Prospective Evaluation of Clinico-pathological Predictors of Post-operative Atrial Fibrillation: An Ancillary Study from the OPERA Trial

Running title: *Corradi & Saffitz et al.; Predictors of post-operative atrial fibrillation*

Domenico Corradi, MD[1]*; Jeffrey E. Saffitz, MD, PhD[2]*; Deborah Novelli, PhD[3]; Angeliki Asimaki, PhD[2]; Caterina Simon, MD[4]; Emanuela Oldoni, PhD[3]; Serge Masson, PhD[3]; Jennifer M.T.A. Meessen, PhD[3]; Rodolfo Monaco, MD[1]; Roberta Manuguerra, MD[1]; Roberto Latini, MD[3]; Peter Libby, MD[5]; Luigi Tavazzi, MD[6]; Roberto Marchioli, MD[7]; Luca Dozza, MSB[8]; Laura Cavallotti, MD[9]; Aneta Aleksova, MD[10]; Renato Gregorini, MD[11]; Dariush Mozaffarian, MD, PhD[12, 13]

[1]Dept of Medicine & Surgery, Unit of Pathology, Univ of Parma, Parma, Italy; [2]Beth Israel Deaconess Medical Center & Harvard Medical School, Boston, MA; [3]Istituto di Ricerche Farmacologiche Mario Negri IRCCS, Milan; [4]USC Cardiochirurgia ASST Papa Giovanni XXIII Bergamo, Italy; [5]Brigham and Women's Hospital, Harvard Medical School, Boston, MA; [6]GVM Hospitals of Care & Rsrch, Villa Maria Cecilia Hospital, Cotignola; [7]Cardiovascular, Renal, & Metabolic Medical & Scientific Services; IQVIA Milan; [8]Cardiothoracic & Vascular Dept, Maria Cecilia Hospital, GVM Care & Rsrch, Cotignola; [9]Centro Cardiologico Monzino, IRCCS, Milan; [10]Ospedali Riuniti & Univ of Trieste, Trieste; [11]Città di Lecce Hospital GVM Care & Rsrch, Lecce, Italy; [12]Brigham and Women's Hospital; [13]Friedman School of Nutrition Science & Policy, Tufts Univ, Boston, MA
*contributed equally as senior co-authors

Correspondence:
Domenico Corradi, MD
Department of Medicine and Surgery,
Unit of Pathology, University of Parma
via Gramsci 14
43126, Parma, Italy
Tel: +39 0521 702390
E-mail: domenico.corradi@unipr.it

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Abstract:

Background - Post-operative atrial fibrillation (POAF) occurs in 30-50% of patients undergoing cardiac surgery and is associated with increased morbidity and mortality. Prospective identification of structural/molecular changes in atrial myocardium that correlate with myocardial injury and precede and predict risk of POAF may identify new molecular pathways and targets for prevention of this common morbid complication.

Methods - Right atrial appendage (RAA) samples were prospectively collected during cardiac surgery from 239 patients enrolled in the OPERA trial, fixed in 10% buffered formalin and embedded in paraffin for histology. We assessed general tissue morphology, cardiomyocyte diameters, myocytolysis (perinuclear myofibril loss), accumulation of perinuclear glycogen, interstitial fibrosis, and myocardial gap junction distribution. We also assayed NT-proBNP, hs-cTnT, CRP, and circulating oxidative stress biomarkers (F2-isoprostanes, F3-isoprostanes, isofurans) in plasma collected before, during, and 48h after surgery. POAF was defined as occurrence of post-cardiac surgery atrial fibrillation or flutter of at least 30 seconds duration confirmed by rhythm strip or 12-lead ECG. The follow-up period for all arrhythmias was from surgery until hospital discharge or post-operative day 10.

Results - 35.2% of patients experienced POAF. Compared to the non-POAF group, they were slightly older and more likely to have chronic obstructive pulmonary disease or heart failure. They also had a higher EuroSCORE and more often underwent valve surgery. No differences in left atrial size were observed between POAF and non-POAF patients. The extent of atrial interstitial fibrosis, cardiomyocyte myocytolysis, cardiomyocyte diameter, glycogen score or Cx43 distribution at the time of surgery was not significantly associated with incidence of POAF. None of these histopathologic abnormalities were correlated with levels of NT-proBNP, hs-cTnT, CRP or oxidative stress biomarkers.

Conclusions - In sinus rhythm patients undergoing cardiac surgery, histopathological changes in the RAA do not predict POAF. They also do not correlate with biomarkers of cardiac function, inflammation, and oxidative stress.

Key words: pathology; atrial fibrillation; remodeling; biomarker; postoperative complication arrhythmia; fibrosis; cardiomyocyte; right atrial appendage

Nonstandard Abbreviations and Acronyms

AF: atrial fibrillation

BNP: brain natriuretic peptide

CRP: C-reactive protein

Cx43: connexin 43

ECG: electrocardiogram

EuroSCORE: European system for cardiac operative risk evaluation

n-3 PUFA: long-chain ω-3 polyunsaturated fatty acid

NT-proBNP: N-terminal pro brain natriuretic peptide

OPERA: omega-3 fatty acids for prevention of post-operative atrial fibrillation

PAS: periodic acid–Schiff

PCI: prior percutaneous coronary intervention

POAF: post-operative atrial fibrillation

RAA: right atrial appendage

Introduction

Post-operative atrial fibrillation (POAF) is the most common complication of cardiac surgery ranging from 30% to 50% of cases and usually occurring within 2 to 4 days after the surgical procedure (with a peak incidence on post-operative day 2).[1-4] POAF, in its turn, is associated with increased post-operative thromboembolic risk and stroke, hemodynamic compromise, ventricular dysrhythmias, as well as significantly higher both in-hospital and 6-month mortality.[5] In addition to the type of surgery, the only well-established predictor for the development of POAF is advanced age.[6-11]

Remarkably, despite decades of recognition of POAF and attempts to mitigate its development, the underlying pathophysiologic mechanisms for POAF remain unclear. One

hypothesis holds that both initiation and maintenance of this arrhythmia depend on a structural substrate that arises as a result of the multiple risk factors listed above[3]. However, a major limitation in developing preventive strategies has been a lack of understanding of the specific preceding anatomic and molecular changes in the atrial myocardium at the time of cardiac surgery that might influence the risk of POAF.

Prior pathological studies have typically been retrospective (with tissue collected after onset of atrial fibrillation, AF), and have combined tissues from patients who had already experienced AF for variable intervals (e.g. new onset, paroxysmal, and persistent). These studies have also relied on what were likely non-representative samples obtained at convenience rather than pre-specified sampling by protocol. These limitations have made it impossible to identify and define rigorously the critical tissue substrates of AF, and to distinguish changes in tissue structure that increase the risk of AF from those caused by AF itself.

