar to those associated with an American diet to levels similar to those of individuals consuming a Mediterranean diet. The fish oil diet supplement decreased the heart rate following cardiac surgery, lowered the neutrophil to lymphocyte ratio and atrial neutrophil infiltration, and eliminated the inducibility of post-operative AF (POAF). In addition to slowing post-operative heart rate, fish oil also improved heart rate variability. This was consistent with enhanced parasympathetic and/or attenuated sympathetic tone after surgery. Fish oil also reduced the atrial expression of iNOS protein.

15. Three weeks of dietary fish oil supplement reduced ET-1 protein abundance in plasma, in the LAA and PVs. Fish oil attenuated the increase in IP3R-II protein associated with surgery. As these elements have a critical role in atrial calcium cycling and atrial conduction, preoperative \( \omega 3 \)-PUFA supplements may suppress POAF by modulating ET-1/IP3 signaling.
6.2. Translational Perspective

To improve human health, scientific discoveries must be translated into practical applications. Discoveries that facilitate the study of human disease at the molecular or cellular level must progress to the clinical level to improve patient care and clinical outcomes.

AF increases risk of stroke, morbidity, and mortality. Current treatments for AF are primarily empiric, and have significant limitations. Studies that identify pathways by which we can prevent the development or progression of AF may lead to improved treatment. At present, little is known about the mechanisms that underlie atrial ectopy and remodeling. Numerous studies have focused on changes in ion channel activity to explore the mechanisms of atrial electrical remodeling. However, most of these studies have ignored the possible role of neuro-hormonal stimuli that can modulate the activity of these channels.

ET-1 is the strongest vasoconstrictor peptide known. ET-1 expression is enhanced by conditions associated with ischemia and wall stress. Here, we show that ET-1 is increased in the atria and the PVs in experimental canine models of cardiac surgery and HF. Cardiac surgery is associated with ischemic injury and autonomic activation that promotes ET-1 expression. Ventricular tachypacing increases atrial wall stress. Both ischemia and increased wall stress promote atrial inflammation and dilatation/fibrosis, important elements of a substrate for AF.

ET-1 peptide modulates intracellular Ca^{2+} handling via activation of PLC coupled to IP3/PKC/MAPK signaling pathways. Enhanced IP3-dependent Ca^{2+} release likely increases spontaneous Ca^{2+} release, Ca^{2+} spark frequency, and ectopic electrical activity.
In our cardiac surgery and HF models, increased ET-1 expression was also associated with increased expression of IP3Rs. The increase of atrial ET-1 and IP3R-I was markedly higher and occurred earlier than in the ventricles. These changes were associated with a similar and parallel increase of atrial fibrosis. This suggests that enhanced ET-1 signaling in the atria and the PVs correlates with AF inducibility and atrial structural remodeling. ET-1 signaling is a pathway that may promote atrial ectopy, reentry and structural/electrical remodeling. Thus, ET-1 synthesis, signaling pathways and/or receptors may all be rational therapeutic targets for pharmacologic intervention in AF, HF and other cardiac diseases associated with abnormal Ca$^{2+}$ cycling.

We have documented that dietary fish oil prevents AF inducibility after cardiac surgery, reduces inflammatory markers, and decreases ET-1 protein expression in plasma, PVs and LAA. Our experimental diet shifted the $\omega$-3-HUFA content of the atria from a level similar to that of individuals regularly consuming an American diet to one close to that of individuals consuming a Mediterranean diet. Thus, in addition to the possible use of ET-1 antagonists, dietary $\omega$-3-PUFA supplementation may be a useful therapeutic intervention with the potential to lower the incidence of POAF, as well as that of AF resulting from other wall stress or ischemia related disorders (e.g., pulmonary HTN, HTN and HF).

AF imposes a metabolic and hemodynamic strain on the atria. Increased hemodynamic burden (as a result of hypertension, mitral regurgitation or heart failure) promotes atrial enlargement and fibrosis -- a critical element of a substrate for AF. Left atrial enlargement is associated with poor clinical outcomes. Previous studies showed that ET-1 is increased in the plasma of AF patients with underlying structural heart disease. In this study, we provide new evidence documenting that both ET-1 gene expression and
processing are enhanced in the LAA of AF patients, and that increased ET-1 content is associated with increased left atrial size and volume. More broadly, atrial ET-1 content is increased in conditions associated with increased left atrial hemodynamic burden such as HTN, HF and MVD. Importantly, the increment in ET-1 expression in a canine HF model induced by ventricular tachypacing paralleled the increase in atrial pressure. This further supports the concept that hemodynamic load is a primary modulator of atrial ET-1 expression.

