

USE OF MONOAMINE OXIDASE AND REDOX ENZYMES
IN ATRIAL TISSUE AS NOVEL PREDICTORS OF
POSTOPERATIVE ATRIAL FIBRILLATION

by

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Postoperative atrial fibrillation (POAF) occurs in approximately 30% of cardiac surgery patients. The complication occurs despite advances in surgical procedures. It is associated with increased mortality, morbidity, and healthcare cost. The exact pathogenesis of this complication is unknown, but oxidative stress and inflammation are considered to be significant factors.

A precisely controlled balance between reactive oxygen species (ROS) generation and ROS scavenging influence the oxidative environment within cells and tissues. Increased activity of monoamine oxidase (MAO) activity, a ROS generating enzyme, can produce a more oxidative environment. A decrease in glutathione (GSH), a ROS scavenging molecule, can also yield an oxidative environment. The enzymes GSH-peroxidase (GPx) and GSH-reductase (GR) are responsible for maintaining the cellular redox environment within a range that is compatible with favorable homeostasis. Despite the well-characterized role that these enzymes play in

maintaining redox environment, a comprehensive evaluation of these enzymes in human myocardium has never been attempted.

Furthermore, since reports have recently documented the association between oxidative stress in atrial tissue and the incidence of POAF, we tested the hypothesis that these enzymes are associated with POAF, in a cohort of patients undergoing cardiac surgery at Vidant Medical Center, East Carolina Heart Institute (ECHI).

Human right atrial appendage tissue were obtained from 244 patients undergoing elective coronary artery bypass graft surgery at ECHI between January, 2010, and December, 2012. The generation of ROS were determined using assays on MAO, NADPH oxidase (NOX), along with the activity of glutathione reductase (GR) and glutathione peroxidase (GPx). Patient outcomes, including POAF development, were analyzed in relation to the assays performed. A statistical model was then created to measure the association for risk of POAF development.

This was the first study to determine that MAO activity is a major source of ROS in human atrial tissue. MAO activity is significantly associated with POAF. Total glutathione (GSHt) activity is inversely related to POAF development. GPx is also significantly associated with POAF as well, but the trend is not a linear. GR activity is not correlated with POAF.

MAO, GSHt and GPx are enzymes that contribute to the atrial oxidative environment and increased risk of POAF development. Because this is a pilot study, further exploration is needed to validate our study and to determine if and where these enzymes fit in the etiology of POAF.

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List of Abbreviations:

ACEI	Angiotensin Converting Enzyme Inhibitor
AF	Atrial Fibrillation
ARB	Angiotensin Receptor Blocker
BMI	Body Mass Index
CABG	Coronary Artery Bypass Graft
CAD	Coronary Artery Disease
CI	Confidence Interval
COPD	Chronic Obstructive Pulmonary Disease
CPB	Cardiopulmonary bypass
CPBT	Cardiopulmonary bypass time
CRP	C-reactive protein
FAD	Flavin Adenine Dinucleotide
GSSG	Oxidized Glutathione
GPx	Glutathione Peroxidase
GR	Glutathione Reductase
GSH	Reduced Glutathione

GSHt	Total Glutathione
HF	Heart failure
LOS	Length of stay
MAO	Monoamine oxidase
MI	Myocardial infarction
NOS	Nitric oxide synthase
NOX	NADPH oxidase
PmFB	Permeabilized myofibers
POAF	Postoperative atrial fibrillation
POSR	Postoperative sinus rhythm
PUFA	N-3 polyunsaturated fatty acids
Q1	First quartile
Q2	Second quartile
Q3	Third quartile
Q4	Fourth quartile
RAA	Right Atrial Appendage
Ref	Referent

ROS Reactive Oxygen Species

RR Relative risk

SD Standard deviation

SOD Superoxide dismutase

TNF- α Tumor Necrosis Factor- α

XO Xanthine oxidase

1. INTRODUCTION

Heart disease is the leading cause of death in the United States. Approximately 11% of the adult population in the U.S. will develop this disease, and this rate rises to 30.5% of patients over the age of 65. It accounted for approximately 307,000 deaths in men and 290,000 deaths in women in 2010. More specifically, it was determined that coronary heart disease is the main cause of approximately 1 in 6 deaths in the United States in 2008.¹

Yet despite the pronounced increase in heart disease in recent years, modern medicine has been able to keep pace and has made tremendous strides as well. In particular, advancements in cardiac surgery have allowed patients with heart disease to live far longer, with far better quality of life, than ever before. A recent assessment has documented that cardiac surgery was the most commonly performed surgery in the United States in 2010. Approximately 15% of all surgical procedures that year were conducted on the cardiovascular system, accounting for almost 20% of all surgeries on patients over the age of 45.² Cardiac catheterization was the most commonly-performed cardiac surgery, followed by angioplasty, coronary artery stent insertion, and coronary artery bypass graft (CABG) surgery.² CABG surgery is the second-most expensive type of surgery for all patients over 44 years of age.³ This surgery is recommended in patients with severe or unstable angina, ST-segment elevation myocardial infarction, and non-ST-segment elevation myocardial infarction, among other symptoms.⁴

Although striking improvements in cardiac surgery have indeed changed the outcome of millions of individuals afflicted with heart disease, there are many adverse

consequences that have come along with those improvements. Along with the high financial costs of the surgery itself, there are many postoperative complications associated with cardiac surgery, including respiratory failure, renal failure, and gastrointestinal failure.⁵ However, the most common complication is postoperative atrial fibrillation (POAF), occurring in 30% to 60% of all patients undergoing cardiac surgery worldwide.⁶ It is important to emphasize that even in non-cardiac surgical procedures, POAF is a common complication as well, although more variable and generally at a lower rate of incidence (0.3% - 29%).⁷

The development of POAF can result in increased mortality, morbidity, and hospital cost. Patients with POAF have an increased number of postoperative days in the hospital (5.8 vs. 4.4) and ICU (1.5 vs. 1.2) compared to patients who stayed in sinus rhythm. There is also a higher readmission to the ICU (11% vs. 1%) and number of laboratory tests ordered (53.7 vs. 38.6). Upon discharge, physicians sent fewer POAF patients to their home (29.4% vs. 34.6%) and more to a post-hospital care facility (19.7% vs. 13.1%). In total, a patient with POAF has additional hospital charges that approached \$12,000 in 2009.⁸ With almost 400,000 people having CABGs in 2010, it can be extrapolated that POAF adds an additional \$1.4- to \$2.9-billion in extra hospital charges, each year.²

POAF is closely related to atrial fibrillation (AF), which is the most common arrhythmia found in clinical practice.⁹ AF is characterized as a disorder in which the atria do not contract in a synchronized process. Instead it quivers, leading to hemostasis in the atrial compartment, ultimately putting the patient at increased risk of thromboembolism and stroke. It was first described by Garrey¹⁰ as an “incoordinated

[sic], disorderly and extremely bizarre contractile process in which normal systole and diastole no longer occur". He continued by stating that it is a "group of fibers [that] are contracting independently".¹⁰ Garrey hypothesized the pathogenesis as coming from a "subacute or chronic inflammatory change leading to fibrosis."¹⁰ Cohnheim concluded a "metabolic poison" was the link between the lag of fibrillation after ligation of a coronary artery, but the actual mechanism of action was unknown.¹⁰ The etiology of AF has evolved since its first depiction, but some characteristics have remained. Patients with a history of AF have a higher incidence of POAF.

There are myriad factors that contribute to the development of POAF. At a macro level, these factors can be either acute or chronic. Acute factors occur during a time period immediately surrounding the surgery, while chronic factors are typically considered to be events and clinical conditions that lead up to surgery. Age and race are well-documented set of non-modifiable risk factors. Invariably, studies have repeatedly shown that the patient's age is the strongest predictor of POAF. In one study, almost 20% of all thoracic surgical patients ≥ 60 years old developed POAF, while only 7% of the patients < 60 years old developed it.¹¹ Caucasian race is another independent predictor of POAF.^{12,13} Other chronic factors, such as structural heart disease, congestive heart failure, and hypertension, are known variables that contribute to atrial remodeling. Atrial remodeling is a diffuse term that describes a persistent change in the structure or function of the atria at the anatomic and/or physiological level. These factors all play a part in increasing the susceptibility for a patient to develop POAF.

Of the acute physiological factors that are associated with POAF, inflammation and oxidative stress are the two factors that have been studied the most, and are the

factors generally considered to be the most significant. One widely-held theory is that the use of tubing and instrumentation of the atria, such as occurs with a cardiopulmonary bypass (CPB) machine or aortic cross-clamp, increases systemic inflammation and myocardial oxidative stress, thereby contributing to the probability of atrial injury and POAF.^{14, 15} Reperfusion that occurs after CBP causes the generation of reactive oxygen species (ROS) in the myocardium. The increased oxidative stress is thought to interfere with the electrical conduction pathways within the atria. The “metabolic poison,” associated with oxidative stress and inflammation first mentioned by Cohnheim, is now thought to acutely contribute to the etiology of POAF. Regardless of the specific mechanism, the collective effect of inflammation and oxidative stress associated with cardiac surgery has a pronounced impact on the patient in the immediate post-operative period (1-5 days). Other acute risks are the endogenous pro- and anti-inflammatory molecules in circulation. Their precise role in POAF is poorly understood. The flux in the concentration of these molecules can come from anesthesia or trauma acquired from the surgical procedure. There is a positive correlation between an increase in proinflammatory cytokines and morbidity after cardiac surgery.¹⁶

1.1. *Inflammation and POAF*

Cardiac surgery provokes a powerful inflammatory response that can often lead to adverse consequences to the patient. Inflammation is the body’s response to the disruption within the tissues, involving a series of controlled humoral and cellular reactions which elicit pro-inflammatory responses within the body. Systemic inflammation is typically confirmed in the clinical setting by assessing the increase of circulating C-reactive protein (CRP), which indicates complement activation.

The intensity of atrial inflammation is correlated to the invasiveness of the surgery. Heart valve surgery has the highest incidence, followed by CABG and lastly non-cardiac surgery. Atrial inflammation, as determined by myeloperoxidase activity and neutrophil infiltration, is associated with an increase in the incidence and duration of AF. Some studies found that postoperative white blood cell counts were increased in POAF patients. Also CRP levels increase postoperatively until it peaks at postoperative day two. This time course matches the same pattern displayed by the majority of cardiac surgery patients developing POAF. However, although a generalized atrial tissue inflammation is known to be linked to POAF, there is not a single pro-inflammatory marker that correlates with POAF independent of other known risk factors. In some studies, however, white blood cell count has been shown to be an independent predictive marker for POAF development.^{17, 18}

One of the greatest known triggers of inflammatory responses is CPB. The use of the CPB machine causes two-step complement activation. During CPB intervention, the ‘alternative pathway’ becomes activated via Tumor necrosis factor- α (TNF- α) due to the blood mixing with the machine. The second phase of activation is via the ‘classical pathway’ which is initiated by the anti-coagulant protamine sulfate. POAF development usually overlaps with activation of the ‘classical pathway’. Retrospective epidemiological studies have documented that anti-inflammatory drugs are effective in lowering POAF incidence.⁷ Some classes of drugs, such as steroids and HMG-CoA reductase inhibitors, can inhibit tissue inflammation associated with surgery. This inhibition results in decreased CRP concentration and AF duration. Other studies have

demonstrated that drugs with anti-inflammatory effects are effective at lowering the incidence of POAF by the inhibition of cytokine release.