Therefore, we set out to assess structural and molecular changes in atrial myocardium and circulating biomarkers collected prospectively in a large number of cardiac surgery patients to determine if any might be independently associated with the subsequent development of POAF.

Methods

The data that support the findings of this study are available from the corresponding author upon reasonable request.

OPERA patient population and ω-3-PUFA supplementation

The Omega-3 Fatty Acids for Prevention of Post-Operative Atrial Fibrillation (OPERA) study was a prospective, double-blind, placebo-controlled, multinational, randomized clinical trial that tested the hypothesis that perioperative long-chain ω-3 polyunsaturated fatty acid (n-3 PUFA)

supplementation reduces the occurrence of POAF after cardiac surgery.[4, 12] The 1,516 patients enrolled in this trial were in sinus rhythm at the time of surgery. POAF was defined as "any documented AF or atrial flutter following cardiac surgery of at least 30 seconds duration and documented by rhythm strip or 12-lead electrocardiogram (ECG). If only a shorter duration ECG is available, then the diagnosis of AF/atrial flutter is based on the arrhythmia being present at onset or termination". The time period of follow-up for all arrhythmia endpoints was from the time of cardiac surgery until hospital discharge or post-op day 10, whichever occurred first.[4] The protocol specified collection of blood samples before, during, and after surgery, and biopsy of the right atrial appendage (RAA) at the time of surgery.

As a pre-specified substudy, RAA samples were prospectively collected during cardiac surgery from 292 OPERA patients. Out of these, 239 cases had RAA biopsies suitable for a reliable histopathological analysis and were the object of the present study. The baseline characteristics of this subsample was similar to the overall OPERA trial population (Supplementary Table 1). All histological and biochemical analyses were performed centrally by skilled personnel unaware of patient characteristics, study treatment or outcomes.

This study was approved by the IRB of all participating institutions and conducted according to international standards of Good Clinical Practice (FDA Title 21 part 312, International Conference on Harmonization guidelines). All patients provided written informed consent.

Additional details are reported in the *Data Supplement*.

Histopathologic and morphometric methods

After excision, tissue specimens were fixed in 10% buffered formalin solution for at least 24 hours and then embedded in paraffin tissue blocks. From each block, 4 μm-thick histological

sections were cut for histopathological analyses aimed at exploring the most common changes observed in the setting of AF structural remodeling. RAA samples were initially observed in hematoxylin-eosin stained sections to evaluate general tissue morphology (Figures 1A-1B). To quantitate cardiomyocyte hypertrophy, the transverse diameters of 100 longitudinally oriented cardiomyocytes were measured (through the nucleus region) in PAS-stained sections using Cell^F software (Olympus Soft Imaging Solutions, version 2.6) (Figures 1C-1D)[13]. The presence of severe perinuclear myofibril loss in cardiomyocytes (so-called "myocytolysis") was also determined in the same PAS-stained sections[13]. The percentage of cardiomyocytes showing myocytolysis was assessed at a magnification of x400. Accumulation of glycogen in the perinuclear area in cells showing myocytolysis is an indicator of a metabolic shift from the use of fatty acids to glucose. The presence of glycogen granules was evaluated at a magnification of x400 and scored semi-qualitatively into three categories: *none* (absent), *mild-moderate*, *severe*. Examples are shown in Figure 2.

The percentage of myocardium occupied by interstitial fibrosis was assessed at a magnification of x400 in Van Gieson-stained sections (Figure 3). To quantify fibrosis, a light microscope equipped with an ocular reticule containing 42 sampling points was used. The fraction of points overlying interstitial fibrosis was accurately counted and multiplied by 100.[14]

Connexin 43 distribution

A primary antibody against the major myocardial gap junction protein connexin 43 (Cx43; SIGMA Aldrich, polyclonal, code C6219, dilution 1:400, overnight at 4°C) was used to characterize the distribution of gap junctions in cardiomyocytes.

Detailed descriptions of the standard immunofluorescence methods have previously been published.[15, 16] Immunopositive signals were detected using a Cy3-conjugated goat anti-rabbit

(H+L) (Jackson Immunoresearch, Cambridgeshire, UK; catalog number 111-165-144; dilution 1:400) and the localization of Cx43 was qualitatively evaluated through a confocal microscope (Zeiss 510, Belgium) at an original magnification of x40. No attempt was made to quantify immunoreactive signals. Rather, we optimized the staining protocol to identify the lowest antibody concentration that shows a strong signal at intercalated disks in normal myocardial samples. We then assessed changes in the amount and/or distribution of signal in the tissue samples. As shown in Figure 4, Cx43 signal was categorized as a) *control-like*, when strong signal occurred in a normal junctional distribution; b) *depressed*, when the amount of signal was reduced; c)*heterogeneous*, if both a) and b) conditions coexisted; or d) *absent*. The extent to which Cx43 immunoreactive signal was redistributed to the lateral borders of cardiomyocytes was categorized as either *minimal/absent* or *present*.

Statistical analyses

Multivariate linear and logistic regression analyses were employed to assess the relation of circulating markers [brain batriuretic peptide (BNP), troponin, C-reactive protein (CRP), plasma and urine F2-isoprostanes, F3-isoprostanes, isofurans] with the atrial histopathological endpoints. Detailed description of the performed analyses is described in the *Supplement Material.*

Supplementary Methods

Methods regarding assay of circulating cardiac markers and oxidative stress biomarkers are detailed in the *Supplement Material*.

Results

Characteristics of patients undergoing atrial biopsy

RAA tissue samples were obtained from 239 patients, of whom 141 were American participants and 98 Italian patients. Characteristics of the overall population and those in the current histological sub-study were similar and are summarized in Supplementary Table 1.

The mean age of study patients was 64.8±12.6 years; 72.4% were male, and 53/239 patients (21.2%) had heart failure, 8% had experienced previous AF, 17.2% had prior percutaneous coronary interventions (PCI), and 4.3% had undergone previous cardiac surgery. The median logistic EuroSCORE (European System for Cardiac Operative Risk Evaluation) value was 3.8 (interquartile range [IQR], 1.9-8.4). Valve surgery was performed in 58.8% of patients; see also Table 1.

Eighty-four patients (35.2%) developed POAF. Compared to patients who did not develop POAF, these patients were older (67.2 vs. 63.5, p=0.03) and more likely to have chronic obstructive pulmonary disease (22.6% vs 12.3%, p=0.04). Further, they presented more often with heart failure (29.3% vs 18.2%, p=0.04), experienced more prior AF episodes (17.9% vs 2.6%, p<0.001), and had a higher EuroSCORE (4.8 vs 3.1, p=0.04). They were also likely to have undergone valve surgery (69.1% vs 53.3%, p=0.02), compared to those in the non-POAF group. Left atrial size (41.5 vs 42.2, p=0.56) and left ventricular ejection fraction (58.4 vs 57.3, p=0.47) were comparable between the two groups (Table 1).