In human studies, we showed that ET-1 levels are associated with AF persistence. Atrial ET-1 mRNA levels are correlated with the mRNA expression of fibrotic mediators (PDGF, CTGF) as well as with atrial BNP mRNA expression (a predictor of AF). These associations suggest that ET-1 promotes atrial enlargement and fibrosis and contributes to increased AF persistence.

Genome-wide association studies have yielded a growing number of genetic markers associated with AF. The SNP rs2200733, located on chromosome 4q25, has been strongly associated with risk of AF in both European and Chinese populations. As this is an intergenic region, the mechanism(s) linking this region with AF risk are unclear.

In a cohort of lone AF patients of known genotype, I tested the hypothesis that plasma ET-1 is associated with rs2200733 SNP genotype and that lone AF patients with the risk allele have higher plasma ET-1 levels. I found that plasma ET-1 is strongly associated with the minor risk allele of the rs220733 SNP in lone AF patients. Having one or two copies of the rs2200733 risk allele was associated with a 47% and 116% increase in plasma ET-1, respectively. Plasma ET-1 was also associated with age, increased RV pressure (a precursor of pulmonary hypertension) and left atrial enlargement - all
important and well known risk factors for AF. This study provides novel insights regarding the pathophysiological significance of one of the SNPs most strongly linked to AF, yet which is located in a non-coding region.

The association of the rs2200733 SNP with circulating ET-1 levels suggests the intriguing possibility that interventions which attenuate ET-1 synthesis, and/or the use of ET-1 antagonists may improve outcomes for AF patients with this genotype.

Collectively, our studies reveal a strong relation of ET-1 to the creation of an AF substrate.

*It would be of great interest to determine if agents or procedures that reduce ET-1 production or block its receptors have a therapeutic benefit with respect to prevention of human AF, or slowing its progression.*
6.3. Future Directions

In the few decades since its discovery, it has become apparent that ET-1 is not merely a vasoconstrictor peptide, but that it is also a multifunctional factor that can affect many aspects of cardiac function. Efforts to better understand the molecular basis of ET-1 signaling in human heart are still needed. Although clinical studies have been conducted to evaluate the benefits of ET-1 antagonists in HF and PHTN, additional efforts that are focused on AF are warranted.

While my study has provided new insights into the role of ET-1 in the atria, several questions have arisen that will need to be addressed in future studies:

1. We have found that ET-1 protein is increased in canine cardiac surgery and HF models, and in AF patients with structural heart disease. It is of interest to assess plasma and atrial ET-1 content in post-surgical and lone AF patients, to evaluate the extent to which the ET-1 system is involved in the development of AF in the absence of structural heart disease. Both plasma and atrial tissues should be assessed in the same individual, when possible, in order to better understand the relation and source of plasma and myocardial ET-1.

2. Functional studies on human tissues and in relevant experimental models are required to define the molecular mechanisms of ET-1 actions on the atria and ligand-receptor interaction relationship. The observation that endothelin receptors are downregulated after surgery in our canine cardiac surgery model requires pharmacodynamic studies to
determine the significance of this regulation. Downregulation of ET-1 receptors may be mediated by the ligand as adaptive mechanism to attenuate signaling. Time course and dose-titration experiments must be performed to test if ET-1 regulates receptor expression in a time- or concentration-dependent manner. In contrast, receptor downregulation might enhance signaling and may be modulated independent of ET-1, as suggested by our human AF data.

3. Detailed functional studies are warranted to examine the cellular impact of ET-1 on IP3 signaling, Ca\textsuperscript{2+} transients and triggers in both atrial and ventricular myocytes. Simultaneous cellular electrophysiology and Ca\textsuperscript{2+} fluorescence imaging studies will be required to assess and compare the impact of ET-1 on the amplitude and kinetics of atrial and ventricular Ca\textsuperscript{2+} transients and currents. In ventricular myocytes, ET-1 promotes intracellular and perinuclear Ca\textsuperscript{2+} release through IP3Rs. This serves as an additional pathway to enhance fibrosis and hypertrophy independent of the PKC/MAPK pathways. The role of ET-1 in promoting perinuclear Ca\textsuperscript{2+} release and fibrosis in atrial myocytes also needs to be explored.