CRP is closely related to systemic inflammation and the development of AF.¹⁹ The peak concentration of CRP occurs on the second or third postoperative day. That timing also correlates with the highest frequency of POAF.²⁰ Overall, the protein is significantly increased in atrial arrhythmia patients, compared to patients who stay in sinus rhythm.²¹ Yet despite its tight correlation with POAF, CRP isn't a good diagnostic molecule in predicting POAF.²¹ TNF- α is another cytokine strongly associated with POAF. Increased levels of TNF- α are associated with severe leukocyte infiltration and increased fibrosis compared to patients who stay in sinus rhythm. Increased levels are also found in patients with persistent AF compared to paroxysmal AF.²² The chance of a patient developing POAF has been significantly associated with increased leucocyte levels, an inflammatory marker.²³ Interleukins are a subgroup of cytokines that are also related to POAF development. Low levels of interleukin-2 (IL-2) are associated with a decreased incidence of POAF.²² Interleukin-6 (IL-6) is associated with both pro- and anti-inflammatory functions. Among other tasks, it helps in the synthesis of CRP, and mediates the proliferation of TNF- α . High levels of serum IL-6 are one of the best predictors associated with POAF and AF.^{21,22} Interleukin-8 (IL-8) helps with the recruitment of neutrophils, and pro-thrombotic and inflammatory states. Higher concentrations of IL-8 are found in patients with permanent AF, compared to paroxysmal AF.²²

1.2. *Oxidative Stress and POAF*

Oxidative stress occurs when ROS generation in a cell or tissue overwhelms the ROS scavenging capacity leading to a disruption in homeostasis.²⁴ When an oxygen radical reacts with a non-radical, it can often initiate a chain reaction, or vicious cycle that results in the production of more free radicals. Because of the high reactivity of oxygen radicals, persistent oxidative stress can lead to damage to biological systems. Current literature has documented a role for oxidative stress in numerous chronic human diseases, particularly ischemic and inflammatory diseases of the cardiovascular system.²⁵ Specifically, the increased production of atrial ROS are associated with atrial remodeling and a greater probability of AF development.²⁶

With regard to POAF, the use of CPB has been suggested to be a causal factor in ROS generation during cardiac surgery due to complement activation. This systemic inflammation caused by CPB is a factor in POAF development. There is a local inflammation from the actual incision that increases the incidence of POAF. However, current literature has failed to show consistent evidence of an association between POAF and CPB.⁷ Since the current data are retrospective, and have a limited sample size, further exploration is needed to establish what specific role CPB has in myocardial or system ROS generation, if any.²¹

1.2.1. *Sources of ROS related to POAF*

Free radicals are generated from numerous biochemical processes, including the reduction of molecular oxygen during aerobic respiration and the oxidation of catecholamines, thereby resulting in the incomplete reduction of molecular oxygen to

form superoxide.²⁴ A physiologically normal function of free radicals is the capability of signaling and communicating. Abnormally generated free radicals are centered on many diseases.²⁵ Major sources of free radicals in cells and tissues include the mitochondria, xanthine oxidase (XO), NADPH oxidase (NOX), and nitric oxide synthase (NOS).²⁷

Within the mitochondria, molecular oxygen is reduced to water in order to generate energy in the form of ATP. The energy-producing process is not perfect and <5% of the molecular oxygen is incompletely reduced. This sub-species of oxygen, known as a superoxide radical, is thought to be the main source of ROS during ischemia and reperfusion, and has been suggested to be a source of ROS in the myocardium during the ‘warm-up’ period of reperfusion following cardioplegic arrest.²⁷

The membrane-bound enzyme NOX has been shown to be an important source of ROS in cardiomyocytes. Atrial NOX activity has been reported to be an independent predictor of POAF.²⁸ Iravanian and Dudley hypothesized perioperative inflammation stimulated NOX within the atria, lead to a surge in oxidative stress that subsequently triggered an arrhythmia. Both NOX gene expression and protein level are up-regulated in patients with POAF compared to patients who stay in sinus rhythm.²⁹ Current evidence shows inhibitors of NOX could provide a method to reduce or prevent the development of POAF.³⁰

Another significant generator of ROS is XO.²⁷ During an ischemic event, the concentration of hypoxanthine is increased and this enzyme is used to oxidize hypoxanthine to xanthine.²⁷ Gene expression has been found to be up-regulated in patients with POAF compared to patients who stay in sinus rhythm.²⁹ Although this

enzyme system has been implicated as a source of ROS in numerous cardiovascular diseases, at present no connection between this enzyme and the development of an arrhythmia has been established in either experimental or clinical studies.

Different isoforms of NOS are well known to be expressed in cardiac tissue. NOS1 and 3 are found under homeostatic conditions, while NOS2 is induced under pathological conditions. During heart failure, NOS has been shown to become uncoupled and produce superoxide radicals.³¹ There are also both preclinical and clinical data suggesting NOS signaling has a role in the pathogenesis of AF.²⁸

Another source of ROS in cardiomyocytes that has been largely ignored until recent years is the enzyme monoamine oxidase (MAO). This enzyme is tethered to the outer mitochondrial membrane, and is responsible for oxidative metabolism of catecholamines, in the process generating an oxidized catechol aldehyde, ammonia, and a hydrogen peroxide molecule. Even in experimental models, there is a very limited amount of literature on MAO in regards to the heart. One group found the isozyme MAO-B is significantly up-regulated in patients with persistent AF, compared to sinus rhythm patients.²⁹ Investigation and characterization of this source of ROS in human atrial myocardium was part of the rationale for this thesis project.

1.2.2. *Antioxidants and scavengers of ROS*

Antioxidant molecules and ROS-scavenging enzyme systems are designed to scavenge or reduce oxidant molecules (e.g., oxygen radicals, peroxides) before they are able to damage vital components in the cell.²⁴ Antioxidant enzymes become up-regulated when the cell is exposed to a certain ROS threshold.²⁷ During healthy homeostasis,

endogenous antioxidant systems are able to match the generated ROS. However, a pathological event, such as ischemia-reperfusion, elicits a surge of ROS that develops over a short period of time.²⁷ If ROS overwhelms the endogenous antioxidant system, tissue damage, atrial remodeling and electro-mechanical disruption could occur.³² The clinical use of antioxidant therapy has repeatedly established antiarrhythmic benefit in humans.⁷

1.2.2.1. *Enzymatic ROS scavenging*

Catalase is an enzyme responsible for reducing H_2O_2 to H_2O and O_2 . This enzyme, when overexpressed in heart, has been previously shown to increase the lifespan in mice due to its positive effects on cardiac aging.³³ However, the gene expression of this enzyme is down-regulated in patients with persistent AF, compared to patients who stay in sinus rhythm.²⁹

Superoxide dismutase (SOD) is an antioxidant that catalyzes the dismutation of superoxide into oxygen and hydrogen peroxide. It is the primary enzyme responsible for the breakdown of

superoxide.²⁷ The gene expression of this enzyme has also been shown to be down-regulated in AF patients compared to patients who stay in sinus

rhythm.²⁹ When animal models are given SOD during ischemia-reperfusion, the infarct

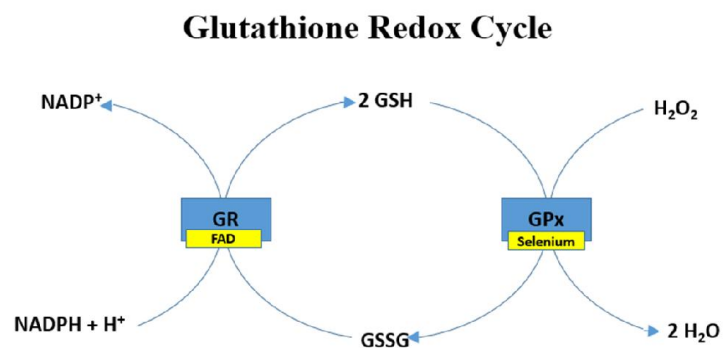


Figure 1.1 A simplified scheme of the redox cycle involving GSH and related enzymes GPx and GR

size is significantly decreased. Despite showing promise in experimental models, clinical trials have not matched these results.²⁷

Glutathione is the main intracellular redox antioxidant.³⁴ The ratio of oxidized (GSSG) to reduced glutathione (GSH) in patients with AF development shows a positive association.³⁵ The GSH/GSSG ratio is decreased by 50-70% in patients with ischemia, heart failure, and type II diabetes. Those morbidities are all associated with arrhythmias.³⁶ GSH is oxidized to GSSG by GPx, with the help of the cofactor selenium. The gene expression of GPx has been shown to be down-regulated in AF patients compared to patients who stay in sinus rhythm.²⁹ Glutathione Reductase (GR) reduces GSSG back to GSH, with the cofactor flavin adenine dinucleotide (FAD) (Figure 1). This antioxidant enzyme is also downregulated in AF patients compared to those in sinus rhythm.²⁹

1.2.2.2. *Non-enzymatic ROS scavenging*

Many vitamins are other antioxidants utilized by the body. A study of rapid atrial pacing in a canine model reported that vitamin C decreased superoxide formation in heart. The same study found that it reduced the incidence of POAF in humans.⁷ Vitamin E, a lipid-soluble antioxidant, has also been shown to reduce the development of ischemia/reperfusion-induced arrhythmia. N-3 polyunsaturated fatty acids (PUFA) are also being studied for their ability to quell the imbalance of oxidative stress, and thus reduce POAF incidence. A recent study determined a combination of PUFA and vitamins C and E increases the antioxidant capacity, thus decreasing POAF incidence. It does this by inducing lower ROS production levels compared to the control group.³⁷

1.3. *Hypothesis*

Since Cohnheim first postulated a “metabolic poison” as a cause of AF, many studies have been performed to investigate the relationship between oxidative stress and the development of POAF.¹⁰ The exact pathophysiological events that occur during POAF are still unknown. This thesis seeks to address the hypothesis that MAO activity with other redox molecules from the right atrial appendage can be used as predictive biomarkers of POAF risk.

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2. METHODS

2.1. *Patient enrollment and inclusion/exclusion criteria*

Approval for this study was granted by the Institutional Review Board of the Brody School of Medicine at East Carolina University. A total of 244 patients undergoing primary, non-emergent CABG or CABG/valve surgery between January 2009 and December 2012 were enrolled. Patients with severely enlarged atria (>4.0 cm diameter), a history of arrhythmias, prior cardiac surgery, a left ventricular ejection fraction (LVEF) < 30% and/or history of anti-arrhythmic medication were excluded from this study.

2.2. *Atrial tissue collection and processing*

Following median sternotomy, but prior to institution of cardiopulmonary bypass, a sample of the right atrial appendage (RAA) was resected and immediately rinsed in ice-cold Buffer X.¹ The sample was then blotted on gauze to remove excess buffer, trimmed of the epicardial layer, and frozen in liquid N₂. This method ensured that all samples obtained were predominantly myocardium and rapidly processed and frozen (< 90 seconds from time of removal) to minimize protein and mRNA degradation. In some cases viable atrial myocardium was transferred to the laboratory and used for preparation of permeabilized myofibers (PmFBs) and analysis of mitochondrial function.