Histopathological, morphometric and Connexin 43 analyses

None of the histological variables (amount of interstitial fibrosis, cardiomyocyte myocytolysis, cardiomyocyte diameter, glycogen score, Cx43 distribution analysis**)** significantly differed between POAF and non-POAF patients (Figures 1-4 and Table 2). In a multivariable logistic

regression model adjusted for all statistically significant clinical variables and including all histological features, the following factors independently associated with incident POAF: age (OR: 1.05 95% CI: 1.01-1.09, p=0.021), history of prior AF (OR: 11.6, 95% CI: 2.26-59.0, p=0.003), and congestive heart failure (OR=0.37, 95%CI:0.16-0.85, P=0.018). None of the histological features (e.g. fibrosis, myocytolysis, cardiomyocyte diameter, glycogen accumulation, Cx43 patterns) reached statistical significance. A non-significant trend was seen between presence of Cx43 lateralization at the time of cardiac surgery and lower future risk of POAF (OR=0.460, 95% CI=0.191-1.104, P=0.084).

We also explored the univariate relationships between baseline characteristics of the population and the histological features of the right atrium at the time of cardiac surgery. Patients with angina pectoris had a higher fibrosis percentage (p=0.034; Supplementary Table 2); men had more myocytolysis than women (p=0.022); and patients with diabetes mellitus showed more perinuclear myofibril loss and moderate accumulation of glycogen (p=0.022, p=0.003 respectively). Cx43 junctional signal (Figure 4) was depressed at intercalated disks in patients with COPD (p=0.006) and with higher EuroSCORE index (p=0.043). Interestingly, patients randomized to the n-3 PUFA treatment had less frequent lateralization of Cx43, compared with the placebo group (p=0.035) (data not shown). These exploratory analyses were not corrected for multiple comparisons and should be interpreted with caution.

Since the administration of n-3 PUFA correlated with reduced Cx43 lateralization, we determined if it also impacted POAF development in the subgroup in which Cx43 lateralization was present. However, compared with placebo, n-3 PUFA treatment did not significantly alter the POAF risk in the 202 patients with Cx43 present (p=0.656)

Histopathological features and biomarker concentrations

In multivariable-adjusted analyses, biomarker levels of cardiac strain, inflammation, and oxidative stress were generally not significantly associated with histopathological features of the right atrium at the time of cardiac surgery (Tables 3 and 4). The most notable association was an inverse association between N-terminal pro brain natriuretic peptide (NT-proBNP) levels and those of myocytolysis (p=0.001): mean NT-proBNP levels were about twice as high in the lowest tertile of myocytolysis, compared to the highest tertile.

Discussion

POAF and its currently-known predisposing factors

POAF commonly complicates cardiac surgery, and represents a major contributor to post-operative morbidity and increased length of stay and other healthcare utilization.[1] Its incidence has increased significantly over the few past decades, likely because of the aging of the patient populations undergoing cardiac surgery. Despite substantial investigation, the pathologic and pathophysiologic variables that can reliably predict development of POAF and might provide mechanistic insight into its pathogenesis remain elusive.[17]

POAF onset peaks on the second post-operative day, which suggests a key role for acute surgery-induced factors.[17] Inflammation and oxidative stress related to atrial trauma during surgery, systemic inflammation, and stress related to cardiopulmonary bypass may all contribute to supraventricular arrhythmogenesis. Atrial enlargement due to intravenous fluid administration post-operatively may also predict POAF. All these early changes have the potential of creating atrial conduction disturbances and enhancing triggers for POAF.

In addition to the above-mentioned factors directly related to surgery, various features reflecting chronic cardiac remodeling might increase POAF risk by creating anatomical substrates that promote arrhythmia maintenance. Aging itself correlates strongly with POAF presumably due, at least in part, to age-related collagen deposition within atrial myocardium which may favor fragmentation and reentrant arrhythmogenesis. Associated co-morbidities, such as systolic dysfunction, hypertension, COPD, and obesity, may also increase POAF risk.

Major strengths of the present investigation

The present investigation – an ancillary study to the large, placebo-controlled, double-blind randomized OPERA clinical trial – is the most comprehensive prospective evaluation to-date of how key structural and molecular characteristics of the right atrial myocardium at the time of surgery relate to the subsequent risk of POAF. Prior studies of myocardial histopathology and AF have nearly always been cross-sectional, i.e., with myocardial sampling occurring after AF is already present; and utilized convenience samples rather than a more general, prospectively ascertained population of patients undergoing cardiac surgery. Major strengths of the present study include (a) analysis of the largest number of prospectively collected (prior to AF onset) atrial tissue samples; (b) analysis of a defined, well characterized multi-center patient population in sinus rhythm at the time of cardiac surgery (previously, it has been impossible to distinguish changes in tissue structure that increased risk of AF from those associated to persistent AF); and (c) the broad inclusion criteria of OPERA and its multi-center, multi-country design which maximized generalizability of our findings to the real-world population of patients, while minimizing potential bias from patient selection. Other strengths include participation by two experienced cardiac pathologists who were members of the OPERA Biological Studies Committee and have expertise in morphological/immuno-histochemical analysis of atrial

myocardium and anatomic substrates of cardiac arrhythmias, ensuring a coordinated and rigorous approach; and a generalizable patient population with overall characteristics and incidence of POAF resembling those reported in numerous previous large patient samples.

POAF occurrence and the levels of circulating cardiac/oxidative stress biomarkers are not correlated with the structural remodeling of the RAA

Despite the above-mentioned strengths, this investigation did not demonstrate any consistent statistically significant relationships between structural remodeling of the RAA (e.g., interstitial fibrosis, percentage of myocytolytic cardiomyocytes, muscle-cell hypertrophy, Cx43 distribution) and development of POAF. Similarly, levels of circulating cardiac biomarkers (e.g., NT-proBNP, high-sensitivity cardiac troponin T, high sensitivity C-reactive protein)[18] and oxidative stress biomarkers (e.g., F2-isoprostanes, F3-isoprostanes, isofurans)[19] did not correlate with the presence of these histopathologic abnormalities. One exception is the counterintuitive highly significant relation (Table 3, p=0.003) between plasma F2-isoprostanes and fibrosis. This is, most likely, a chance finding attributable to the multiplicity of comparisons.

To our knowledge, the present study represents the largest prospective investigation to-date of these crucial relationships in cardiac surgery patients. Yet, while the sample size of nearly 250 patients is impressive for a prospective collection, it may have been insufficient to convincingly detect more subtle relationships between structural remodeling of the RAA, biomarkers of cardiac stress and inflammation, and risk of POAF. The findings relating to Cx43 lateralization, randomized n-3 PUFA treatment, and POAF; to NT-proBNP and myocytolysis; and to other associations that did not achieve statistical significance, provide an important foundation for further hypothesis generation and subsequent prospective studies to elucidate the crucial unanswered questions on atrial structural remodelling and risk of POAF.