4. The current studies have revealed the presence of regional differences in ET-1 protein in different chambers and regions of the heart. These studies also suggest that ET-1 signaling is enhanced in normal and failing atria and PVs, relative to the ventricles. It would be of interest to compare regional gene expression (mRNA), protein expression (immunohistochemistry) and functional studies (as in #3, above) in available donor and transplant hearts. These coordinated studies will help to assess the significance of ET-1-
signaling pathways on both electrical and structural properties of cardiac myocytes. Higher atrial ET-1 may influence receptor expression, and the threshold at which atrial myocytes respond to ET-1 or other stimuli. Such studies might lead to the discovery of agents that can selectively treat atrial arrhythmia. The specific electrophysiologic role of ET-1 signaling in atrial myocytes of the PVs needs to be tested. ET-1 may initiate ectopic triggers or may promote reentries in the PVs and surrounding atrial areas.

5. The precise mechanisms by which ET-1 promotes fibrosis in human atria need to be further explored. We showed that the PDGF and the CTGF mRNA are associated with ET-1 mRNA expression in human LAA. Functional and interventional studies would be helpful to evaluate functional relation of ET-1 to the PDGF and the TGF-β pathways. Both pathways are potential mediators of ET-1 induced atrial fibrosis. Cell culture and biochemistry studies are helpful to evaluate the cellular mechanisms in vitro on isolated myocytes and fibroblasts. However, interventional studies using in vivo models of AF are more physiologic. Our human and canine studies have defined an association of ET-1 with AF rhythm and LA enlargement/fibrosis, however, it is unknown whether there is an ET-1 threshold that results in AF-fibrosis relationship, versus an effect on atrial ectopy. ET-1 may acutely promote atrial ectopy, but persistent elevation may be needed to promote fibrosis.

6. Well-designed experimental and clinical studies are warranted to evaluate and verify the therapeutic potentials of ET-1 antagonists as a new class of medications to treat AF. It seems likely that these drugs may be particularly useful for human AF in the setting of
concomitant cardiovascular disease. To test this hypothesis, extensive biomarker, electrophysiologic and pharmacogenetic studies are needed in patients with AF and concomitant cardiovascular conditions, in order to identify which patients are most likely to benefit from ET antagonists.

7. Changes in plasma and atrial lipid composition in response to dietary lipid supplements are time-and dose-dependent. Our studies demonstrate that large doses of fish oil were needed to change myocardial lipid content and confer protection against AF inducibility. The doses used were greater than those typically consumed. Additional studies are needed to characterize the relationship between plasma and tissue levels, in order to predict the minimum dose needed to achieve clinical benefit. Well controlled clinical and experimental studies with sufficient dose and pre-treatment duration are needed to assess potential therapeutic role of ω3-PUFA supplements to prevent human AF following cardiac surgery. Similarly, additional studies are needed define the threshold ω6/ω3 ratio needed to impact atrial arrhythmogenesis.

8. The mechanisms by which rs220733 SNP modulates plasma ET-1 expression needs to be further investigated. Studies are warranted in order to replicate our findings in a lone AF cohort and in AF patients with underlying cardiac disease. Collection of both atrial and plasma tissues from same patients would be extremely helpful to understand the relation and the source of plasma ET-1. The relation of changes in ET-1 to changes in Ang-II should be explored. Similarly, additional studies are needed to carefully explore the role of PITX2, ENPEP and/or microRNAs that may mediate the effects of SNPs in
this region. Genotype-phenotype studies are helpful to gain broad understanding of the molecular mechanisms by which the rs220733 SNP modulates ET-1 expression and increases AF risk.
Appendix 1: Subcellular localization of canonical transient receptor potential (TRPC1, 3, 6; green) in isolated canine right atrial myocytes (RA) and right ventricular myocytes (RV). Sections were also stained with mouse anti α-actinin (red) to stain for cardiac myocytes and dapi to stain nuclei (blue).
Appendix 2: Subcellular localization of ET receptor type A and B (ETAR & ETBR, green), inositol triphosphate receptor type I and II (IP3R-I & IP3R-II, green) in isolated canine right atrial myocytes. Sections were also stained with mouse anti α-actinin (red) to stain for cardiac myocytes and dapi to stain nuclei (blue).
Appendix 3: Distribution of endothelin receptors type A (ETAR, A), type B (ETBR, B) and IP3 receptors type I (IP3R-I, C) and type II (IP3R-II, D) in atrial myocytes and fibroblasts. Sections were stained with vimentin (purple) to stain fibroblasts and α-actinin (red) to stain cardiomyocytes, and DAPI to stain nuclei (blue). Note that both ET-1 receptors and IP3Rs are expressed in the cytosol and nuclei of atrial fibroblasts.
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