2.3. *Permeabilized fiber preparation*

Portions of this technique have been described elsewhere,¹ but have been adapted for application in human cardiac muscle and for specific measurements made in this

study. After RAA tissue harvest, myocardium was removed and placed in ice-cold Buffer X, containing (in mM: 7.23 K₂EGTA, 2.77 CaK₂EGTA, 20 Imidazole, 20 Taurine, 5.7 ATP, 14.3 PCr, 6.56 MgCl₂·6H₂O, 50 MES; pH 7.1). Muscle was then cut into strips ~4-6 mm L x 2-3 mm wide and placed in a solution of Buffer X containing 3 mg/ml collagenase Type I (Sigma-Aldrich, St. Louis, MO), and incubated for 30-45 minutes at 4°C. Fiber bundles were then carefully trimmed of vascular and connective tissue, separated along their longitudinal axis, and permeabilized for 30 minutes in Buffer X + 50 µg/ml saponin at 4°C. We used 30 µg/ml saponin in female patients, for reasons described elsewhere.² Following permeabilization, PmFBs were washed in ice-cold Buffer Z containing (in mM): 110 K-MES, 35 KCl, 1 EGTA, 5 K₂HPO₂, 3 MgCl₂·6H₂O, and 5 mg/ml BSA (pH 7.4, 295 mOsm) and remained in Buffer Z on a rotator at 4°C until analysis (<2 hours). We have observed that PmFBs exhibit a very strong Ca²⁺-independent contraction that is temperature sensitive and can occur even at 4°C,²⁰ therefore, 20 µM Blebbistatin (Sigma-Aldrich, St. Louis, MO) was added to the wash buffer and the respiration medium during experiments to prevent contraction as previously described.

2.4. *Measurement of mitochondrial H₂O₂ emission in cardiac PmFBs*

All mitochondrial H₂O₂ measurements were performed at 37°C. Hydrogen peroxide coming from mito-ETS as a result of palmitoyl-L-carnitine, glutamate and succinate oxidation was determined in PmFB's with 100 µM ADP, 5 mM glucose and 1 U/ml hexokinase present to keep the mitochondria in a permanent, submaximal phosphorylating state (i.e. most physiological).^{3,4} Hydrogen peroxide emission rate was determined in real time by continuous monitoring of Amplex Red oxidation in the

presence of horseradish peroxidase (1 U/ml) and SOD (25 U/ml) using a spectrofluorometer (Photon Technology Instruments, Birmingham, NJ,) equipped with a thermo-jacketed cuvette chamber.

2.5. *MAO and NOX activity*

Myocardial samples frozen in liquid N₂ were homogenized in 10X (wt./vol) TEE buffer containing (in mM: 10 Tris base, 1 EDTA, 1 EGTA), and 0.5% Tween-20, using a glass grinder (Kimble Chase, Vineland, NJ). All enzyme activity and glutathione assays were performed on the same day as the protein extraction. We have empirically determined that glutathione and enzyme activity must be assessed immediately in protein extractions to obtain accurate results, and that freezing samples or keeping them at 4°C overnight will cause dramatic loss of content and activity. Hydrogen peroxide generation from MAO and NOX was determined in real time by continuous monitoring of Amplex Red oxidation in presence of horseradish peroxidase (1 U/ml) and SOD (25 U/ml) using a spectrofluorometer (Horiba Jobin Yvon, Ann Arbor, MI) equipped with a thermo-jacketed cuvette chamber maintained at 37°C. MAO activity was determined by continuous monitoring of clorgyline-sensitive H₂O₂ production supported by 1 mM Tyramine or 2 μM Norepinephrine as previously described.⁵ NOX activity was determined by continuous monitoring of apocynin-sensitive H₂O₂ production supported by 0.5 mM NADPH.⁶

2.6. *GSHt, GPx and GR activity*

All enzyme activity and glutathione assays were performed on the same day as the protein extraction. Total glutathione measurements were performed as described

previously^{7,8} using a modified Tietze method.⁹ GR activity in myocardial tissue was measured in TEE buffer containing 1 mM GSSG and 0.5 mM NADPH, where activity was calculated from the linear decrease in NADPH absorbance with time.¹⁰ GPx activity was determined in TEE buffer containing 1 mM GSH, 100 mU/ml GR enzyme, and 0.5 mM NADPH. The reaction is initiated with a nominal amount of tert-Butyl-Hydroperoxide and the activity of GPx was calculated from the linear decrease in NADPH absorbance with time.¹¹

2.7. *Determination of POAF*

Postoperatively, patients' heart rates and rhythms were continuously monitored with telemetry until discharge. POAF was defined by a sustained episode of AF lasting ≥ 1 minute, or for any length of time requiring intervention for hemodynamic compromise.

2.8. *Statistical Analysis*

Categorical variables were reported as frequency and percentage while continuous variables were reported as mean \pm standard deviation, median, and interquartile range. Variables not previously categorized were divided into quartiles prior to statistical analysis. Quartile categorization is advantageous because it limits the influence of outliers and allows for the assessment of trends across categories.

Statistical significance of group comparisons for categorical variables was determined using Fisher's Exact and chi-square (χ^2) procedures and for continuous variables was determined using the Deuchler-Wilcoxon method. Relative risk and 95% confidence intervals were computed using log-binomial or robust Poisson regression.

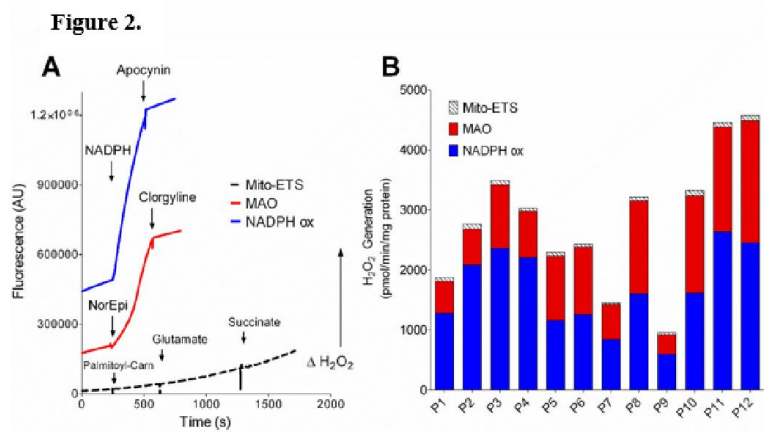
P-values for trends were computed using a likelihood ratio test (or score test when convergence was not achieved). Assays were performed using a missing by design sampling strategy. The iterative expectation-maximization (EM) algorithm was used to impute missing values.¹²⁻¹⁴ The relative imputation efficiency ranged from 96% to 99% (Variance inflation: MAO=0.38, GSht=0.02, GPx=0.15, GR=0.54; Fraction missing information: MAO=0.29, GSht=0.02, GPx= 0.14, GR=0.37). Patients with and without missing data did not differ by key demographic characteristics (i.e., age, sex, race; Hochberg adjusted $p > 0.05$).¹⁵ Furthermore, a complete-case analysis was performed and it did not substantively change the results of the study. The multivariable models included variables that have been previously reported to be associated with POAF, regardless of their statistical significance in our dataset. These included age, sex, race, diabetes, hypertension, ACEI/ARB use, statin use, and CPBT.¹⁶⁻²⁰ Statistical significance was defined as $p < 0.05$. SAS Version 9.3 (Cary, NC) was used for all analyses.

3. RESULTS

3.1. *Analysis of major ROS sources in human atrial myocardium*

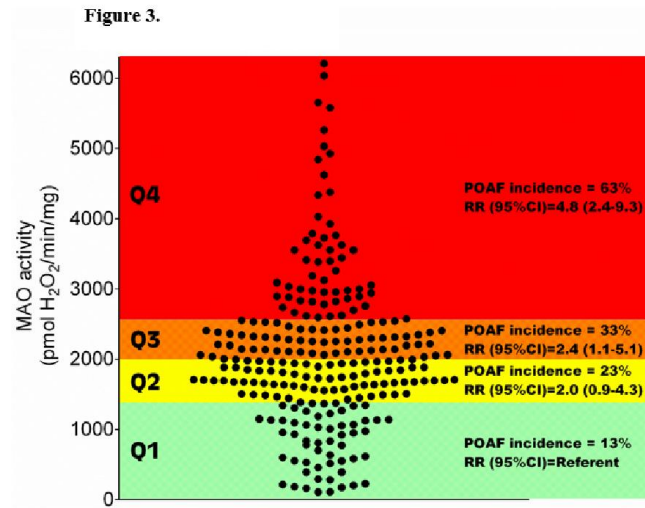
An assessment of three major ROS sources in atrial myocardium was performed from RAA biopsies of 12 individual patients (demographic and

clinical characteristics of these 12 patients are provided in Table 1). Rates of H₂O₂ production in the myocardial tissue homogenate was confirmed to be derived from MAO and NOX based on the sensitivity to their inhibitors clorgyline and apocynin, respectively (Figure 2A). Hydrogen peroxide production derived from the mito-ETS was driven by oxidation of substrates as they were individually titrated into the respiration medium containing the PmFBs (Figure 2A). Total rates of H₂O₂ production from these three sources were individually quantified and combined within each of the 12 patients (Figure 2B). The rate of H₂O₂ originating from mito-ETS was determined to be at least 10-fold lower than either MAO or NOX alone. As previously reported by our group, diabetic patients had significantly higher rates of H₂O₂ from mito-ETS compared with non-diabetic patients.^{3,4}



3.2. *Patient characteristics, biochemical markers, and relationship to POAF*

A total of 80 (33%) patients developed POAF. Patients with POAF were older and presented with hypertension more frequently (Table 2). Additionally, they experienced longer CPBT. Mean MAO levels were significantly higher among patients with POAF ($P < 0.0001$) and



a linear trend across quartile levels was observed ($P_{\text{trend}} < 0.0001$), with the incidence of POAF being the highest in quartile 4 compared with quartile 1 (Figure 3). POAF was not associated with GSht, GPx, and GR (Figures 4, and 5B & C) in the univariable analysis. In multivariable analysis, MAO remained statistically significant after adjusting for age, sex, race, diabetes, hypertension, ACEI/ARB use, statin use, and CPBT ($P_{\text{trend}} = 0.009$, Table 3). A statistically significant linear trend also was observed for GSht in multivariable analysis ($P_{\text{trend}} = 0.014$).

4. DISCUSSION

Several reports have documented the inverse association of POAF and β -blocker use, illustrating the underlying etiologic role of catecholamines and excessive sympathetic discharge.^{21,22} Others have used prophylactic amiodarone,²³ sotalol,²⁴ magnesium,²⁵ and statins,²⁶ all of which were successful at reducing the incidence of POAF to varying

degrees; however, all patients were treated regardless of POAF status. These studies illustrate the importance of investigating biological factors that may predispose patients to POAF.

The findings of this study demonstrate for the first time that MAO is a major source of ROS in human atrial myocardium, and its activity varies across a 50-fold range among patients. It also provides evidence that atrial MAO activity serves as an independent predictor of POAF and lends further support to current theory that redox imbalance (i.e., oxidative stress) in the atrial myocardium is a significant factor in the etiology of POAF, particularly with respect to our myocardial GSht-related data. Furthermore, the results collectively integrate a number of perioperative factors known to contribute to the etiology of POAF (e.g., catecholamine overload and oxidative stress).