Review of the literature

With these issues in mind, we reviewed 49 clinical and experimental studies focusing on the relationship between RAA structural remodeling and, broadly speaking, AF (either post-operative or non-surgery associated; Table 5 and Table 6, respectively). This included 19 studies on POAF and 30 (5 in animal models) on non-POAF supraventricular arrhythmia. Studies on POAF assessed the predictive value of histological alterations for incident AF, while in the other 30 studies associations between ongoing AF and histological alterations were assessed. Specific experimental designs differed, and relatively few studies comprehensively investigated multiple aspects of structural remodeling of the RAA including fibrosis, myocytolysis, cardiomyocyte hypertrophy, and connexins. The results varied considerably regarding specific histopathological features that correlated with AF. None of these previous studies were double-blind, placebo-controlled, multinational, and randomized in nature as the present OPERA sub-study was and in which these biases were minimized. These methodological differences may be largely responsible for the higher frequency of significant relationships between histological features and risk of POAF seen in previous studies. Another concern involves use of automatic measurements, used in many prior studies, which may incorrectly classify and overestimate the extent of histological changes. In this regard, 19 previous studies (39%) used automatic morphometry methods, 8 (16%) used semi-automatic measurements, and 22 (49%) involved non-automatic (manual) histopathological evaluations. Only 13 of 19 investigations on POAF (68%) and 6 of 30 studies on non-POAF (20%) employed multivariate data analysis. Finally, the sample size of the various populations under investigation differed substantially between studies (96±55, range 24-259 in POAF manuscripts; 72±59, range 8-245 in non-POAF papers).

Limitations of the study

One potential limitation was the RAA sampling site, selected for both ethical and practical reasons in this large trial. While the RAA would be expected to undergo remodeling in response to AF, which involves the entire atrial myocardium, it may not exhibit the full range of pathogenic changes in a pre-AF stage as it may be more protected than the left atrium from the effects of many AF-associated conditions. Only a minority of OPERA patients had isolated right heart dysfunction. In addition, AF usually arises in the pulmonary veins and then progressively affects the left atrium, especially its posterior wall.[20] Thus, the RAA may not be an ideal biopsy site to identify structural and molecular determinants of POAF in patients with mainly left-sided cardiac disease.

Conclusions

In conclusion, in this large, prospective clinico-pathological analysis of patients in sinus rhythm undergoing cardiac surgery, histopathological features of the RAA at the time of surgery did not significantly correlate with the subsequent development of POAF, and baseline biomarkers of cardiac inflammation and oxidative stress also generally did not associate with the RAA histopathological features. These novel findings provide an important foundation for further hypotheses and prospective studies to elucidate the crucial unanswered questions on atrial structural remodeling and POAF risk.

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Table 1. Baseline characteristics prior to cardiac surgery in relation to Post-Operative Atrial Fibrillation.

Variables		Total	Non-POAF	POAF	P*
N (%)		239	155 (64.8%)	84 (35.2%)	
Age		64.8 ± 12.6	63.5 ± 13.3	67.2 ± 10.7	**0.028**
Sex	Female	66 (27.6%)	41 (26.5%)	25 (29.8%)	0.585
	Male	173 (72.4%)	114 (73.6%)	59 (70.2%)	
Treatment	Placebo	122 (51.0%)	80 (51.6%)	42 (50.0%)	0.812
	n-3 PUFA	117 (49.0%)	75 (48.4%)	42 (50.0%)	
Current smoking		25 (10.6%)	17 (11.1%)	8 (9.6%)	0.726
COPD		38 (15.9%)	19 (12.3%)	19 (22.6%)	**0.037**
Diabetes Mellitus		72 (30.1%)	45 (29.0%)	27 (32.1%)	0.617
Hypertension		196 (82.0%)	123 (79.4%)	73 (86.9%)	0.147
Chronic renal failure		11 (4.6%)	8 (5.2%)	3 (3.6%)	0.751
Prior myocardial infarction		57/236 (24.2%)	38/154 (24.7%)	19/82 (23.2%)	0.797
Angina pectoris		51/208 (24.5%)	30/134 (22.4%)	21/74 (28.4%)	0.336
Heart Failure		53 (22.3%)	28 (18.2%)	25 (29.8%)	**0.040**
Previous PCI		41 (17.2%)	25 (16.1%)	16 (19.1%)	0.568
Previous AF		19 (8.0%)	4 (2.6%)	15 (17.9%)	**<0001**
Prior cardiac surgery†		10 (4.3%)	6 (3.9%)	4 (4.8%)	0.744
Coronary bypass		5 (2.1%)	5 (3.2%)	0	1.000
Valve surgery		3 (1.3%)	1 (0.7%)	2 (2.4%)	0.283
Other cardiac surgery		3 (1.3%)	1 (0.7%)	2 (2.4%)	0.283
LA size (mm)		41.8 ± 7.2	41.5 ± 7.1	42.2 ± 7.5	0.562
LVEF(%)		58.0 ± 10.4	58.4 ± 11.0	57.3 ± 9.3	0.469
B-Blockers		113 (53.6%)	70 (51.1%)	43 (58.1%)	0.330
Statins		113 (53.6%)	71 (51.8%)	42 (56.8%)	0.493
ACE or ARB		116 (55.0%)	74 (54.0%)	42 (56.8%)	0.702
Diuretics		58 (27.5%)	40 (29.2%)	18 (24.3%)	0.449
ASA		128 (60.7%)	81 (59.1%)	47 (63.5%)	0.533
Antiarrhythmics		9 (4.3%)	3 (2.2%)	6 (8.1%)	0.069
EuroSCORE	Add-scale	5.1 ± 3.0	4.8 ± 3.1	5.7 ±2.7	**0.041**
	Log-scale	3.8 [1.9 – 8.4]	3.1 [1.6 – 7.9]	4.8 [2.3 – 8.6]	**0.040**
Valve Surgery	No	98/238 (41.2%)	72/154 (46.8%)	26/84 (30.9%)	0.018
	Yes	140/238 (58.8%)	82/154 (53.3%)	58/84 (69.1%)	
Pump time (hours)		1.64 ± 0.69	1.59 ± 0.71	1.74 ± .64	0.105
Cross time (hours)		1.20 ± 0.54	1.16 ± 0.57	1.29 ± 0.48	0.072
Cardioplegia		232 (97.5%)	148 (96.1%)	84 (100.0%)	0.093
Blood transfusion		98 (41.2%)	63 (40.9%)	35 (41.7%)	0.910

* P-value for Chi^2 Test (discrete variables), ANOVA (normally distributed continuous variables) or Kruskal-Wallis (not normally distributed continuous variables)
† Prior cardiac surgery ≠ coronary bypass + valve surgery + other cardiac surgery, one patient had multiple surgeries.