Investigation into the etiology of POAF has largely focused on systemic inflammation and oxidative stress in the post-operative period. Redox modifications of ion channels and proteins have been observed to directly impact cardiomyocyte electrical^{27,28} and mechanical²⁹ function and has been implicated in the early stages of electrical remodeling which accompanies the onset of AF.³⁰ Inflammation is interconnected with myocardial oxidative stress.^{31,32} Circulating cytokines and electrophilic lipids increase strain on antioxidant mechanisms in cardiomyocytes, a system already burdened with buffering oxidants originating from endogenous sources (e.g., MAO, NOX, and mitochondria) (Figure 2). The most important buffer of ROS in mammalian cells and tissues is GSH, which is converted to its oxidized form by GPx in the presence of hydroperoxides, and recycled back to its reduced form by NADPH-dependent GR. The GSH/GSSG (reduced/oxidized) redox couple is considered to be the

key indicator of the cellular redox environment.³³ Also important to the cellular/tissue redox environment is the total amount of GSH (GSht), defined as the additive amount of free GSH and GSSG. A decrease in GSht potentially increases the cell susceptibility to the adverse outcomes associated with oxidative stress (e.g., oxidative modifications of proteins, lipids, and DNA). Our findings that GSht and GPx are inversely correlated with POAF (Figures 4 & 5) suggests that a greater antioxidant capacity should lead to a lower incidence of POAF because of a greater buffering capacity of ROS during the post-operative period. Clinical trials have shown that anti-inflammatory/antioxidant therapies lead to a decreased incidence of POAF.^{34, 35} For example, pre-operative PUFAs and concentrated antioxidant supplementation have been observed to enhance anti-inflammatory/antioxidant capacity in atrial myocardium at the cellular level.³⁶ A follow-up clinical trial with this therapy led to a substantial decrease in POAF.³⁷ Use of n-3 PUFAs alone as prophylactic therapy to mitigate the incidence of POAF has led to mixed results. For example, the omega-3 fatty acids for prevention of post-operative atrial fibrillation (OPERA) trial showed that very high dose (8-10 g/day) of n-3 PUFAs for 2 to 5 days pre-operatively did not reduce the incidence of POAF.³⁸ Nevertheless, use of n-3 PUFAs as prophylactic therapy for arrhythmia and other cardiovascular diseases remains a viable therapeutic option due to the pleiotropic, beneficial effects of these fatty acids in the heart.

Mitochondria, as a consequence of their intracellular volume and density, are considered the predominant source of intracellular ROS in myocardium.³⁸ However, the total ROS that escapes (i.e. ROS emission) from the mitochondria is minimized by the reducing environment within the matrix of this organelle in addition to its redox enzyme

network.^{40, 41} These characteristics potentially explain our observation that H₂O₂ originating from mito-ETS was markedly lower than that from either MAO or NOX alone (Figure 2). ROS derived from NOX in the atrial myocardium, and downstream ROS (e.g., peroxynitrite and reactive aldehydes) have been shown to be significantly correlated with POAF.^{42, 43} Our findings further support the clinical importance of ROS-generating enzymes in atrial tissue and provide novel evidence that ROS derived from MAO may be a key determinant of myocardial redox balance in the postoperative period.

While MAO is an enzyme physically tethered to the outer mitochondrial membrane, MAO-derived ROS typically is not considered to be “mitochondrial ROS.” Our findings support a paradigm shift in the way this enzyme is viewed within the context of cellular redox balance. The wide range in MAO activity (~50-fold) across patients is a significant feature of our findings (Figure 2). Theoretically, the expression and activity of cardiac MAO should reflect sympathetic tone; however, there is considerable variation in promoter activity and transcriptional control of MAO genes in humans.⁴⁴ This may explain the underlying variation in enzyme activity seen in our patient cohort. Conceivably, high levels of catecholamines in the postoperative period may lead to increased concentrations inside cardiomyocytes by neuronal monoamine transporters in the sarcolemmal membrane.⁴⁵ Thus, in patients where MAO activity is high (Q3 and Q4, Figure 2), MAO-derived ROS may in turn be increased, leading to oxidative stress and potentially triggering POAF. In the remodeled myocardium, where fibrosis and altered ion channel expression are present, oxidative stress and inflammation only comprise a portion of the arrhythmogenic substrate. Accordingly, therapeutic strategies to mitigate POAF need to account for all of these possibilities.

Our study is strengthened by its prospective and systematic data collection. Additionally, biomarkers were obtained from myocardial tissue and reflect local cardiac versus serum levels. However, several limitations should be noted. Only the right atrial myocardium was collected in this study, and this may not be the best anatomic site to represent cardiac remodeling and oxidative stress pathways in the heart, given the known importance of the left atrium as a site of arrhythmogenesis. Moreover, studies have shown that as the pathology of AF progresses, a gradient of cardiac remodeling occurs starting in left atrium and ending in right atrium.⁴⁶ The temporality and spatial heterogeneity of this remodeling may be missed by capturing only the right atrium. However, since this patient cohort did not have any prior history of arrhythmias or cardiac surgery, it is unlikely that any remodeling that may exist would be due to the presence of an atrial arrhythmia.

Saturating concentrations of substrate (e.g., tyramine, NADPH, glutamate, etc) were used to measure enzyme activities in our assays. This rarely exists *in vivo*. However, the use of saturating substrate concentration was appropriate because the objective was to compare the maximal capacity for ROS generation and scavenging from myocardial enzymes. Biopsies were obtained at a single point in time, therefore it was not possible to determine the temporality of biomarker levels. Limited longitudinal information was available in our dataset. We were unable to determine the dose, duration and frequency of β -blocker use prior to surgery for each patient. Furthermore, our sample size was small and residual confounding may have been present.

In conclusion, our study suggests that MAO is a major ROS source in the human atrial myocardium and is an important biomarker for POAF, providing clinicians with the

ability to predict which patients are predisposed to this postoperative complication.

Advanced knowledge of POAF risk will enable appropriate prophylactic treatment to be initiated at the time of or prior to surgery, potentially leading to reduced hospital stay and healthcare costs associated with this complication. Additional investigation is needed to elucidate the role of MAO in arrhythmogenesis and to validate our findings in other populations and disease processes.

4.1. References – Methods, Results, Discussion

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5. Conclusions and Future Directions

This thesis has explored the use of redox-associated enzymes/molecules as novel biomarkers for predicting risk for POAF in a cardiac surgery patient population. Importantly, this is the first study identifying MAO as a major source of ROS within human atrium. Current literature states that redox imbalance is potentially a major causal factor in the etiology of POAF. Therefore, it follows that from a mechanistic perspective either an increase in ROS production and/or a decrease in antioxidant capacity could lead to an increase in oxidative stress and consequently, the risk of POAF. This research has the potential to positively impact both the patient and hospital in the very near future.

Prophylactic treatment of patients with anti-arrhythmic medication prior to surgery has shown efficacy at reducing the incidence of POAF. However, it is expensive and the risk of side effects strongly limits the will on the part of surgeons to administer this medication to every patient. Thus, having the ability to stratify patients into either a 'low risk' or a 'high risk' category would be very beneficial as only those patients at the highest risk for POAF would be treated. Physicians using these biomarkers will have an additional tool in their clinical arsenal to decide if prophylactic anti-arrhythmic medication should be included in a particular patient.

There are many directions for follow-up studies, because of the unique parameters measured and analyzed in this thesis project. Future studies can focus on determining which tissue and time course develop the strongest correlation to POAF development. For example, the data collected in this paper came from the right atrial appendage following median sternotomy, but prior to the institution of cardiopulmonary bypass.

Further work into the possible use of whole blood and its constituents in regard to our biomarkers is needed, as both MAO and GSH are indeed present in blood. If similar predictive trends between these markers in blood and POAF incidence are confirmed, this would give a less invasive technique to determine risk in a patient, and could be extrapolated to the general population of non-surgical patients who may also be at high risk for arrhythmia (atrial or ventricular). Repeating the study in a larger patient cohort is needed to further confirm and validate these biomarkers as predictive of POAF. Further research into the potential role of MAO, GSH and other redox enzymes in the pathophysiology of POAF is needed to better understand whether and to what extent these enzymes might be involved in arrhythmogenesis.

A recent paper by *Mariscalco et al* derived a regression model to predict the likelihood of POAF using eight independent factors, including age and other co-morbidities.¹ The independent markers do not include any biochemical markers, such as MAO.

The addition of our independent factors could add power and increase its predictive nature.

Risk of POAF in three hypothetical patients

Time		Patient A	Patient B	Patient C
- 20 years		Began smoking	-	-
-15 years		Hypertension	-	-
-10 years		Diabetes	Hypertension	-
-5 years		Atrial flutter	First detected AF	-
Surgery Day	Age:	83	76	58
	Sex:	Male	Female	Male
	Race:	White	White	Black
	MAO Activity (pmol H ₂ O ₂ /min/mg):	3500 (High, Q4)	2300 (Medium, Q3)	1900 (Low, Q1)
	GSHt (µmol/g):	14 (High, Q1)	17 (Medium, Q2)	19 (Medium, Q2)
POAF Risk?		High risk	Medium risk	Low risk

Figure 6: Hypothetical comparison of chronic and perioperative factors contributing to POAF risk in three individuals. Patient A has a greater risk of POAF due to the multiple chronic conditions in the years before surgery. In addition, the MAO activity & GSHt results increase the incidence rate.

MAO is a novel example of a biomarker able to predict POAF. Further research of enzymes and molecules within cardiac tissue should be studied to determine if those molecules are more strongly correlated enzymes to this complication.

6. Limitations and Alternative Interpretations

Due to the novel nature of this study, there were several limitations that need to be taken into account when interpreting the results. First, patients who had a history of arrhythmia (AF or other), cardiac surgery, implantable defibrillator, chronic renal insufficiency, and the use of anti-arrhythmic drugs other than beta blockers were excluded from this novel study. Prior episodes of these conditions are risk factors for POAF. Future studies should have a goal of being more inclusive and have a more diverse cohort including these excluded diseases.

Atrial tissue biopsies were collected from January 2009 – December 2012. The samples were stored in a -70°C freezer, but some of the enzyme assays were not conducted until many months after biopsy. The lag from collection to experimentation could result in a reduction of bioactive molecules. The procedure dictates biopsy and enzyme assay on the same day. Once a large enough data set is developed, further statistical correlations can be determined.

The patient population from this study came exclusively from eastern North Carolina. Consisting of the eastern 29-counties in North Carolina, this area is very diverse in its demographics and disease states.^{2,3} Over 60% of the population is white, 30% is black, and 3% Native American.² Included in the disease states are a high rate of

cardiovascular disease and other co-morbidities, compared to the rest of North Carolina and the United States.³ Eastern North Carolina has a higher rate of obesity (31.4% vs. 27.0%), diabetes (10.6% vs. 8.6%), and hypertension (32.9% vs. 28.7%), compared to the rest of North Carolina.³ The heterogeneity of the population and maladies could provide a stronger correlation to the demographics of United States as a whole, compared to other studies that focus on a homogenous community. Thus there is a greater probability of replicating our results in other regions of the country.

6.1 ***References: Conclusions and Future Directions, & Limitations and Alternative Interpretations***

- 1) Mariscalco, Giovanni , Fausto Biancari, et al. "Bedside Tool for Predicting the Risk of Postoperative Atrial Fibrillation After Cardiac Surgery: The POAF Score." *Journal of the American Heart Association*. 3 (2014): 1-9. Web.
- 2) Suzanne Lea, P. M., May, C., Miller, E., Smiley, R., & Barrett, E. (2012). *Cancer Prevention in Eastern North Carolina: A Cancer Profile of Eastern North Carolina* . Greenville, NC: East Carolina University.
- 3) Satomi Imai, P. (2011). *Disparities in Health Status and Health Risk Factors in Eastern North Carolina: Data from the Behavioral Risk Factor Surveillance System, 2005-2009 Aggregated*. Greenville, NC: East Carolina University.

Appendix A: Tables

TABLE 1

<u>Pt. #</u>	<u>Age</u>	<u>Sex</u>	<u>Race</u>	<u>Diabetes</u>	<u>HbA1c</u>	<u>HF</u>	<u>POAF</u>	<u>Tobacco</u>	<u>COPD</u>	<u>Prior MI</u>	<u>HTN</u>
1	79	F	AA	Y	6.9	N	N	N	N	N	Y
2	63	F	C	Y	10	N	N	Y	Y	Y	Y
3	67	F	AA	Y	7.2	Y	Y	Y	N	N	Y
4	62	F	AA	N	-	N	N	N	N	N	Y
5	60	F	C	Y	9.2	N	N	N	N	N	Y
6	69	F	AA	N	-	N	N	Y	N	N	Y
7	47	M	C	N	-	N	N	N	N	N	N
8	56	M	C	N	-	N	N	N	N	N	Y
9	52	M	AA	N	-	N	N	Y	N	N	Y
10	44	M	C	N	-	N	N	Y	N	Y	Y
11	58	M	C	N	-	N	Y	N	N	N	Y
12	52	M	C	Y	8.9	N	Y	Y	N	Y	Y

Absent values (-) for glycated hemoglobin (HbA1c) indicate that levels were within normal range (4.5 – 5.9%) for that particular patient (Pt); C, Caucasian; AA, African-American; HF (history of heart failure); POAF, post-operative atrial fibrillation; COPD, history of chronic obstructive pulmonary disease; HTN, history of hypertension

TABLE 2: Patient and Operative Characteristics Stratified by Postoperative Rhythm Class and Univariable Relative Risk for POAF (N=244)

Variables		POAF n (%)	POSR n (%)	P-Value	Univariable RR (95% CI)
Overall		80 (33)	164 (67)	---	---
Demographics/Comorbidities					
Age					
Mean ± SD	66 ± 8.7	62 ± 10		0.0019	---
Median (IQR)	67 (14)	62 (16)			
Q1 (≤56)	10 (13)	54 (33)			Referent
Q2 (56-64)	20 (25)	39 (24)		0.0045	2.2 (1.1-4.2)
Q3 (64-71)	23 (29)	37 (23)			2.5 (1.3-4.7)
Q4 (>71)	27 (34)	34 (21)			2.8 (1.5-5.3)
					P _{trend} =0.0008
Sex					
Female	14 (18)	41 (25)		0.19	Referent
Male	66 (83)	123 (75)			1.4 (0.84-2.2)
Race					
White	69 (86)	132 (80)		0.27	Referent
Black	11 (14)	32 (20)			1.3 (0.78-2.3)
Diabetes					
No	50 (63)	84 (51)		0.096	Referent
Yes	30 (38)	80 (49)			0.73 (0.50-1.06)
Hypertension					
No	7 (9)	34 (21)		0.019	Referent
Yes	73 (91)	130 (79)			2.1 (1.05-4.2)
BMI*					
Mean ± SD	30 ± 5.7	30 ± 6.2		0.74	---
Median (IQR)	30 (7.4)	30 (7.2)			
Q1 (≤26)	23 (29)	39 (24)			Referent
Q2 (26-30)	17 (22)	43 (26)		0.69	0.76 (0.46-1.3)
Q3 (30-33)	22 (27)	40 (25)			0.96 (0.60-1.5)
Q4 (>33)	18 (23)	42 (26)			0.81 (0.49-1.3)
					P _{trend} =0.60
Smoking					
No	59 (74)	107 (65)		0.18	Referent
Yes	21 (26)	57 (35)			0.76 (0.50-1.2)
COPD					
No	60 (75)	136 (83)		0.14	Referent
Yes	20 (25)	28 (17)			1.4 (0.92-2.02)
Prior Stroke					
No	74 (93)	154 (94)		0.68	Referent
Yes	6 (8)	10 (6)			1.2 (0.60-2.2)
Prior MI					
No	45 (56)	75 (46)		0.12	Referent
Yes	35 (44)	89 (54)			0.75 (0.52-1.08)
HF					
No	79 (99)	155 (95)		0.089 [#]	Referent
Yes	1 (1)	9 (5)			0.30 (0.046-1.9)
Ejection Fraction*					
Mean ± SD	54 ± 11	53 ± 14		0.30	---
Median (IQR)	58 (10)	54 (15)			
Q1 (≤48)	17 (22)	52 (32)			Referent
Q2 (48-55)	19 (24)	42 (26)		0.066	1.3 (0.72-2.2)
Q3 (55-62)	25 (31)	28 (17)			1.9 (1.2-3.2)
Q4 (>62)	19 (24)	42 (26)			1.3 (0.72-2.2)
					P _{trend} =0.15
CAD Severity*					
1-Vessel	3 (4)	11 (7)		0.20	Referent
2-Vessel	15 (19)	44 (27)			1.2 (0.40-3.5)
3-Vessel	62 (77)	109 (67)			1.7 (0.61-4.7)
					P _{trend} =0.079
Left Main Disease					
No	65 (81)	135 (82)		0.84	Referent
Yes	15 (19)	29 (18)			1.0 (0.66-1.7)
					1.1

Preoperative Medications				
Beta-Blockers				
No	12 (15)	28 (17)	0.68	Referent 1.1 (0.67-1.9)
Yes	68 (85)	136 (83)		
ACEI/ARBS				
No	70 (88)	128 (78)	0.076	Referent 0.61 (0.34-1.1)
Yes	10 (13)	36 (22)		
Statins				
No	18 (23)	33 (20)	0.67	Referent 0.91 (0.60-1.4)
Yes	62 (78)	131 (80)		
Intraoperative Characteristics				
CPB				
No	3 (4)	10 (6)	0.44	Referent 1.4 (0.53-4.0)
Yes	77 (96)	154 (94)		
CPBT (min)				
Mean ± SD	120 ± 33	110 ± 37	0.012	---
Median (IQR)	115 (37)	104 (48)		
Q1 (≤87)	9 (12)	49 (32)	0.0092	Referent 2.4 (1.2-4.8) 2.7 (1.4-5.4) 2.4 (1.2-4.9)
Q2 (87-108)	22 (29)	37 (24)		
Q3 (108-134)	26 (34)	35 (23)		
Q4 (>134)	20 (26)	33 (21)		
Biomarkers				
MAO*				
Mean ± SD	2843 ± 1987	1725 ± 1130	<0.0001	---
Median (IQR)	2608 (2038)	1691 (1286)		
Q1 (≤1344)	8 (10)	54 (33)	<0.0001	Referent 2.0 (0.90-4.3) 2.4 (1.1-5.1) 4.8 (2.4-9.3) P _{trend} <0.0001
Q2 (1344-2035)	15 (19)	44 (27)		
Q3 (2035-2820)	19 (23)	42 (25)		
Q4 (>2820)	38 (47)	24 (15)		
GSH*				
Mean ± SD	19 ± 6.0	20 ± 6.5	0.15	---
Median (IQR)	18 (7.6)	20 (7.9)		
Q1 (≤16)	24 (30)	36 (22)	0.36	Referent 0.90 (0.57-1.4) 0.69 (0.41-1.1) 0.70 (0.42-1.2) P _{trend} =0.099
Q2 (16-20)	22 (28)	39 (24)		
Q3 (20-23)	17 (21)	45 (27)		
Q4 (>23)	17 (22)	44 (27)		
GPX*				
Mean ± SD	17 ± 5.6	17 ± 6.9	0.42	---
Median (IQR)	17 (6.6)	16 (9.4)		
Q1 (≤12)	13 (16)	48 (29)	0.024	Referent 1.9 (1.1-3.3) 2.0 (1.2-3.6) 1.2 (0.65-2.3) P _{trend} =0.46
Q2 (12-17)	25 (31)	37 (22)		
Q3 (17-21)	26 (32)	34 (21)		
Q4 (>21)	16 (20)	45 (27)		
GR*				
Mean ± SD	4.7 ± 2.5	4.7 ± 2.6	0.42	---
Median (IQR)				
Q1 (≤3.8)	21 (26)	40 (24)	0.64	Referent 0.98 (0.60-1.6) 1.1 (0.66-1.7) 0.76 (0.44-1.3) P _{trend} =0.40
Q2 (4.8)	21 (26)	41 (25)		
Q3 (5.6)	22 (28)	38 (23)		
Q4 (>5.6)	16 (20)	45 (27)		

Test of Statistical Significance (Chi-Square for Categorical Variables, Deuchler-Wilcoxon for Continuous Variables). #Statistical significance computed using Fisher's Exact. P_{trend} computed using likelihood ratio trend test. *Missing values imputed using EM algorithm (n=10 simulations). ACEI=angiotensin converting enzyme inhibitor; ARB=angiotensin receptor blocker; BMI=body mass index; CAD=coronary artery disease; CI=confidence interval; COPD=chronic obstructive pulmonary disease; CPBT=cardiopulmonary bypass time; GPX=glutathione peroxidase; GR=glutathione reductase; GSH=glutathione; HF=heart failure; LOS=length of stay; MAO=monoamine oxidase; MI=myocardial infarction; POAF=postoperative atrial fibrillation; POSR=postoperative sinus rhythm; Q1=first quartile; Q2=second quartile; Q3=third quartile; Q4=fourth quartile; Ref=referent; RR=relative risk; SD=standard deviation.

TABLE 3: Multivariate Analysis of Independent Risk Factors Predictive of POAF[‡]

Models	ARR 95% CI
MAO* Q1 (≤ 1344) Q2 (1344-2035) Q3 (2035-2820) Q4 (> 2820)	Referent 1.8 (0.83-4.0) 2.1 (0.99-4.3) 3.8 (1.9-7.5) $P_{trend}=0.009$
GSH* Q1 (≤ 16) Q2 (16-20) Q3 (20-23) Q4 (> 23)	Referent 0.93 (0.60-1.4) 0.62 (0.36-1.1) 0.56 (0.34-0.93) $P_{trend}=0.014$
GPX* Q1 (≤ 12) Q2 (12-17) Q3 (17-21) Q4 (> 21)	Referent 1.9 (1.1-3.3) 2.4 (1.4-4.2) 1.4 (0.75-2.7) $P_{trend}=0.21$

[‡]Models adjusted for age, sex, race, diabetes, angiotensin converting enzyme inhibitor and angiotensin receptor blocker use, statin use, and hypertension. *Missing values imputed using EM algorithm (n=10 simulations). P_{trend} computed using score trend test. CI=confidence interval; GPX=glutathione peroxidase; GSHt=total glutathione; MAO=monoamine oxidase; Q1=first quartile; Q2=second quartile; Q3=third quartile; Q4=fourth quartile; ARR=adjusted relative risk.

Appendix B: Figure Reproduction:

Glutathione Redox Cycle

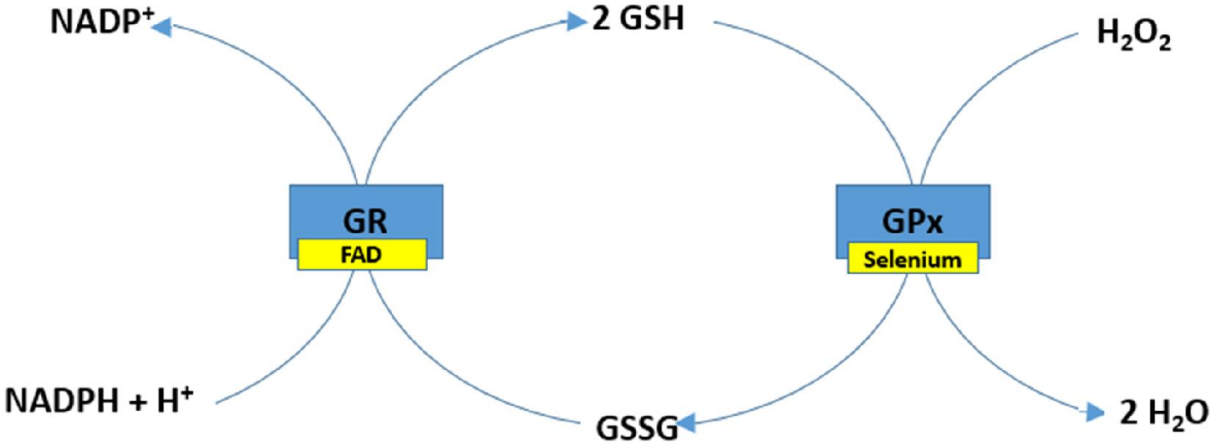


Figure 1.1 A simplified scheme of the redox cycle involving GSH and related enzymes GPx and GR

Figure 2.