Table 2. Histological characteristics at the time of cardiac surgery in relation to subsequent risk of Post-Operative Atrial Fibrillation.

		All Patients	Non-POAF	POAF	P* univariate	OR (95% CI) multivariable	P†
Fibrosis (%)		8.21 [5.02–12.65]	8.34 [5.28–12.65]	7.55 [4.51–12.50]	0.356	0.995 (0.959-1.032)	0.793
Myocytolysis (%)		3.9 [1.7–8.4]	4.0 [1.8–8.8]	3.7 [1.3–6.9]	0.277	0.974 (0.914-1.039)	0.427
Cardiomyocytes Diameter (µm)		14.8 ± 2.6	14.9 ± 2.6	14.7 ± 2.6	0.559	0.915 (0.810-1.034	0.155
Glycogen accumulation	None	58 (26.1%)	35 (23.3%)	23 (31.9%)	0.346	Reference	
	Mild-Moderate	147 (66.2%)	104 (69.3%)	43 (59.7%)		0.628 (0.312-1.263)	0.192
	Severe	17 (7.7%)	11 (7.3%)	6 (8.3%)		0.865 (0.256-2.921)	0.815
Cx43 Laterization	Little/Absent	30 (12.9%)	17 (11.3%)	13 (16.1%)	0.300	Reference	
	Present	202 (87.1%)	134 (88.7%)	68 (83.9%)		0.460 (0.191-1.104)	0.082
Cx43 Junctional	Control-like	139 (59.9%)	97 (64.2%)	42 (51.8%)	0.141	Reference	
	Depressed	77 (33.2%)	46 (30.5%)	31 (38.3%)		1.503 (0.785-2.877)	0.219
	Heterogeneous	16 (6.9%)	8 (5.3%)	8 (9.9%)		2.174 (0.682-6.932)	0.189
Connexin 43	Normal + Normal	11 (4.8%)	9 (6.0%)	2 (2.5%)	0.566	Reference	
	Normal + Abnormal	127 (64.8%)	87 (64.9%)	40 (64.5%)		1.139 (0.222-5.840)	0.876
	Abnormal + Abnormal	58 (29.6%)	38 (28.4%)	20 (32.3%)		1.264 (2.33-6.865)	0.786

* P-value for Chi^2 (discrete variables), ANOVA (normally distributed continuous variables) or Kruskal-Wallis (not normally distributed continuous variables).
† P-value for multivariable Logistic regression adjusted for age, COPD, atrial fibrillation, congestive HF, valvular surgery and log EuroSCORE.
Three classes of Connexin 43: normal pattern (patients with control Cx43 junctional and little/absent Cx43 lateralization); normal + abnormal pattern (patients with control Cx43 junctional and present Cx43 lateralization or patients with depressed Cx43 junctional and little/absent Cx43 lateralization); abnormal pattern (patients with depressed Cx43 junctional and present Cx43 lateralization; or patients with heterogeneous Cx43 junctional and present Cx43 lateralization).

Table 3. Pre-operative levels of biomarkers of cardiac strain, inflammation, and oxidative stress in relation to tertiles of continuous histopathological features of the right atrium at the time of cardiac surgery.

Fibrosis (%)		**Tertile 1 N=79** (2.00-5.99) Median [IQR]	**Tertile 2 N=80** (6.00-10.59) Median [IQR]	**Tertile 3 N=80** (10.59-65.83) Median [IQR]	**P***	Adjusted unit difference **Beta (95%CI)****	**P†**
plasma	NT-proBNP	271 [120-853]	255 [95 - 615]	463 [188 - 888]	0.152	1.50 (-0.74-3.74)	0.187
	Hs cTnT	10.7 [5.9-18.5]	10.8 [6.3 – 21.1]	14.3 [9.0 - 23.8]	0.109	1.71 (-0.87–4.29)	0.193
	Hs-CRP	2.7 [1.0-5.2]	2.1 [1.0 - 5.9]	2.8 [1.1 - 6.9]	0.426	1.00 (-1.16–3.17)	0.363
	F2-isoprostanes	41 [31-52]	40 [31 - 56]	41 [31 - 51]	0.962	-7.71 (-12.84-2.59)	**0.003**
	F3-isoprostanes	1.6 [0.5-3.3]	1.0 [0.5 – 3.0]	1.5 [0.5 – 4.0]	0.852	1.94 (-0.73–4.60)	0.154
	Isofurans	37 [28-51]	35 [25 - 50]	36 [20 - 53]	0.578	-0.99 (-4.49–2.52)	0.580
urine	F2-isoprostanes	1.16 [0.77-2.03]	1.19 [0.95 - 1.96]	1.15 [0.81 - 1.47]	0.297	-1.81 (-6.71–3.10)	0.468
	F3-isoprostanes	0.11 [0.05-0.19]	0.12 [0.07 - 0.23]	0.13 [0.07 - 0.19]	0.748	1.69 (-0.82–4.19)	0.185
	Isofurans	1.79 [1.19-2.60]	1.74 [1.28 - 2.43]	1.80 [1.08 - 2.29]	0.774	-1.11 (-5.65–3.43)	0.629
Myocytolysis (%)		**Tertile 1 N=74** (0.00 – 2.22) Median [IQR]	**Tertile 2 N=74** (2.23 – 6.05) Median [IQR]	**Tertile 3 N=74** (6.05 – 26.85) Median [IQR]	**P***	Adjusted unit difference **Beta (95%CI)****	**P†**
plasma	NT-proBNP	403 [175-1036]	292 [140 - 951]	203 [69 - 542]	**0.012**	-2.24 (-3.59—0.90)	**0.001**
	Hs cTnT	11.3 [6.9-16.6]	10.0 [5.9 - 20.9]	12.5 [7.7 - 21.6]	0.529	-0.08 (-1.67 – 1.51)	0.919
	Hs-CRP	2.1 [1.0-5.7]	2.9 [0.9 – 7.0]	2.1 [1.0- 5.7]	0.868	0.07 (-1.27 – 1.41)	0.922
	F2-isoprostanes	44 [33-55]	39 [30 - 50]	38 [29 - 52]	0.181	-0.68 (-4.36 – 2.99)	0.714
	F3-isoprostanes	1.0 [0.5–4.0]	2.0 [0.5 – 4.0]	1.0 [0.5 - 3.2]	0.144	-0.45 (-2.16 – 1.25)	0.602
	Isofurans	35 [25-54]	38 [23 - 53]	35 [27 - 49]	0.783	0.09 (-2.09 – 2.28)	0.934
urine	F2-isoprostanes	1.16 [0.83-1.57]	1.16 [0.80 - 1.79]	1.18 [0.82 - 1.88]	0.859	1.58 (-1.31 – 4.46)	0.281
	F3-isoprostanes	0.12 [0.07-0.22]	0.13 [0.06 - 0.21]	0.10 [0.05 - 0.19]	0.404	-1.25 (-2.68 – 0.19)	0.089
	Isofurans	1.74 [1.19-2.45]	1.88 [1.45 - 2.60]	1.74 [1.11 - 2.29]	0.672	-0.10 (-2.78 – 2.58)	0.942
Cardiomyocyte diameter, µm		**Tertile 1 N=74 (8.44 – 13.75) Median [IQR]**	**Tertile 2 N=74 (13.76 – 15.67) Median [IQR]**	**Tertile 3 N=74 (15.72 – 23.42) Median [IQR]**	**P***	Adjusted unit difference **Beta (95%CI)****	**P†**
plasma	NT-proBNP	246 [124 - 861]	271 [120 - 888]	336 [158 - 713]	0.860	-0.36 (-1.08 – 0.36)	0.325
	Hs cTnT	10.1 [6.2 - 16.1]	9.3 [5.7 - 19.9]	15.6 [8.3 - 22.4]	**0.016**	0.78 (-0.04 – 1.60)	0.063
	Hs-CRP	1.3 [0.8 - 6.6]	2.1 [1.1 - 5.2]	3.1 [1.7 - 9.2]	**0.030**	0.68 (-0.02 – 1.37)	0.056
	F2-isoprostanes	42 [32 - 63]	40 [31 - 49]	40 [31 - 51]	0.337	-0.35 (-2.27 – 1.56)	0.715
	F3-isoprostanes	2.0 [0.5 – 4.0]	1.0 [0.5 – 3.0]	1.0 [0.5 - 3.3]	0.214	-0.26 (-1.14 – 0.63)	0.568
	Isofurans	36 [28 - 54]	33 [21 - 50]	37 [26 - 50]	0.428	-0.22 (-1.36 – 0.91)	0.697
urine	F2-isoprostanes	1.18 [0.84 - 1.59]	1.14 [0.75 - 1.75]	1.29 [0.94 - 1.92]	0.261	0.94 (-0.60 – 2.48)	0.230
	F3-isoprostanes	0.13 [0.06 - 0.24]	0.09 [0.05 - 0.15]	0.14 [0.09 - 0.22]	0.056	0.58 (-0.22 – 1.37)	0.153
	Isofurans	1.81 [1.20 - 2.56]	1.56 [1.16 - 2.17]	2.03 [1.46 - 2.61]	0.136	0.55 (-0.88 – 1.99)	0.446

* P for Kruskall Wallis test. † All biomarkers were log-transformed and included in linear regression analysis with histopathological feature as dependent adjusted for age, COPD, history of atrial fibrillation, heart failure, valve surgery and EuroSCORE log. Beta represents the % unit difference in histopathologic features for continuous outcomes for a doubling in levels of each biomarker.

Table 4. Pre-operative levels of biomarkers of cardiac strain, inflammation, and oxidative stress in relation to categories of histopathological features of the right atrium at the time of cardiac surgery.

Glycogen accumulation		**None N=58**	**Mild-Moderate N=147**	**Severe N=17**	**P***	**Adjusted OR (95% CI)** Mild-Moderate+Severe vs none	**P†**
plasma	NT-proBNP	308 [132 - 674]	266 [123 - 853]	625 [77 – 1132]	0.966	0.85 (0.47-1.54)	0.600
	Hs cTnT	9.3 [5.7 - 14.5]	14.0 [7.4 - 23.2]	9.0 [5.2 - 45.9]	**0.023**	2.46 (1.02-5.92)	**0.045**
	Hs-CRP	1.4 [0.9 - 5.4]	2.7 [1.1 - 6.7]	2.6 [1.6 - 6.6]	0.118	1.76 (0.96-3.22)	0.068
	F2-isoprostanes	44 [32 - 55]	39 [30 - 51]	42 [34 - 52]	0.565	0.36 (0.07-1.80)	0.214
	F3-isoprostanes	1.0 [0.5 – 4.0]	1.6 [0.5 - 3.6]	2.0 [0.5 – 4.0]	0.865	1.22 (0.59-2.55)	0.591
	Isofurans	34 [20 - 59]	36 [27 - 50]	45 [32 - 53]	0.371	1.57 (0.59-4.22)	0.371
urine	F2-isoprostanes	1.18 [0.88 - 1.59]	1.18 [0.81 - 1.66]	1.12 [0.88 - 2.02]	0.960	0.78 (0.22-2.76)	0.695
	F3-isoprostanes	0.13 [0.08 - 0.24]	0.11 [0.05 - 0.19]	0.07 [0.01 - 0.12]	0.054	0.29 (0.13-0.67)	**0.004**
	Isofurans	1.77 [1.36 - 2.25]	1.82 [1.17 - 2.53]	1.75 [1.48 - 2.47]	0.908	1.05 (0.32-3.44)	0.940
Cx43 Laterization		**Little / absent N=30**	**Present N=202**		**P***	**Adjusted OR (95% CI)**	**P†**
plasma	NT-proBNP	459 [237 - 760]	267 [125 - 847]		0.368	0.78 (0.21 -1.11)	0.087
	Hs cTnT	12.7 [5.9 - 15.7]	11.3 [6.9 - 21.8]		0.269	1.47 (0.51-4.21)	0.471
	Hs-CRP	3.1 [1.0 - 5.7]	2.3 [1.0 - 6.3]		0.977	1.05 (0.47-2.35)	0.904
	F2-isoprostanes	39 [33 - 53]	41 [31 - 52]		0.683	0.27 (0.04-1.78)	0.174
	F3-isoprostanes	3.0 [0.5 – 4.0]	1.2 [0.5 - 3.2]		0.191	0.67 (0.26-1.70)	0.400
	Isofurans	39 [28 - 59]	36 [25 - 50]		0.233	0.42 (0.13-1.36)	0.145
urine	F2-isoprostanes	1.21 [0.8 - 2.02]	1.18 [0.83 - 1.64]		0.751	1.13 (0.20-6.44)	0.888
	F3-isoprostanes	0.19 [0.1 - 0.34]	0.12 [0.06 - 0.19]		**0.039**	0.31 (0.08-1.16)	0.081
	Isofurans	1.61 [1.01 - 2.60]	1.78 [1.23 - 2.30]		0.330	2.68 (0.59-12.27)	0.205
Cx43 Junctional		**Control like N=139**	**Depressed N=77**	**Heterogeneous N=16**	**P***	**Adjusted OR (95% CI)** Depressed vs Control-like	**P†**
plasma	NT-proBNP	273 [108 - 759]	323 [158 - 875]	304 [180 – 1024]	0.406	1.41 (0.77-2.56)	0.262
	Hs cTnT	12.7 [7.0 - 22.4]	10.9 [7.1 - 20.3]	5.8 [5.0 - 16.5]	0.351	0.90 (0.46-1.78)	0.767
	Hs-CRP	2.6 [1.1 – 7.0]	2.6 [1.0 - 6.5]	1.4 [0.7 - 2.4]	0.142	0.85 (0.48-1.48)	0.558
	F2-isoprostanes	40 [31 - 51]	41 [31 - 57]	41 [30 - 44]	0.479	2.45 (0.56-10.80)	0.235
	F3-isoprostanes	1.0 [0.5 – 4.0]	2.0 [0.5 – 4.0]	0.5 [0.5 – 3.0]	0.125	1.50 (0.75-3.02)	0.251
	Isofurans	36 [23 - 50]	36 [29 - 56]	28 [22 - 43]	0.371	1.30 (0.49-3.42)	0.598
urine	F2-isoprostanes	1.23 [0.94 - 1.72]	1.08 [0.64 - 1.94]	0.99 [0.55 - 1.52]	0.133	0.32 (0.09-1.17)	0.084
	F3-isoprostanes	0.12 [0.07 - 0.20]	0.12 [0.06 - 0.24]	0.12 [0.07 - 0.16]	0.943	0.95 (0.49-1.86)	0.889
	Isofurans	1.86 [1.31 - 2.31]	1.73 [1.00 - 2.74]	1.46 [1.05 - 1.77]	0.211	0.81 (0.25-2.58)	0.717
Connexin 43		**Normal+normal N=11**	**Normal + Abnor N=146**	**Ab + Abnormal N=74**	**P***	**Adjusted OR (95% CI)** Normal+Abnor vs Ab+Abnormal	**P†**
plasma	NT-proBNP	459 [273 - 674]	255 [115 - 791]	308 [174 - 1012]	0.408	1.41 (0.72-2.75)	0.310
	Hs cTnT	7.6 [4.9 - 16.0]	12.7 [7.0 - 21.6]	10.1 [6.4 - 20.7]	0.564	1.11 (0.51-2.45)	0.788
	Hs-CRP	5.2 [0.7 - 5.7]	2.3 [1.1 – 7.0]	2.3 [1.0 - 5.2]	0.720	0.88 (.048-1.64)	0.696
	F2-isoprostanes	39 [35 - 51]	41 [31 - 54]	42 [31 - 52]	0.928	2.34 (0.40-13.86)	0.349
	F3-isoprostanes	3.0 [0.5 – 4.0]	1.0 [0.5 – 4.0]	2.0 [0.5 - 3.3]	0.764	1.61 (0.74-3.48)	0.231
	Isofurans	35 [20 - 55]	37 [25 - 53]	35 [25 - 51]	0.872	1.11 (0.36-3.40)	0.862
urine	F2-isoprostanes	1.55 [1.21 - 2.02]	1.18 [0.85 - 1.58]	1.11 [0.69 - 1.75]	0.131	0.46 (0.11-1.97)	0.296
	F3-isoprostanes	0.32 [0.16 - 0.34]	0.11 [0.07 - 0.19]	0.12 [0.06 - 0.19]	0.064	0.97 (0.48-1.98)	0.939
	Isofurans	1.83 [1.08 - 2.60]	1.83 [1.31 - 2.29]	1.73 [1.00 - 2.43]	0.615	0.66 (0.16-2.70)	0.566

*P for Kruskal Wallis test. † Biomarkers were log-transformed and included in logistic regression analysis with the histopathological feature dependent adjusted for age, COPD, history of Atrial Fibrillation, heart failure, valve surgery and EuroSCORE log. Three classes of Connexin 43: normal pattern (patients with control Cx43 junctional and little/absent Cx43 lateralization); normal + abnormal pattern (patients with control Cx43 junctional and present Cx43 lateralization or patients with depressed Cx43 junctional and little/absent Cx43 lateralization); abnormal pattern (patients with depressed Cx43 junctional and present Cx43 lateralization; or patients with heterogeneous Cx43 junctional and present Cx43 lateralization).

Table 5. Literature analysis of papers focused on Post-Operative Atrial Fibrillation, subdivided by methods of histological evaluations.

	Total	Methods of histological analysis		
		Automatic	Semi-automatic	No Automatic
Nr of papers, n	19	4	2	13
Nr of patients, n, mean±SD (total)	96±55 (1828)	64±34 (257)	84±72 (168)	108±58 (1403)
Nr of patients with POAF, n (%), mean±SD	530 (29), 28±17	81 (32) 20±8	98 (58) 49±48	351 (25) 27±12
Age overall (years), mean±SD	63±7	62±13	58±12	65±3
Age of POAF (years), mean±SD	68±7	74±0	61±17	69±4
Sex (Male), n (%)	1200 (66)	189 (74)	80 (48)	931 (66)
Type of surgery, **Coronary bypass, n/n** **Valve surgery, n/n**	 17/19 10/19	 3/4 3/4	 1/2 2/2	 13/13 5/13
Multivariate analysis, n/n	13/19	2/4	2/2	9/13
Biomarkers, n	12/19	1/12	2/2	9/13
Histological variables analyzed				
Fibrosis, n/n	15/19	3/4	2/2	10/13
Fibrosis association with POAF, n/n	8/15 Yes 6/15 No 1/15 not clear	2/3 Yes 1/3 No	2/2 Yes	4/10 Yes 5/10 No 1/10 not clear
Myocytolysis, n/n	8/19	1/4	1/2	6/13
Myocytolysis association with POAF, n/n	7/8 Yes 1/8 No	1/1 Yes 0/1 No	1/1 Yes 0/1 No	5/6 Yes 1/6 No
Hypertrophy of cardiomyocytes, n/n	6/19	1/4	0/2	5/13
Hypertrophy of cardiomyocytes association with POAF, n/n	1/6 Yes 5/6 No	1/1 No	-	4/5 Yes 1/5 No
Cx43, n/n	3/19	2/4	0/2	1/13
Cx43 association with POAF, n/n	1/3 Yes 2/3 No	1/2 Yes 1/2 No	-	0/1 Yes 1/1 No

Automatic: the measurements were performed by using a computer image analyzer; semi-automatic: the measurements were performed manual and with a computer-assisted image analyzer; No Automatic: the measurements were performed manual by the operators.

Table 6. Literature analysis of papers focused on Other Types of Atrial Fibrillation, subdivided by methods of histological evaluations.

	Total	Methods of histological analysis		
		Automatic	Semi-automatic	No Automatic
Nr of papers, n	30	15	6	9
Nr of patients (without animals), n, mean±SD (total)	72±59 (1877)	69±43 (831)	73±58 (367)	75±82 (679)
Nr of animals, n, mean±SD	123, 21±13	87 17±12	36	-
Age(years) of patients, mean±SD	56±9	53±9	57±10	60±7
Age of AF (years), mean±SD	61±11	57±8	70±18	62±9
Sex (Male), n (%)	1061 (57)	486 (58)	229 (62)	346 (51)
Type of surgery **Coronary bypass, n/n** **Valve surgery** **Others**	 6/30 19/30 11/30	 1/15 7/15 6/15	 4/6 2/6	 5/9 8/9 3/9
Multivariate analysis, n/n	6/30	3/15	2/6	2/9
Biomarkers, n	7/30	4/15	2/6	1/9
Histological variables analyzed				
Fibrosis, n/n	20/30	11/15	3/6	6/9
Fibrosis association with POAF, n/n	18/20 Yes 2/20 No	11/11 Yes	3/3 Yes	4/6 Yes 2/6 No
Myocytolysis, n/n	6/30	-	2/6	4/9
Myocytolysis association with POAF, n/n	5/6 Yes 1/6 not clear	-	2/2 Yes	3/4 Yes 1/4 not clear
Hypertrophy of cardiomyocytes, n/n	5/30	1/15	1/6	3/9
Hypertrophy of cardiomyocytes association with POAF, n/n	5/5 Yes	1/1 Yes	1/1 Yes	3/3 Yes
Cx43, n/n	1/30	-	1/6	-
Cx43 association with POAF, n/n	1/1 No	-	1/1 No	-

Automatic: the measurements were performed by using a computer image analyzer; semi-automatic: the measurements were performed manual and with a computer-assisted image analyzer; No Automatic: the measurements were performed manual by the operators.

Figure Legends:

Figure 1. A. Low-power histopathological view of a RAA biopsy with mild-to-moderate hypertrophy of the pectinate muscles (arrow) and moderate subepicardial fibro-adipose thickening (arrowhead). B. A detail of the region in A marked by an asterisk, with moderate-to-severe interstitial fibrosis (arrow). C. Intermediate-power view of a RAA biopsy with moderate interstitial fibrosis and cardiomyocytes with transverse diameters within the normal limits. D. C. Intermediate-power view of a RAA biopsy with moderate interstitial fibrosis and cardiomyocytes with enlarged transverse diameters. Stainings: A-B hematoxylin-eosin; C-D Periodic acid–Schiff. Original magnifications: A x2 (scale bar: 2mm), B-D x20 (scale bar: 150μm).

Figure 2. RAA biopsies including cardiomyocytes (arrows) A. without glycogen deposition, B. with mild glycogen deposition, C. with moderate glycogen deposition, and D. with severe glycogen deposition. Staining: A-D Periodic acid–Schiff (PAS). Original magnifications: A-D x40 (scale bar: 100μm).

Figure 3. RAA myocardial fibrosis. A. Interstitial fibrosis within the normal limits and sparse myocytolytic cardiomyocytes (arrow). B. Mildly increased perivascular fibrosis around deep-penetrating myocardial blood vessels (arrow). C. Mild-to-moderate myocardial interstitial fibrosis (arrow). D. Severe myocardial interstitial fibrosis (arrow). Staining: A-D Van Gieson staining method. Original magnifications: A-B x10 (scale bar: 300μm); C-D x20 (scale bar: 150μm).

Figure 4. Connexin 43 immunosignal. A. Control distribution of Connexin 43 at the intercalated disks. B. Depressed signal at the intercalated disks. C. Profound lateralization of Connexin 43. D. Near-absence Connexin 43 immunosignal. A-D confocal microscopy. Original magnifications: A-D x40 (scale bar: 100μm).

What is Known:

- Post-operative atrial fibrillation (POAF) occurs in 30-50% of patients undergoing cardiac surgery and is associated with increased morbidity and mortality.
- The lack of understanding of the specific preceding anatomic and molecular changes in the atrial myocardium at the time of cardiac surgery that might influence the risk of POAF is a major limitation in developing preventive strategies.

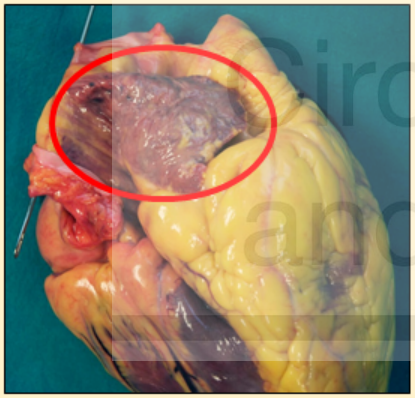
What the Study Adds:

- In 239 patients undergoing cardiac surgery (35.2% developed POAF), right atrial appendage (RAA) was excised, fixed, paraffin embedded and analyzed.
- On top of well-known risk factors for POAF (i.e. advanced age, heart failure, COPD, higher EuroSCORE, valve surgery), none of the histological features (i.e. fibrosis, myocytolysis, cardiomyocyte diameter, glycogen score, Connexin 43 distribution) was independently associated with risk of POAF.
- RAA structural remodeling does not reflect changes in other parts of the atria, shown to contribute to the pathogenesis of POAF.

Graphic Abstract

ClinicalTrials.gov Identifier: NCT00970489

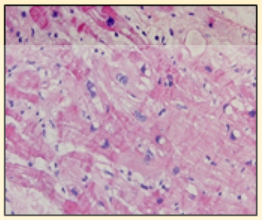
TO INVESTIGATE THE HISTOPATHOLOGY OF RIGHT ATRIAL APPENDAGE (RAA) THAT MIGHT PREDICT POST-OPERATIVE ATRIAL FIBRILLATION (POAF)



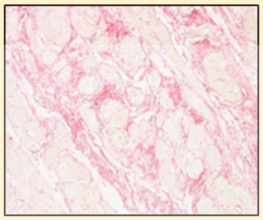
239 RAA BIOPSIES AND BLOOD SAMPLES FROM PATIENTS IN SINUS RHYTHM UNDERGOING CARDIAC SURGERY

HISTOPATHOLOGICAL ANALYSIS

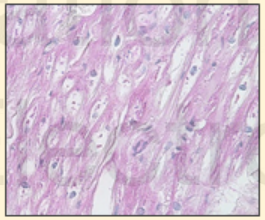
GENERAL MORPHOLOGY



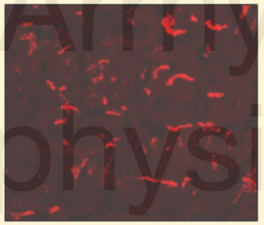
INTERSTITIAL FIBROSIS



HYPERTROPHY MYOCYTOLYSIS



CONNEXIN 43



CIRCULATING BIOMARKERS

HISTOPATHOLOGICAL CHANGES IN THE RAA AND CIRCULATING BIOMARKERS DO NOT PREDICT POAF OCCURRENCE