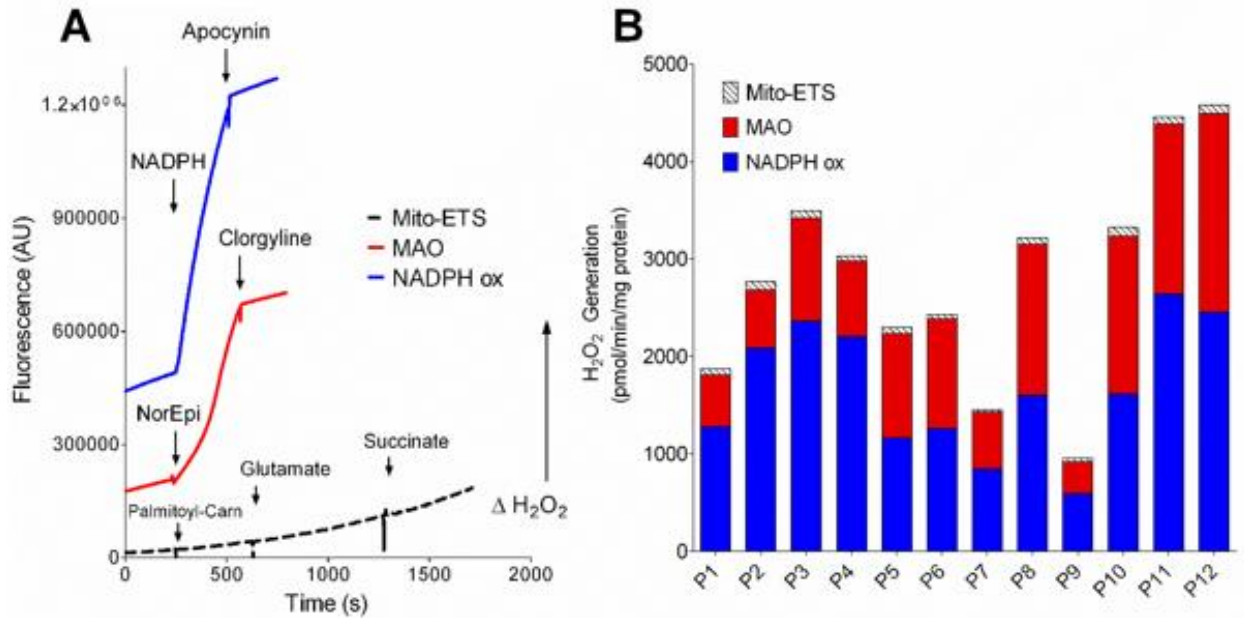


Figure 2. Comparative analysis of major ROS sources in human atrial myocardium.

Shown in **A** are representative H₂O₂ production traces from NOX (blue), MAO (red) and mito-ETS (black dash) in RAA tissue obtained from one individual patient. PmFBs were used for determining H₂O₂ from mito-ETS, while homogenate was used for NOX and MAO. Substrates were added to cuvette where indicated. Apocynin and Clorgyline are administered where indicated to confirm the source of H₂O₂ production to be NOX and MAO, respectively. In **B** are the quantified rates from each of these three sources in RAA tissue obtained from 12 individual patients.

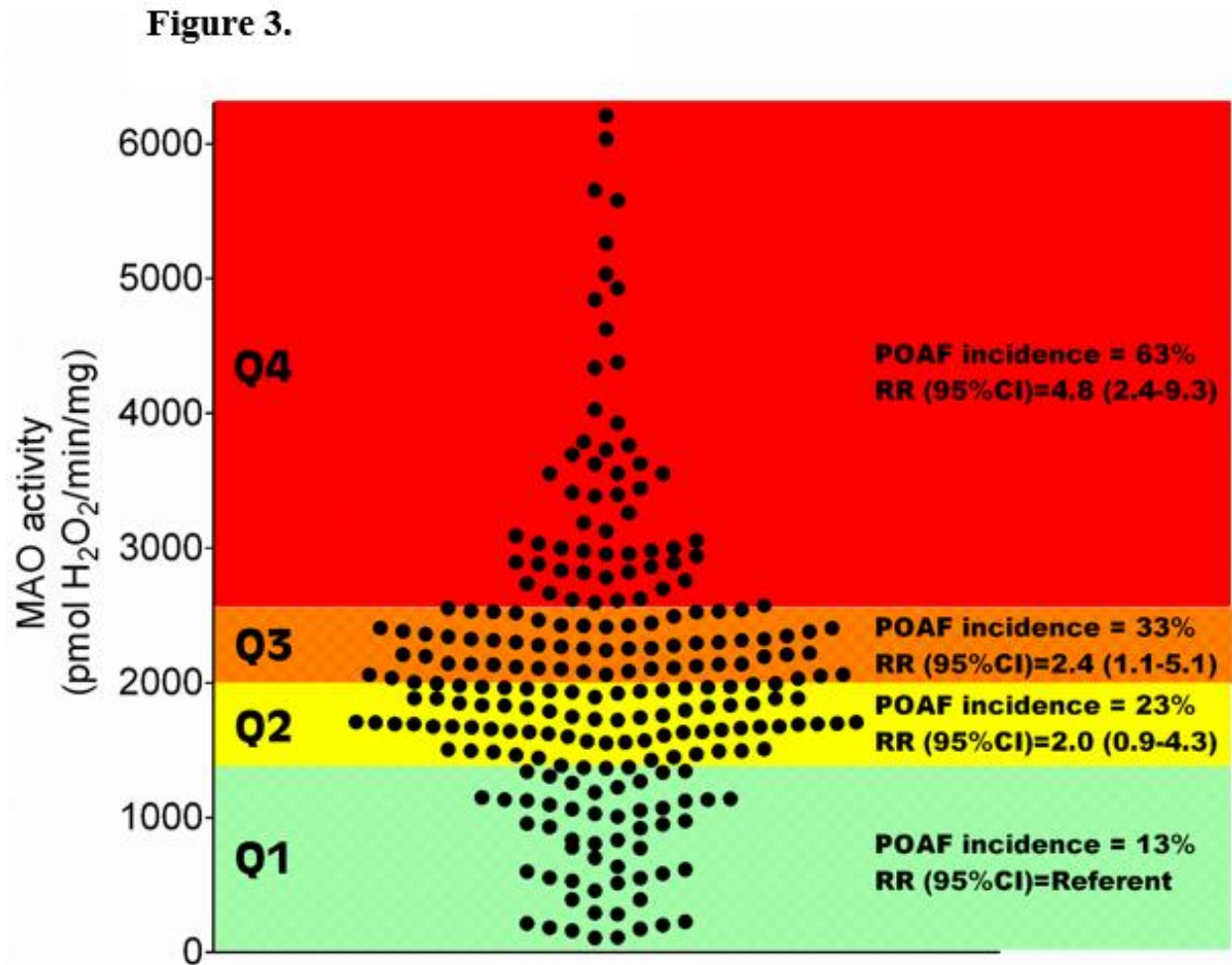


Figure 3. MAO activity in atrial myocardium and incidence of POAF. All rates of MAO activity from entire cohort of patients recruited for this study are shown (1 circle = 1 patient). Quartiles of pooled data were generated, and univariable analysis performed with POAF as the outcome variable using Poisson regression. Each quartile is delineated with color shading to illustrate the risk of POAF within that particular quartile (Green = <15%, Yellow = 15-30%, Orange = 30-40%, Red = >40%). Within each quartile, POAF incidence = number of patients in that particular quartile experiencing POAF. RR = Relative Risk, with 95% confidence interval (CI).

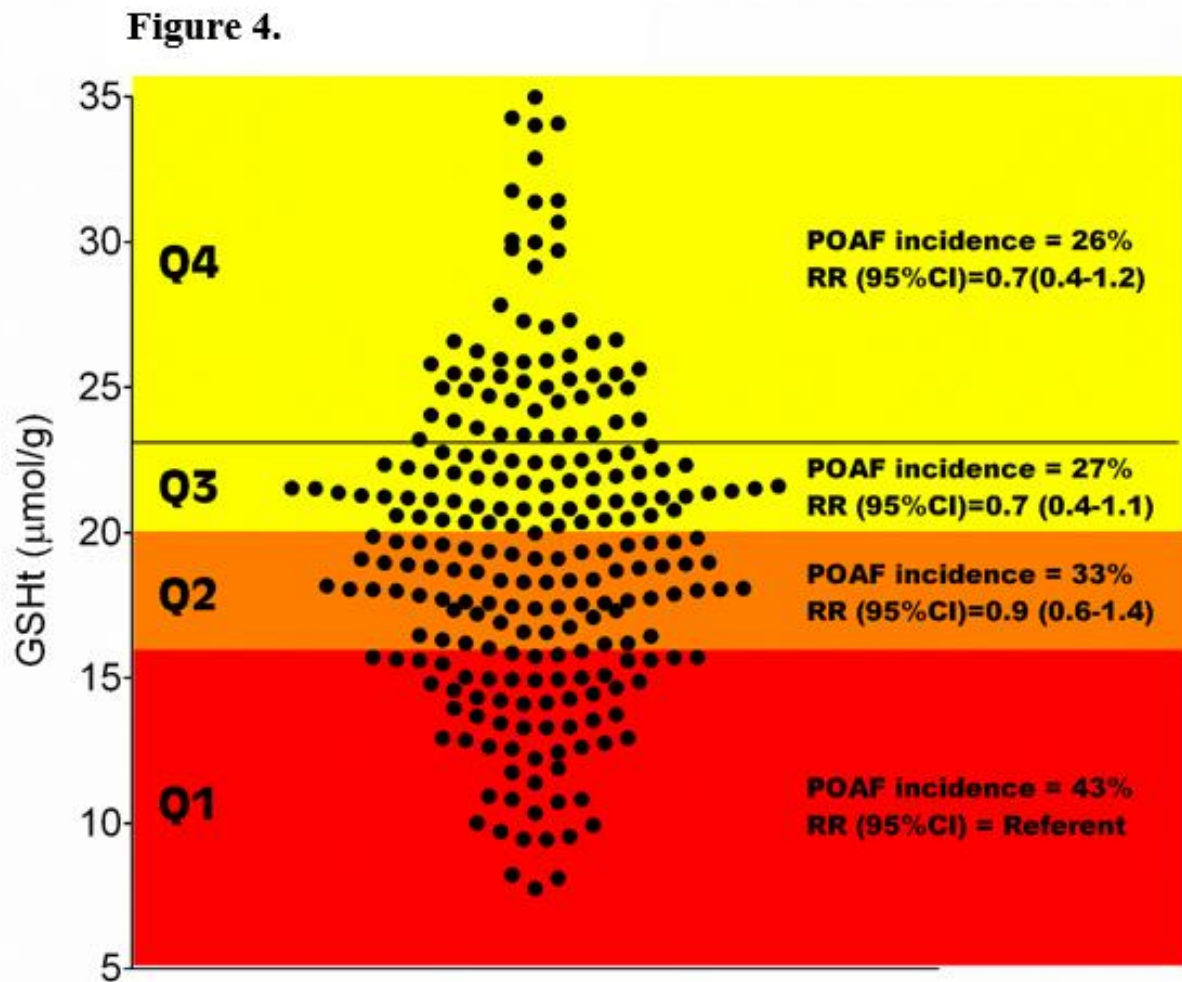


Figure 4. Total GSH (GSht) in atrial myocardium and incidence of POAF. Data shown in this figure is GSht for the entire cohort of patients recruited for this study.

Quartiles of pooled data were generated, and univariable analysis performed with POAF as the outcome variable using Poisson regression. Each quartile is delineated with color shading to illustrate the risk of POAF within that particular quartile (Red = >40%, Yellow = 15-30%, Orange = 30-40%). Within each quartile, POAF incidence = number of patients in that particular quartile experiencing POAF. RR = Relative Risk, with 95% confidence interval (CI).

Figure 5.

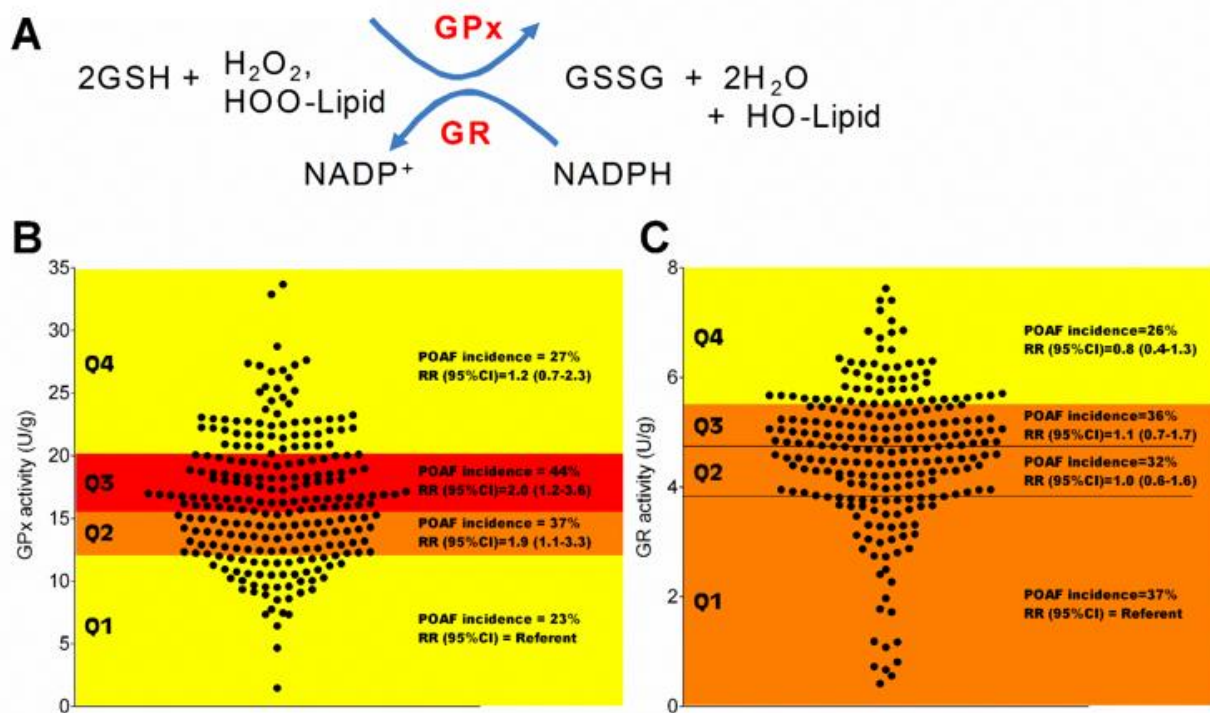


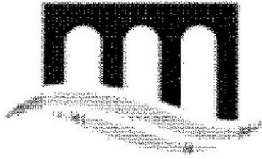
Figure 5. GPx-GR activity in atrial myocardium and incidence of POAF. Shown in panel A is a simplified schematic of the redox cycle involving GSH and related enzymes GPx and GR (HOO-Lipid = Lipid peroxide). Data shown in panel B are GPx activity and C GR activity for the entire cohort of patients recruited for this study. Quartiles of pooled data were generated, and univariable analysis performed with POAF as the outcome variable using Poisson regression. Each quartile is delineated with color shading to illustrate the risk of POAF within that particular quartile (Red = >40%, Yellow = 15-30%, Orange = 30-40%). Within each quartile, POAF incidence = number of patients in that particular quartile experiencing POAF. RR = Relative Risk, with 95% confidence interval (CI).

Risk of POAF in three hypothetical patients

Time		Patient A	Patient B	Patient C
- 20 years		Began smoking	-	-
-15 years		Hypertension	-	-
-10 years		Diabetes	Hypertension	-
-5 years		Atrial flutter	First detected AF	-
Surgery Day	Age:	83	76	58
	Sex:	Male	Female	Male
	Race:	White	White	Black
	MAO Activity (pmol H ₂ O ₂ /min/mg):	3500 (High, Q4)	2300 (Medium, Q3)	1900 (Low, Q1)
	GSHt (μmol/g):	14 (High, Q1)	17 (Medium, Q2)	19 (Medium, Q2)
POAF Risk?		High risk	Medium risk	Low risk

Figure 6: Hypothetical comparison of chronic and perioperative factors contributing to POAF risk in three individuals. Patient A has a greater risk of POAF due to the multiple chronic conditions in the years before surgery. In addition, the MAO activity & GSHt results increase the incidence rate.

**Appendix C: East Carolina University and Medical Center Institutional Review Board
Letter of Approval**



EAST CAROLINA UNIVERSITY

University & Medical Center Institutional Review Board Office

1L-09 Brody Medical Sciences Building • 600 Moye Boulevard • Greenville, NC 27834

Office 252-744-2914 • Fax 252-744-2284 • www.ecu.edu/irb

TO: Ethan Anderson, PhD, Department of Pharmacology & Toxicology, BSOM, ECU
FROM: UMCIRB *STC*
DATE: October 27, 2010
RE: Full Committee Approval for Continuing Review of a Research Study Requiring Modifications
TITLE: "Linking Redox Chemistry and Mitochondria in Atrium Post-Operative Arrhythmia"

UMCIRB #09-0669

The above referenced research study was initially reviewed by the convened University and Medical Center Institutional Review Board (UMCIRB) on **9/23/09 & 10/14/09**. The research study underwent a subsequent continuing review for approval on **10/13/10** by the convened UMCIRB. Requested modifications were prescribed and received final approval on **10/27/10** by the convened UMCIRB. The UMCIRB deemed this NIH sponsored study **more than minimal risk** requiring a continuing review in **12 months**. Changes to this approved research may not be initiated without UMCIRB review except when necessary to eliminate an apparent immediate hazard to the participant. All unanticipated problems involving risks to participants and others must be promptly reported to the UMCIRB. The investigator must submit a continuing review/closure application to the UMCIRB prior to the date of study expiration. The investigator must adhere to all reporting requirements for this study.

The above referenced research study has been given approval for the period of **10/27/10** to **10/26/11**. The approval includes the following items:

- Continuing Review Form (dated 9/14/10)
- Research protocol
- Informed consent (version 3 dated 10/19/10)

The following UMCIRB members were recused for reasons of potential for Conflict of Interest on this research study:
None

NOTE: The following UMCIRB members with a potential Conflict of Interest did not attend this IRB meeting:
None

The UMCIRB applies 45 CFR 46, Subparts A-D, to all research reviewed by the UMCIRB regardless of the funding source. 21 CFR 50 and 21 CFR 56 are applied to all research studies under the Food and Drug Administration regulation. The UMCIRB follows applicable International Conference on Harmonisation Good Clinical Practice guidelines.

INFORMED CONSENT

Title: LINKING REDOX CHEMISTRY AND MITOCHONDRIA IN ATRIUM TO POST-OPERATIVE ARRHYTHMIA

Principal Investigator: Ethan J. Anderson, PhD

Institution: Brody School of Medicine

Address: 6S-11 BSOM

Telephone #: (252) 744-2757

This consent form document may contain words that you do not understand. You should ask the study doctor or the study coordinator to explain any words or information in this consent form that you do not understand.

INTRODUCTION

You have been asked to participate in a research study being conducted by Ethan Anderson, PhD, Alan Kypson, MD, and fellow researchers at East Carolina University designed to determine if there are certain types of proteins in your heart tissue that predisposes you to developing an irregular heartbeat after your surgery. Atrial fibrillation (irregular heartbeat) is one of the most common complications after open heart surgery. Little is known about why or how this occurs, and more importantly why it occurs in some patients and not in others. The studies proposed here are directed towards identifying a biochemical marker in your heart tissue which could help to identify patients in the future who are at increased risk of developing postoperative atrial fibrillation, so that we can prevent this from happening. Approximately 500 individuals will be enrolled in this study.

PLANS AND PROCEDURES

At the time of your heart surgery, your doctors will take a small piece of your atrial tissue, approximately the size of a button. **Even if you do not consent to this study, this same exact piece of atrial tissue will be clipped out and discarded as a routine part of your heart surgery.** There is nothing unusual or modified about your heart surgery in order to obtain this tissue and the taking of this tissue does not increase your risks in any way. A small sample (~ 1 tablespoon) of your blood will also be taken in the operating room at this time.

The blood and heart tissue samples collected from you for this study will be taken to a laboratory, where certain types of biochemical analysis will be performed either immediately or at a later time. A portion of this tissue will be stored for up to 20 years to research scientific questions specifically related to post-operative atrial fibrillation, and other types of disease. You need to be aware that this research may include doing tests to look at specific types of genes, proteins, or other molecules present in your heart that will help the researchers know more about different types of diseases so that treatments for these diseases can be developed in the future. However, none of the information the researchers find from these tests will be made available to you, nor will they affect your treatment or prognosis. Your identity and clinical information will remain completely confidential. Also, you will continue to be the owner of the samples after they are taken, and you retain the right to have the sample material destroyed at any time by contacting the study Principal Investigator. During this study the samples will be stored with number identifiers only; however, the number identifier will be linked with a specific name and will be kept on file in the possession of the Principal Investigator. The linked file will be stored password protected on the Principal Investigator's computer with CD backup. No other individuals will have access to these identifying materials unless the Principal Investigator is required by law to provide such identifying information. Data will not be publicly available and participants will not be identified or linked to the samples in publication. If a commercial product is developed from this research project, you will not profit financially from such a product.

UMCIRB
APPROVED
FROM 10-27-10
TO 10-26-11

Subjects initials _____

POTENTIAL RISKS AND DISCOMFORTS

There are no extra risks to you by agreeing to participate in this study.

POTENTIAL BENEFITS

The information gathered from this study may provide valuable insight into findings on the causes and potential treatments for post-operative atrial fibrillation and other cardiovascular diseases. There will be no direct benefits to you from this research.

TERMINATION OF PARTICIPATION

Your participation in this research study may be terminated without your consent if the investigators believe that these procedures will pose unnecessary risks to you. You may also be terminated from participation if you do not adhere to the study protocol.

COST AND COMPENSATION

You will receive no monetary compensation for participating in this study

CONFIDENTIALITY

Only the investigators associated with this study will have access to the data obtained. However, the University Medical Center Institutional Review Board (UMCIRB), governmental, or university officials may require access to the data to determine if the research outlined here complies with all university and governmental statutes. Your identity will be protected by numeric coding. In the reporting of the results, no identifying information will be released.

VOLUNTARY PARTICIPATION

You understand that your participation in this study is voluntary. Refusal to participate will involve no penalty or loss of benefits to which you are otherwise entitled. Furthermore, you may stop participating at any time you choose without penalty, loss of benefits, or without jeopardizing your continuing medical care at this institution.

PERSONS TO CONTACT WITH QUESTIONS

The investigators will be available to answer any questions concerning this research, now or in the future. You may contact the primary investigators Ethan Anderson, PhD at 252-744-2757 (days), or Alan Kypson, MD at 252-744-3552 (days). If questions arise about your rights as a research subject, you may contact the Chairman of the University and Medical Center Institutional Review Board at phone number 252-744-2914 (days). You may contact Risk Management & Quality Programs at 252-328-2380.

RESEARCH PARTICIPANT AUTHORIZATION TO USE AND DISCLOSE INFORMATION

When taking part in research, health information is collected, used, and shared only with others who are involved in the research. Federal laws require that researchers and health care providers protect your identifiable health information. Also, federal laws require that we get your permission to use collected health information for the research. This permission is called authorization.

In order to complete the research project in which you have decided to take part, we need to collect and use some of your health information. Specifically, this information includes:

Select the boxes that identify the types of health information:

- Billing records
- Hospital/medical records (in and out patient)
- Mental Health records
- Lab, pathology and/or radiology results
- Physician/clinic records
- PHI previously collected for research purposes

Subjects initials _____

UMCIRB
APPROVED

FROM 10-27-10
TO 10-25-11

Unique Identifier: 09-0669

Other: Data gathered during course of testing in relation to body composition, plasma lipids, exercise stress test results, exercise training, exercise history, health history.

The members of the research team will conduct the research study at ECU PCMH ECU & PCMH
 Other

Select the boxes that identify who gets the information:

- Sponsor or other funding source to provide oversight for entire research project
- Research investigators to conduct and oversee the research project
- Research team members to participate in the various research activities
- FDA or other regulatory agencies to provide regulatory oversight
- UMCIRB to provide continuing review of the research project
- Institutional officials in connection with duties for monitoring investigatory activity
- Researchers at other sites to participate in the research when more than one research site is involved
- Other

Information about you will be used and released in such a way that will protect your identity as much as possible. The individual/agencies who may receive health information about you also agree to keep this information confidential. However, there is always a chance that your information could be shared in a way that it would no longer be protected. Therefore, although we take precautions to protect your information, confidentiality cannot be absolutely guaranteed.

We are asking your permission to share your health information related to this study with the individuals/agencies listed above upon their request. You may or may not be eligible to begin participating in this study if you do not sign this Authorization form. You have the right to stop or limit the sharing your information. You have the right to limit who may receive this information. You may stop or limit how your protected health information is used for this research study by giving the investigator your request in writing. If you want us to stop using your information, you may be removed from the study. If you are removed from the study it will not affect your ability to receive standard medical care or any other benefits for which you are entitled to receive. Protected Health Information collected for the purpose of the research study collected prior to withdrawing your Authorization will continued to be used for the purposed of the research study.

We will share only the information listed above with the individuals/agencies listed above. If we need to share other information or if we need to send it to other individuals/agencies not listed above, we will ask you permission in writing again. At any time, you can ask us to tell you what information about you has been shared and with whom. However, you may not have access to your information until the study is over.

Research information continues to be looked at after the study is finished so it is difficult to say when use of your information will stop. Currently, there is not an expiration date for the use and disclosure of your information for this study.

If you have questions about the sharing of information related to this research study, call the principal investigator Ethan Anderson at phone number 252-744-2757. Also, you may telephone the University and Medical Center Institutional Review Board at 252-744-2914. In addition, if you have concerns about confidentiality and privacy rights, you may phone the Privacy Officer at Pitt County Memorial Hospital at 252-847-6545 or at East Carolina University 252-744-2030.

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FROM 10-27-10
TO 10-28-11

Subjects initials _____

CONSENT TO PARTICIPATE

I certify that I have read all of the above, asked questions and received answers concerning areas I did not understand, and have received satisfactory answers to these questions. I willingly give my consent for participation in this research study. (A copy of this consent form will be given, upon request, to the person signing as the subject or as the subject's authorized representative.)

Subject's Name (Print)

Signature of Subject Time and Date

Signature of Legally Authorized Representative Time and Date

AUDITOR WITNESS: I confirm that the contents of this consent form were orally presented.

Auditor's Name (Print)

Signature of Auditor Witness Time and Date

Ethan J. Anderson, PhD

Principal Investigator's Name (Print)

Signature of Principal Investigator Time and Date

UMCIRB
APPROVED
FROM 10-27-10
TO 10-25-11

Subjects initials _____

Continuing Review/Closure Obligation

As a investigator you are required to submit a continuing review/closure form to the UMCIRB office in order to have your study renewed or closed before the date of expiration as noted on your approval letter. This information is required to outline the research activities since it was last approved. You must submit this research form even if you there has been no activity, no participant s enrolled, or you do not wish to continue the activity any longer. The regulations do not permit any research activity outside of the IRB approval period. Additionally, the regulations do not permit the UMCIRB to provide a retrospective approval during a period of lapse. Research studies that are allowed to be expired will be reported to the Vice Chancellor for Research and Graduate Studies, along with relevant other administration within the institution. The continuing review/closure form is located on our website at www.ecu.edu/irb under forms and documents. The meeting dates and submission deadlines are also posted on our web site under meeting information. Please contact the UMCIRB office at 252-744-2914 if you have any questions regarding your role or requirements with continuing review.
<http://www.hhs.gov/ohrp/humansubjects/guidance/contrev0107.htm>

Required Approval for Any Changes to the IRB Approved Research

As a research investigator you are required to obtain IRB approval prior to making any changes in your research study. Changes may not be initiated without IRB review and approval, except when necessary to eliminate an immediate apparent hazard to the participant. In the case when changes must be immediately undertaken to prevent a hazard to the participant and there was no opportunity to obtain prior IRB approval, the IRB must be informed of the change as soon as possible via a protocol deviation form.
<http://www.hhs.gov/ohrp/humansubjects/guidance/45cfr46.htm#46.103>

Reporting of Unanticipated Problems to Participants or Others

As a research investigator you are required to report unanticipated problems to participants or others involving your research as soon as possible. Serious adverse events as defined by the FDA regulations may be a subset of unanticipated problems. The reporting times as specified within the research protocol, applicable regulations and policies should be followed.
<http://www.hhs.gov/ohrp/policy/AdvEvtGuid.htm>



EAST CAROLINA UNIVERSITY
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1L-09 Brody Medical Sciences Building · Mail S682
600 Moye Boulevard · Greenville, NC 27834
Office 252-744-2914 · Fax 252-744-2284 · www.ecu.edu/irb

Notification of Continuing Review Approval

From: Biomedical IRB
To: [Ethan Anderson](#)
CC:
Date: 10/27/2011
Re: [CR00000051](#)
[UMCIRB 09-0669](#)
[IMPORTED] Linking Redox Chemistry and Mitochondria in Atrium to Post-Operative Arrhythmia

I am pleased to inform you that at the convened meeting on 10/26/2011 12:00 PM of Biomedical IRB, this research study underwent a continuing review and the committee voted to approve the study. Approval of the study and the consent form(s) is for the period of 10/26/2011 to 10/25/2012.

The Biomedical IRB deemed this study Greater than Minimal Risk.

Changes to this approved research may not be initiated without UMCIRB review except when necessary to eliminate an apparent immediate hazard to the participant. All unanticipated problems involving risks to participants and others must be promptly reported to the UMCIRB. The investigator must submit a continuing review/closure application to the UMCIRB prior to the date of study expiration. The investigator must adhere to all reporting requirements for this study.

The approval includes the following items:

Name	Description	Modified	Version
Informed Consent - POAF study v4 10-11-2011.doc	Consent Forms	10/11/2011 9:24 AM	0.01
Revised protocol for 09-0669 Sept2011.doc	Study Protocol or Grant Application	10/11/2011 9:19 AM	0.01

The following UMCIRB members were recused for reasons of potential for Conflict of Interest on this research study:

None

The following UMCIRB members with a potential Conflict of Interest did not attend this IRB

None

EAST CAROLINA UNIVERSITY
University & Medical Center Institutional Review Board Office
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600 Moye Boulevard · Greenville, NC 27834
Office 252-744-2914 · Fax 252-744-2284 · www.ecu.edu/irb

Notification of Continuing Review Approval

Biomedical IRB
[Ethan Anderson](#)

10/10/2012

[CR00000583](#)

[UMCIRB 09-0669](#)

[IMPORTED] Linking Redox Chemistry and Mitochondria in Atrium to Post-Operative Arrhythmia

I am pleased to inform you that at the convened meeting of the Biomedical IRB on 10/10/2012, this research study underwent a continuing review and the committee voted to approve the study. Approval of the study and the consent form(s) is for the period of 10/10/2012 to 10/9/2013.

The Biomedical IRB deemed this study Greater than Minimal Risk.

Changes to this approved research may not be initiated without UMCIRB review except when necessary to eliminate an apparent immediate hazard to the participant. All unanticipated problems involving risks to participants and others must be promptly reported to the UMCIRB. The investigator must submit a continuing review/closure application to the UMCIRB prior to the date of study expiration. The investigator must adhere

to all reporting requirements for this study.

Consent Forms

10/11/2011 9:24AM 0.01

The approval includes the following items:

Study Protocol or GrantApplication10/11/2011 9:19AM0.01

[Informed Consent - POAF study v4 10-](#)

[11Revised protocol for 09-0669](#)

[Sept2011.doc](#) Description Modified

Version

Name

[2011.doc](#)

The following UMCIRB members were recused for reasons of potential for Conflict of Interest study: None

on this research The following UMCIRB members with a potential Conflict of Interest did not Anderson

attend this IRB meeting: E.

IRB00000705 East Carolina U IRB #1 (Biomedical) IORG0000418
IRB00003781 East Carolina U IRB #2 (Behavioral/SS) IORG0000418 IRB00004973

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Office 252-744-2914 · Fax 252-744-2284 · www.ecu.edu/irb



Notification of Continuing Review Approval

Biomedical IRB

[Ethan Anderson](#)

From: 9/26/2013
To: [CR00001312](#)
CC: [UMCIRB 09-0669](#)
Date: [IMPORTED] Linking Redox Chemistry and Mitochondria in Atrium to Post-
Re: Operative Arrhythmia

I am pleased to inform you that at the convened meeting on 9/25/2013 of the Biomedical IRB, this research study underwent a continuing review and the committee voted to approve the study. Approval of the study and the consent form(s) is for the period of 9/25/2013 to 9/24/2014.

The Biomedical IRB deemed this study Greater than Minimal Risk.

Changes to this approved research may not be initiated without UMCIRB review except when necessary to eliminate an apparent immediate hazard to the participant. All unanticipated problems involving risks to participants and others must be promptly reported to the UMCIRB. The investigator must submit a continuing review/closure application to the UMCIRB prior to the date of study expiration. The investigator must adhere to all reporting requirements for this study.

Approved consent documents with the IRB approval date stamped on the document should ~~tab in the study workspace~~ be used to consent participants (consent documents with the IRB approval date stamp are

found under the Documents The approval includes the following items:

Document	Description
Informed Consent - POAF study v4 10-11-2011.doc(0.01)	Consent Forms
Revised protocol for 09-0669 Sept2011.doc(0.01)	Study Protocol or Grant Application

study:

None

E. Anderson

The following UMCIRB members were recused for reasons of potential for Conflict of Interest on this research

The following UMCIRB members with a potential Conflict of Interest did not attend this IRB meeting: