Peer-reviewed version available at *J. Clin. Med.* **2020**, *9*, 544; doi:10.3390/jcm9020544

Modulation of SERCA in patients with persistent atrial fibrillation treated by epicardial thoracoscopic ablation: the CAMAF Study

Celestino Sardu, MD, MSc, PhD[1, 2]*; Gaetano Santulli, MD, PhD[3,4]; Germano Guerra, MD, PhD[5]; Maria Consiglia Trotta, MD, PhD[1]; Matteo Santamaria, MD, PhD[6]; Cosimo Sacra, MD[7]; Nicola Testa, MD[7];Valentino Ducceschi, MD, PhD[8]; Gianluca Gatta, MD, PhD[1]; Michele D' Amico, MD[6]; Ferdinando Carlo Sasso, MD, PhD[1]; Giuseppe Paolisso, MD[1]; Raffaele Marfella, MD, PhD[1].

1.Department of Advanced Medical and Surgical Sciences, University of Campania "Luigi Vanvitelli"; 2. Department of Medical Sciences, International University of Health and Medical Sciences "Saint Camillus", Rome, Italy; 3. Department of Advanced Biomedical Sciences, International Translational Research and Medical Education Academic Research Unit (ITME), "Federico II" University, Naples, Italy; 4. Department of Medicine, Albert Einstein College of Medicine, Fleischer Institute for Diabetes and Metabolism (FIDAM), Montefiore University Hospital, New York, NY; 5. Department of Medicine and Health Sciences "Vincenzo Tiberio", University of Molise; 6.Department of Experimental Medicine, University of Campania "Luigi Vanvitelli"; 7.Cardiovascular and Arrhythmias Department, Catholic University of Sacred Heart; 8.Cardiovascular and Arrhythmias Department, "Vecchio Pellegrini" Hospital.

Keywords: persistent atrial fibrillation, epicardial ablation, calcium channels, SERCA.

Clinical trial: #NCT04174885.

Ethical Committee: 021.2016

Word count: 3373.

Number of figures: 3.

*** Correspondence:**

Prof. Celestino Sardu, MD, MSc, PhD
Department of Advanced Medical and Surgical Sciences,
University of Campania "Luigi Vanvitelli"
Address: Piazza Miraglia 2, 80131, Naples, Italy.
email**:** drsarducele@gmail.com
Tel: +390815665110. Fax: +390815095303

ABSTRACT

Objectives: To evaluate atrial fibrillation (AF) recurrence and Sarcoplasmic Endoplasmic Reticulum Calcium ATPase (SERCA) levels in patients treated by epicardial thoracoscopic ablation for persistent AF.

Background: Reduced levels of SERCA have been reported in the peripheral blood cells of patients with AF. We hypothesize that SERCA levels can predict the response to epicardial ablation.

Methods: We designed a prospective, multicenter observational study to recruit, from October 2014 to June 2016, patients with persistent AF receiving an epicardial thoracoscopic pulmonary vein isolation.

Results: We enrolled 27 patients; responders patients (n=15) did not present AF recurrence after epicardial ablation at 1-year follow-up. These patients displayed a marked remodeling of the left atrium, with a significant reduction of inflammatory cytokines, B type Natriuretic Peptide (BNP), and over expression of SERCA as compared to baseline and to non-responders ($p<0.05$). Furthermore, mean AF duration (HR 1.235 [1.037-1.471], $p<0.05$), LAV (HR 1.755 [1.126-2.738], $p<0.05$), BNP (HR 1.945 [1.895-1.999], $p<0.05$), and SERCA (HR 1.763 [1.167-2.663], $p<0.05$) were predictive of AF recurrence.

Conclusions: Our data indicate that baseline values of SERCA in patients with persistent AF might be predictive of failure to epicardial ablative approach. Intriguingly, epicardial ablation was associated with increased levels of SERCA in responders. Therefore, SERCA might be an innovative therapeutic target to improve the response to epicardial ablative treatments.

INTRODUCTION

Atrial fibrillation (AF) is the most common arrhythmia worldwide (1). Based on the duration of episodes and the date of onset, AF is defined as paroxysmal, persistent, or permanent (1). Patients with persistent AF have an increased risk of thromboembolic stroke, heart failure, and overall worse prognosis (2). Therefore, in patients with persistent AF catheter ablation is considered a valid therapeutic option to ameliorate clinical outcomes via restoration of sinus rhythm (2,3,4). On the other hand, in patients with persistent AF catheter ablation has a success rate of ~50% at 5 years (5), and a successful epicardial ablation by sinus rhythm restoration induces a reduction of left atrial diameters and volume (6-9). The lack of therapeutic effect in a relatively high percentage of patients limits its clinical application, and might itself be responsible for atrial fibrosis and remodeling, eventually leading to permanent AF (6,7). In this setting, epicardial AF ablation has been proposed to play a role in the modulation of the complex electro-anatomical arrhythmic atrial substrate in patients with persistent AF (6). Multiple hypotheses have been proposed to explain the cases of unsuccessful epicardial ablation. For instance, the advanced atrial electrical/anatomical remodeling (6-9) might enhance abnormal trigger activity and reentry mechanisms (10), which are among the main mechanisms implied in the genesis and perpetuation of persistent AF (11). Intriguingly, alterations in the regulation of intra cellular calcium (Ca^{2+}) have been linked to an abnormal trigger activity and reentry in AF patients (11,12). Moreover, in human atrial myocytes altered Ca^{2+}fluxes cause an increased prevalence of spontaneous events and delayed after depolarizations (DADs) (12). Sarcoplasmic Endoplasmic Reticulum Ca^{2+} ATPase (SERCA) is considered a major player in these processes (12). Indeed, patients

with AF have lower SERCA levels compared to patients with sinus rhythm (13). Of note, SERCA levels can be assayed in peripheral blood lymphocytes, as their levels correlates with SERCA levels obtained in specimens of cardiac tissue (13). Mechanistically, a reduced Ca^{2+} uptake in the endoplasmatic/sarcoplasmatic reticulum, which is mediated by SERCA, results in intra-cytoplasmic Ca^{2+}overload,which is known to be arrhythmogenic (14). Specifically, the abnormal Ca^{2+} handling along with cellular DAD-mediated triggered activity might promote AF persistence, thereby favoring electrical and anatomical reentry (14,15). The persistence of abnormal Ca^{2+}handling can activate ion channels and trigger Ca^{2+}-dependent signaling pathways, eventually promoting atrial remodeling and the progression of AF to more persistent forms (16). Therefore, we speculate that alterations in SERCA levels might play a crucial role in AF persistence and in its recurrence following an epicardial ablation. To our knowledge, these aspects have never been investigated hitherto. Our hypothesis is that reduced SERCA levels might be linked to higher rates of failure of epicardial ablation in patients with persistent AF. To verify such hypothesis, we designed a prospective, multicenter observational study to evaluate AF recurrences during 1 year of follow-up after epicardial ablation, correlating this clinical outcome to SERCA expression in patients with sinus rhythm restoration (responders group) vs. patients with AF (non-responders group) after an epicardial ablative approach.

METHODS

Study design

From October 2014 to June 2016, we recruited consecutive patients with persistent, symptomatic AF, and for ≥ 6 months refractory to ≥1 class 1-3 antiarrhythmic drugs (AADs) in the prospective, multicenter observational study CAMAF (Ca^{2+} ATPase Modulates Atrial Fibrillation) at Catholic University of Sacred Heart, Campobasso, Italy, at University of Molise, Campobasso, Italy, at "Vecchio Pellegrini" Hospital, Naples, Italy, at University of Naples "Federico II", Naples, Italy and at University of Campania "Luigi Vanvitelli", Naples, Italy. Exclusion criteria were: patients aged <18 or >75 years, with structural heart diseases, left ventricle ejection fraction <30%,and left atrium diameter >55 mm; patients affected by any condition that would make survival for 1 year unlikely, and patients with prior cardiac surgery. All enrolled patients received an epicardial thoracoscopic (closed heart) video assisted pulmonary vein isolation (PVI). AF was defined according to the international guidelines for the management of patients with AF (2). Persistent AF was defined as recurrent AF with episodes that lasted more than 7 days (2). Before interventions and at follow-up, baseline laboratory studies, including glycated hemoglobin A1c (HbA1c),lipid panel, and fibrinogen, C reactive protein (CRP), interleukin 6 (IL6), tumor necrosis factor alpha (TNFα), B type Natriuretic Peptide (BNP), and SERCA levels were evaluated. These biomarkers were evaluated in the overall study population, and at 1-year follow-up in responders vs. non-responders. The Institutional Committees on Human Research at Participants Centers approved the study. The study was performed according to the declaration of Helsinki. All patients were informed of the nature of the study and provided written consent.

Peer-reviewed version available at J. Clin. Med. 2020, 9, 544; doi:10.3390/jcm9020544

Anthropometric evaluation

Clinical assessment included physical examination, vital signs, and review of adverse events. Fasting blood (at least 12 hours from last meal) were performed for glycemia, lipid profile (total cholesterol, triglycerides, high-density, and low-density cholesterol), and CRP at every visit.

Pre-operative management

All patients with persistent AF were under oral anticoagulation therapy (dicumarolic oral anticoagulation or new oral anticoagulation drugs). Three-five days before the procedure we discontinued all oral anticoagulation drugs, replaced by subcutaneous fractionated heparin. All patients received a trans-esophageal echocardiography within 48 hours before the procedure to rule out the presence of atrial thrombi. AADs were discontinued for 3-halflife before the ablative treatment.

Epicardial catheter ablation procedure

The goal of the procedure was electrical isolation of pulmonary veins and posterior left atrium wall by creating a continuous circumferential ablation line around all four pulmonary veins (‘‘box-lesion’’) using a fully thoracoscopic, unilateral, off-pump approach (6). In general anesthesia and with selective left lung ventilation, the right thorax was entered by three working ports, a rigid video-thoracoscopic camera with a direct optics was used. Pericardium was widely opened anterior to a phrenic nerve and transverse and oblique sinuses were dissected (6). An insertion of the ablation catheter was a tricky part of the surgery with the first MW catheter, while no special introducer was provided and the big and clumsy flexible triangular endoscopic liver retractor was used (6). Later RF devices were equipped with special flexible introducers, lately also with a magnetic tip

and the positioning of the catheter around PVs became much safer and easier(6).Pericardial fat was bluntly dissected in visible areas and the correct position of catheter was checked also by trans esophageal echocardiography before the ablation (6).We used the COBRA Fusion TM 150 (Estech, San Ramon, CA, USA) ablation system with the use of temperature controlled, both monopolar and bipolar RF energy, and with a suction design that eliminated the heat sink effect. We used a setting of 70°C and performed two cycles of 60 s each. In all systems, a circumferential movement of the catheter was done between the ablation cycles to close the box lesion. The lesion was checked visually and another ablation was performed on the septum, if it did not look continuous. If patients were not in SR after ablation, a direct current cardio version was performed. Then, an exit block was tested by pacing right atrium and right pulmonary veins (2). To test pacing atrial thresholds, and conduction block along the box lesion, we introduced a tetrapolar catheter (Josephson curve catheter, Webster, Diamond Bar, CA USA) by internal right femoral vein access. This catheter was advanced first in the right atrium, and then positioned within the box lesion (at the level of the right pulmonary veins and the posterior aspect of the left atrium).Then we advanced into the right atrium a deflectable curve catheter, Webster, Diamond Bar, CA USA) through the right internal jugular vein, positioning it in the coronary sinus in order to confirm potential captures (exit block). After confirmation of bidirectional(entrance and exit) block, we concluded the procedure. In absence of complications, and with stable hemodynamic conditions (6), patients were transferred to the intensive care unit.

Peri-operative and post-operative care

After epicardial ablation, patients were monitored in the recovery room and then transferred to the cardiothoracic surgery department on the same day of the intervention. We removed chest drains after a chest radiography. After evaluation of bleeding risk, patients were given un-fractionated heparin and continued until the international normalized ratio was >2.0. Oral anticoagulants, and AADs were continued for all blanking period after procedure. In case of AF recurrences, episodes lasting 24 hours were treated with an electric cardioversion. After clinical and instrumental assessment, patients were discharged and followed to outpatient clinic.

Blood sampling

From peripheral veins accesses we extracted blood samples to measure routine serum parameters, inflammatory markers, BNP and SERCA. Blood samples were collected at baseline (before epicardial ablation),and at 12-month follow-up after epicardial ablation to monitor the actual efficacy (responders vs. non-responders) of epicardial ablation in all treated patients.

Cytokines

Peripheral venous blood was drawn after an overnight fast, at breakfast time, before the intervention. We stored serum samples at -80°C. Serum concentrations of TNFα and IL6 were determined in duplicate using a highly sensitive quantitative sandwich enzyme assay (ELISA, Quantikine HS; R&D Systems, Minneapolis, USA).

Isolation of human lymphocytes

We isolated circulating lymphocytes from venous blood and we assayed their SERCA expression, as previously reported (17). T-Lymphocytes were isolated from human blood

donors following the protocol described by Lefort and Kim (17) with some modifications. Briefly, 3 mL of whole blood were added to a density gradient media (1114683 Polymorph Prep™, Progen, Germany) and centrifuged at 500 x g for 30 minutes at 20°C. After centrifugation, the layer containing peripheral blood mononuclear cells was transferred to a fresh tube by using a P1000 pipette, washed twice with phosphate buffer saline, centrifuged at 500 x g for 5 minutes each time, and transferred to a T-75 culture flask in 20 mL RPMI 1640 media (10% FBS and 1% penicillin/streptomycin). Cells were then incubated at 37°C and 5% CO_2 for 1 hour, in order to separate the monocytes adherent to the flask surface from the lymphocytes remaining in suspension. The cell medium containing lymphocytes was removed from the flask and centrifuged at 500 x g for 5 minutes; the pellet was re-suspended and trypan blue dye was added to determine the number of viable lymphocytes present in the suspension.

Protein isolation and ELISA test

Following lymphocytes lysis in RIPA buffer (R0278 Merck, Darmstadt, Germany) containing a complete protease inhibitor and phosphatase cocktail (11873580001 Roche, Basel, Switzerland),cell lysates were centrifuged at 12,000 rpm for 10 min at 4°C (16). Protein concentrations of supernatants were quantified with Bio-Rad protein assay (500-0006 Bio-Rad Laboratories, Hercules, CA) in order to assess SERCA2 protein levels by ELISA assay (Human SERCA2 Elisa kit,LS-F6830 LifeSpan Biosciences, Seattle, WA) following the manufacturer's instructions (17).

Peer-reviewed version available at J. Clin. Med. 2020, 9, 544; doi:10.3390/jcm9020544

Study end points

The end points of the study were the freedom from episodes of AF, defined as the absence of episodes of AF lasting >30 seconds on any ECG or 24-hour ECG Holter monitoring (2), and the identification of cytokines and SERCA levels comparing patients with sinus rhythm restoration (responders) vs. patients with AF recurrence (non-responders) after epicardial ablation at 12-month follow-up.

Follow-up

At outpatient clinic, patients were firstly followed for ten days after the epicardial ablation procedure to evaluate AF recurrence. Moreover, in case of AF recurrence during the first three months, defined as blanking period, patients received a pharmacological and/or an external cardioversion to abolish AF, and to restore sinus rhythm. After a blanking period, we discontinued AADs medications as recommended by international guidelines (2). Six months after epicardial ablation we discontinued oral anticoagulation in case of $CHADS_2$ score <1,(2). Therefore, we detected AF recurrence during the clinical evaluation at outpatient clinic by patients' symptoms, by ECG registration, ECG Holter exams and hospital discharge schedules.

Statistical Analysis

All data were analyzed by two different physicians, and the patients (n=27) have been divided after ablation in responder patients (n=15) vs. non-responders (n=12) to the ablative treatment. Based on our pilot studies and on the prevalence of persistent AF treated via thoracoscopic epicardial AF ablation, we performed a power analysis to determine the optimal sample size: applying an α cut-off of 5% and a β cut-off of 20%, we calculated that a n=12 per group would have been sufficient to reach statistical

Peer-reviewed version available at J. Clin. Med. 2020, 9, 544; doi:10.3390/jcm9020544

significance. We applied the two-tailed Student's t test to test normally distributed variables, for paired or unpaired data. We used one-way analysis of variance (ANOVA) for more than two independent groups of data. Chi-square or Fisher exact test were used to compare categorical variables. We defined as statistical significant a p value <0.05. Overall survival and event-free survival were assessed by Kaplan-Meier survival curves and compared applying the log-rank test, dividing the overall study population into 3 groups (tertiles) according to SERCA values. We performed a multivariable logistic regression analysis for AF recurrence event risk; only variables presenting a p value ≤0.25 at the univariate analysis were included in the model. We used a stepwise method with backward elimination, and we calculated odds ratios (OR) with 95% confidence intervals. The model was evaluated with Hosmer and Lemeshow test. Statistical analysis was performed using the SPSS software (SPSS Inc, Chicago, IL).

RESULTS

We recruited 27 patients (27% female); mean age was 57.1 ± 5.8 years; mean BMI was 28.2± 2 (kg/m^2). Clinical characteristics of our study population are reported in **Table 1**. At baseline, these characteristics were well balanced comparing responders vs. non-responders to the ablative treatment, except the mean AF duration time in months (40.2± 7.5 vs. 50.2 ± 5.7,p<0.05), LA diameter (LAD, in millimeters, mm: 42.2 ± 3.9 vs. 47.8 ± 4.0,p<0.05), and LA volume (LAV, in milliliters, ml: 30.5 ± 2.5 vs. 36.2 ± 2.5,p<0.05). At the end of follow-up, responders to epicardial ablation experienced a significant reduction of inflammatory biomarkers, BNP, and significantly augmented levels of SERCA compared to non-responders (p<0.05). **Table 1**. Equally important, responders also exhibited lower LAD (41.2 ± 2.9 vs. 48.2 ± 4.3 mm, p<0.05), LAV (28.5 ± 2.3 vs. 36.8 ± 2.9 ml, p<0.05), and a

Peer-reviewed version available at J. Clin. Med. 2020, 9, 544; doi:10.3390/jcm9020544

significantly improved myocardial function, assessed in terms of left ventricle ejection fraction (LVEF,52+4 vs. 46+4%,p<0.05), as well as a reduced mitral reflow (MR) compared to non-responder patients. **Table 1**.The different expression of SERCA at baseline and at follow-up in responders vs. non-responders to epicardial ablation is depicted in **Figure 1**. Dividing the overall study population to stratify the risk of AF events into 3 groups (tertiles) according to SERCA values, we observed a significantly different rate of AF recurrence at 3^{th},6^{th} and 12^{th} month of follow-up comparing patients in I vs. II and vs. III tertile of SERCA (p<0.05). **Figure 2**. The Kaplan curve and log-rank tests compared AF recurrent event rates across the tertiles of baseline SERCA values (patients in I vs. II vs. III tertile of SERCA (χ^2=6.241, p<0.05). **Figure 3**. At the end of follow-up, responders to epicardial ablation were taking fewer AADs, dicumarolic, and new oral anticoagulants drugs vs. non-responders (p<0.05). **Table 1**. At the multivariate analysis, we tested by Cox regression the factors influencing AF recurrences at 1-year follow-up after thoracoscopic epicardial AF ablation (CI 95%, p<0.05). As shown in **Table 2**, these factors were: mean AF duration (HR 1.235 [1.037-1.471], p<0.05), LAV (HR 1.755 [1.126-2.738], p<0.05), BNP (HR 1.945 [1.895-1.999], p<0.05), and SERCA (HR 1.763 [1.167-2.663], p<0.05).

DISCUSSION

The main findings of the present study are: 1) responders to epicardial ablation show a significant reduction of BNP values and inflammatory cytokines vs. non-responders; 2) responders display a significant reduction of LAD, LAV, MR and a significant improvement of LVEF vs. non-responders; 3) responders to epicardial ablation have significantly

increased SERCA levels in peripheral lymphocytes vs. non-responders; 4) AF recurrence after epicardial ablation was predicted via AF duration, LAV, SERCA levels and serum BNP.

BNP is a cardiac peptide implied in the pathogenesis and persistence of AF (19). In isolated rabbit cardiomyocytes, BNP infusion increases transient inward Ca^{2+} currents, sodium (Na^{+}) and Na^{+}/Ca^{2+} exchanger currents, and L-type Ca^{2+}currents (19). Such alterations in Ca^{2+} currents increase arrhythmogenesis, amplifying genesis and persistence of AF (19). In fact, increased BNP levels after an ablative approach in AF patients associate with a greater risk of future AF recurrence (20). In our study, patients experiencing sinus rhythm restoration after epicardial ablation (responders) had lower BNP levels compared to non-responders; in addition, increased BNP serum levels at baseline were predictive of AF recurrence after epicardial ablation. Notably, higher BNP values are preeminently expressed in AF patients with left atrium remodeling (20). Therefore, atrial remodeling might make patients with persistent AF less responsive to an ablative approach (20). Similarly, we observed a significant reduction in the serum levels of inflammatory cytokines in responder patients. Consistent with our results, inflammation is considered a key contributor to the pathophysiology of AF, also in patients who do not respond to an ablative approach (21). In our study, non-responders had higher values of LAD and LAV both at baseline and at follow-up. Increased values of LAD and LAV are established markers of atrial remodeling (19,20,21). Atrial remodeling is crucial in the pathogenesis of AF (22) and might underlie the failure of an ablative treatment (23). Indeed, atrial remodeling might cause AF resistance and predispose the patients with persistent AF to higher risk of AF recurrence after an ablative approach (19-25). On the other hand, responders to ablation might experience an amelioration in the

volumetry of cardiac chambers, with consequent improvement of LVEF (26), which, alongside with the significant reduction in MR observed in responders, might be interpreted as an index of mechanical improvement (amelioration in diastolic and systolic cardiac phases) induced by successful epicardial ablation (27,28). AF has been shown to aggravate MR by AF-induced atrial remodeling (27,28). Indeed, AF can alter atrial function and synchrony, affecting annular size, geometry, and function (27,28); these abnormalities can favor AF onset, recurrence, and persistence (27-29). Consequently, patients with long-standing AF might experience a lower rate of sinus rhythm restoration after an epicardial ablative approach (30). Hence, AF duration can affect the outcomes of catheter ablation treatment (30). To date, AF persistence might contribute to alterations in the electrophysiological and anatomical properties of the heart, becoming itself a cause, as well as a resulting effect of the electro-anatomical atrial remodeling, which is a fundamental determinant of AF perpetuation (7,30). These complex electro-anatomical modifications have been shown to make patients more refractory to ablative therapies (16,22).

Finally, at 1-year follow-up, responders patients exhibited significantly higher levels of SERCA values compared to baseline, as well as compared to patients non-responding to epicardial ablation. We also show that a low baseline SERCA expression in patients with persistent AF might increase ~1.8 fold the risk to experience AF recurrences after epicardial ablation. This aspect might help clinicians identifying patients that are likely to not respond to epicardial ablation. Additionally, SERCA might become the therapeutic target of tailored therapies and interventional approaches to reduce the recurrence of persistent AF in patients treated by an ablative approach and in overall population.

Study limitations

One limitation of our prospective multicenter study is the small size of our population of patients with persistent AF treated by epicardial catheter ablation; however, we performed an a priori power analysis revealing that a n=12 per group would have been sufficient to achieve statistical significance. Moreover, due to our relatively short follow-up (12 months), we cannot draw conclusions for long-term prognosis. In this study, we have not systematically used continuous ECG monitoring systems to evaluate AF recurrence, and/or remote monitoring systems, which have been previously reported to affect prognosis (30). We did not report data on cardiac imaging via magnetic resonance to assess cardiac fibrosis. Finally, the low percentage of responders to an epicardial ablative treatment might be due to the fact that we did not perform a combined epicardial and endocardial approach to reduce AF recurrences, as described by other investigators (6).

CONCLUSION

Our data indicate that responders to epicardial ablation display a reduction in BNP and inflammatory markers. They also show a marked reduction in atrium size and MR, with improved LVEF. Intriguingly, non-responders exhibit a significant reduction in SERCA protein level compared to responders, and low baseline SERCA values were predictors of AF recurrence and refractoriness to epicardial ablation. Taken together, our findings suggest that targeting SERCA might represent an effective therapeutic strategy to reduce post-ablative recurrences in patients with persistent AF.

Funding: G.S. is supported by the NIH (R01-DK123259, R00-DK107895, R01-HL146691, R01-DK033823).

Author Contributions Statement: All authors have equally contributed to this original research article.

Conflict of interest Statement: There are not conflict of interests to report.

Figures and tables legends.

Figure 1. In this figure representation of baseline (top part) and follow-up end (inferior part) levels of Sarcoplasmic Endoplasmic Reticulum Calcium ATPase (SERCA) in nanograms/milliliters (ng/ml). In the left part of the figure the SERCA levels are represented by columns with mean values ± standard deviations in overall population (blu color), and responders (green color) vs. non responders (red color) patients. On the right part of the figure, the dispersion graph represents the values of SERCA in responders (green color) vs. non responders patients (red color). The symbol "*" markes a p value <0.05, as statistical significant.

Figure 2. In upper part of figure the number of atrial fibrillation (AF) recurrence for 3, 6 and 12 months of follow-up in study population divided for tertiles of Sarcoplasmic Endoplasmic Reticulum Calcium ATPase (SERCA) values and arranged by columns for number of events in ascending order (in blu the III tertile, in green the II tertile and in yellow the I tertile of SERCA). In the lower part of figure the table to report the number (n) of AF recurrences for I, II and III tertile of SERCA values. The symbol "*" is for p <0.05 comparing I tertile vs. II tertile of SERCA; the symbol "**" is for p<0.05 comparing I vs. III tertile of SERCA; the symbol "***" is for p<0.05 comparing II vs. III tertile of SERCA; p<0.05 is for statistical significant.

Figure 3. Kaplan curve for cumulative survival free from atrial fibrillation (AF) events dividing study population in tertiles of Sarcoplasmic Endoplasmic Reticulum Calcium ATPase (SERCA) values. Yellow: 1° tertile; green: 2° tertile; blue: 3° tertile. We used a Log Rank Test for the equality of survival distributions for the different levels and tertiles of SERCA, with X2 =6.241 and p<0.05, considered as statistical significant.

Table 1. In this table clinical characteristics of study population before treatment (baseline) and at follow-up end. The patients are divided in general population, and differentiated in responders vs. non responders patients to epicardial ablation. AAD is anti arrhythmic drugs; ACE is angiotensin converting enzyme; AF is atrial fibrillation; ARS is angiotensin receptors; BMI is body mass index; BNP is B type Natriuretic Peptide; CAD is coronary artery disease; COPD is chronic obstructive pulmonary disease; CRP is c reactive protein; IL 6 is interleukine 6; LAD is left atrium diameter; LAV is left atrium volume; LVEF is left ventricle ejection fraction; MR is mitral regurgitation; mm is milli meters; ml is milli liters; pg/ml is picogrammes/milliliters; ng/ml is nanogrammes/milliliters; SERCA is for Sarcoplasmic Endoplasmic Reticulum Calcium ATPase; TNF-a is tumor necrosis factor a type. The symbol "–" is indicating a non statistical significant p value comparing responders vs. non responders patients (p value >0.05); the symbol "*" is indicating a p value <0.05 at baseline comparing responders vs. non responders patients; the symbol "**" is indicating a p value <0.05 at follow-up end comparing responders vs. non responders patients.

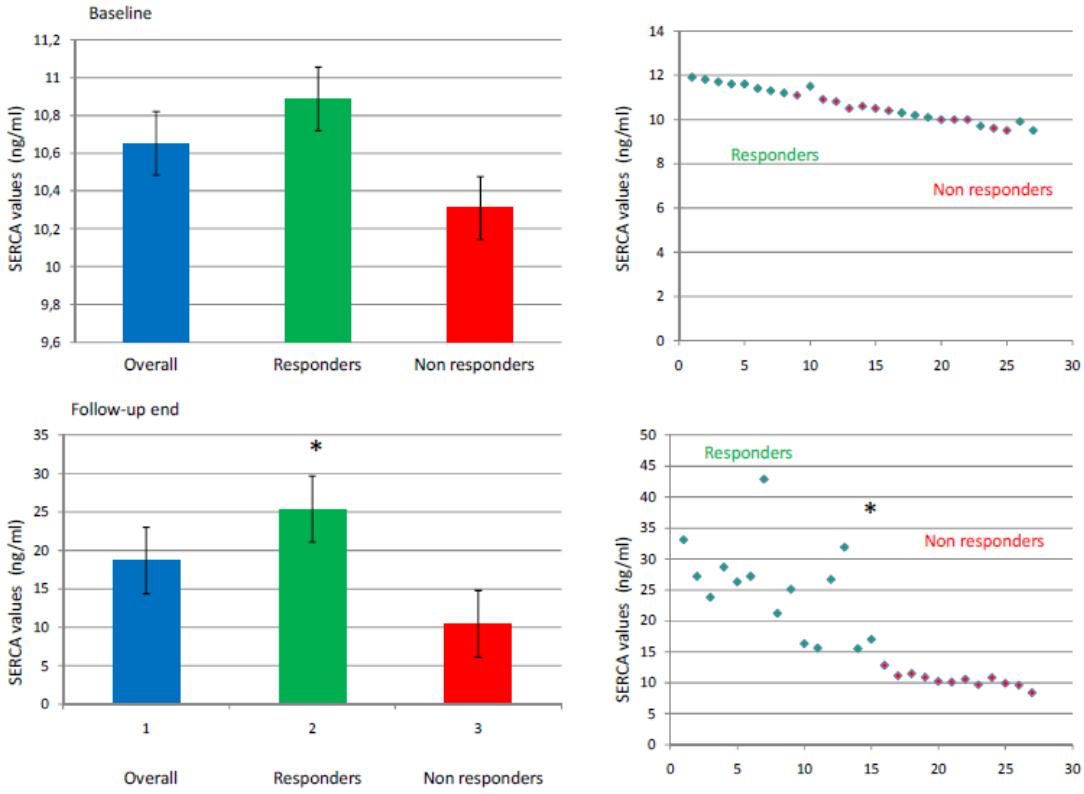
Table 2. In this table representation of Cox Regression analysis for Atrial Fibrillation (AF) recurrence after epicardial thoracoscopic catheter ablation. A p value < 0.05 has been

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 21 January 2020 doi:10.20944/preprints202001.0245.v1

Peer-reviewed version available at *J. Clin. Med.* **2020**, *9*, 544; doi:10.3390/jcm9020544

considered as statistical significant, and marked with the simble *. AF is atrial fibrillation; BNP is B type Natriuretic Peptide; LA is left atrium; LVEF is left ventricle ejection fraction; SERCA is Sarcoplasmic Endoplasmic Reticulum Calcium ATPase. IC is interval of confidence; OR is odd ratio.

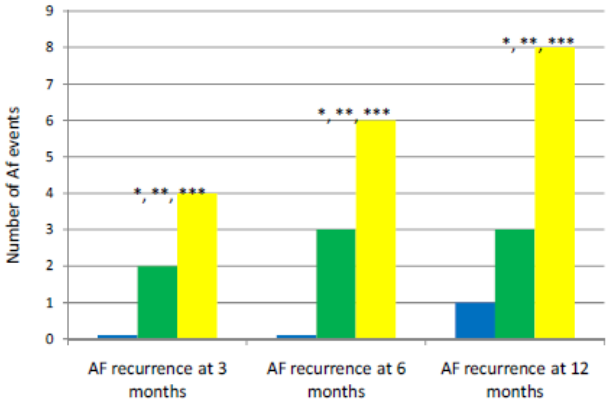
Figure 1. Baseline (top part) and follow-up end (inferior part) levels of Sarcoplasmic Endoplasmic Reticulum Calcium ATPase (SERCA) in nanograms/milliliters (ng/ml).



Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 21 January 2020 doi:10.20944/preprints202001.0245.v1

Peer-reviewed version available at *J. Clin. Med.* **2020**, *9*, 544; doi:10.3390/jcm9020544

Figure 2. In upper part of figure the number of atrial fibrillation (AF) recurrence for 3, 6 and 12 months of follow-up in study population divided for tertiles of Sarcoplasmic Endoplasmic Reticulum Calcium ATPase (SERCA) values and arranged by columns for number of events in ascending order (in blu the III tertile, in green the II tertile and in yellow the I tertile of SERCA). In the lower part of figure the table to report the number (n) of AF recurrences for I, II and III tertile of SERCA values.

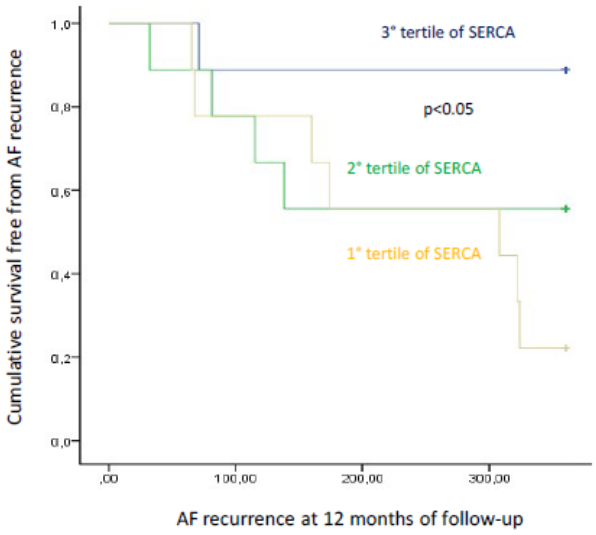


	Number of recurrences for I tertile of SERCA	Number of recurrences for II tertile of SERCA	Number of recurrences for III tertile of SERCA	P value
AF recurrence at 3th month, n 10 (37%)	n 6 (66.7%)	n 3 (33.3%)	n 0	<0.05*, **, ***
AF recurrence at 6th month, n 11 (41%)	n 4 (44.4%)	n 3 (33.3%)	n 0	<0.05*, **, ***
AF recurrence at 12th month, n 12 (44%)	n 8 (88.9%)	n 3 (33.3%)	n 1 (11.1%)	<0.05*, **, ***

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 21 January 2020 doi:10.20944/preprints202001.0245.v1

Peer-reviewed version available at *J. Clin. Med.* **2020**, *9*, 544; doi:10.3390/jcm9020544

Figure 3. Kaplan curve for cumulative survival free from atrial fibrillation (AF) events dividing study population in tertiles of Sarcoplasmic Endoplasmic Reticulum Calcium ATPase (SERCA) values.



Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 21 January 2020 doi:10.20944/preprints202001.0245.v1

Peer-reviewed version available at *J. Clin. Med.* **2020**, *9*, 544; doi:10.3390/jcm9020544

Table 1. Clinical characteristics of study population at baseline and 12 months after epicardial ablation (follow-up end).

		At baseline		At follow-up end		
Clinical general characteristics	**General population**	**Responders**	**Non Responders**	**Responders**	**Non Responders**	**P value**
Number of patients	n 27	n 15 (56%)	n 12 (44%)	n 15 (56%)	n 12 (44%)	
Age (years)	57.1 ± 5.8	55.6 ± 5.8	59 ± 5.3	/	/	--
Gender (male)	n 17 (63%)	n 9 (60%)	n 8 (66%)	/	/	--
BMI (kg/m^2)	28.2 ± 2	27.7 ± 1.8	28.9 ± 2.1	27.9 ± 1.7	28.4 ± 1.9	--
Diabetes	n 4 (15%)	n 2 (13%)	n 2 (16%)	n 2 (13%)	n 2 (16%)	--
CAD	n 10 (37%)	n 6 (40%)	n 4 (34%)	n 6 (40%)	n 5 (41.7%)	--
COPD	n 5 (18.5%)	n 3 (20%)	n 2 (17%)	n 3 (20%)	n 2 (17%)	--
Hypertension	n 6 (22%)	n 3 (20%)	n 3 (25%)	n 4 (26.7%)	n 3 (25%)	--
Mean AF duration (months)	44.7 ± 8.3	40.2 ± 7.5	50.2 ± 5.7	/	/	< 0.05*,
Previous stroke	n 2 (0.7%)	n 1 (0.6%)	n 1 (0.8%)	/	/	--
Biohumoral markers						
Creatinine (mg/dl)	0.98 ± 0.18	1.0 ± 0.17	0.96 ± 0.20	0.98 ± 0.21	1.01 ± 0.19	--
BNP (pg/ml)	198.59 ± 9.6	257.54± 6.12	287. 15 ± 56.26	43.67 + 4.97	303.75 + 51.16	<0.05**
SERCA (ngml)	10.6 ± 0.8	10.9 ± 0.8	10.3 ± 0.6	25.4 ± 6.2	10.6 ± 0.9	<0.05**
IL-6 (pg/ml)	2.8 ± 0.8	2.7 ± 0.9	2.9 ± 0.7	1.6 ± 0.2	2.4 ± 0.3	<0.05**
TNF-a (pg/ml)	9.1 ± 2.3	9.3 ± 2.1	8.6 ± 2.6	6.1 ± 1.7	8.7 ± 2.8	<0.05**
CRP (mg/dl)	4.0 ± 0.2	4.1 ± 0.2	3.9 ± 0.3	1.9 ± 0.4	3.3 ± 0.6	<0.05**
Echocardiographic measurements						
LAD* (mm)	44.15 ± 5.1	42.2± 3.9	47.8 ± 4.0	41.2 ± 2.9	48.2 ± 4.3	<0.05*,**
LAV* (ml)	33 ± 3.8	30.5±2.5	36.2 ± 2.5	28.5 ± 2.9	36.8 ± 2.9	<0.05*,
LVEF	49 ± 5	51± 4	50± 2	52± 4	46± 4	<0.05**
MR low grade	n 19 (70%)	n 10 (66.7%)	n 8 (67%)	n 13 (86%)	n 7 (58.3%)	<0.05**
MR moderate grade	n 9 (33%)	n 5 (33%)	n 4 (33%)	n 2 (13.3%)	n 5 (41.7%)	<0.05**
						--
Drug Therapy						
Beta blockers	n 6 (22.2%)	n 3 (20%)	n 3 (25%)	n 2 (13.3%)	n 2 (16.6%)	--
ACE inhibitors	n 2 (7.4%)	n 1(5%)	n 1 (8%)	n 1 (5%)	n 1 (8%)	--
ARS inhibitors	n 4 (15%)	n 2 (13%)	n 2 (16%)	n 2 (13%)	n 2 (16%)	--
AADs class 1	n 5 (18%)	n 3 (20%)	n 2 (17%)	n 2 (13%)	n 3 (25%)	<0.05**
AADs class 3	n 15 (56%)	n 8 (53%)	n 7 (58%)	n 2 (13%)	n 7 (67%)	<0.05**
Dicumarolic	n 11 (41%)	n 6 (40%)	n 5 (42%)	n 3 (20%)	n 6 (50%)	<0.05**
New oral anticoagulation	n 16 (59%)	n 9 (60%)	n 7 (58%)	n 4 (27%)	n 6 (50%)	<0.05**

Table 2. Univariate and multivariate analysis for AF recurrence after epicardial ablation at 12 months of follow-up.

		Univariate analysis			Multivariate analysis	
Variable	**OR**	**IC 95%**	**P value**	**OR**	**IC 95%**	**P value**
Diabetes	0.933	[0.204-4.274]	0.929	0.338	[0.025-4.637]	0.417
Obesity	1.312	[0.950-1.811]	0.101	1.473	[0.841-2.580]	0.175
Age	1.082	[0.956-1.225]	0.212	0.805	[0.637-1.018]	0.070
Mean AF duration	1.121	[1.032-1.218]	0.007	1.235	[1.037-1.471]	0.018*
LVEF	0.918	[0.817-1.032]	0.153	0.746	[0.555-1.003]	0.053
LA volume	1.264	[1.038-1.540]	0.020	1.755	[1.126-2.738]	0.013*
BNP	1.021	[1.005-1.037]	0.001	1.945	[1.895-1.999]	0.045*
SERCA	1.221	[1.105-1.349]	0.001	1.763	[1.167-2.663]	0.007*

References

1. Chugh SS, Havmoeller R, Narayanan K, Singh D, Rienstra M, Benjamin EJ, Gillum RF, KimYH, McAnulty JH Jr, ZhengZJ, Forouzanfar MH, Naghavi M, Mensah GA, Ezzati M, MurrayCJ. Worldwide epidemiology of atrial fibrillation: a Global Burden of Disease 2010 Study. Circulation 2014;129(8):837-47.

2. Kirchhof P, Benussi S, Kotecha D, Ahlsson A, Atar D, Casadei B, Castella M, Diener HC, Heidbuchel H, Hendriks J, Hindricks G, Manolis AS, Oldgren J, Popescu BA, Schotten U, VanPutte B, Vardas P; Authors/Task Force Members; Document Reviewers: 2016 ESC Guidelines for the management of atrial fibrillation developed in collaboration with EACTS: The Task Force for the management of atrial fibrillation of the European Society of Cardiology(ESC)Developed with the special contribution of the European Heart Rhythm Association(EHRA) of the ESCEndorsed by the European Stroke Organization (ESO). Eur Heart J 2016;37(38):2893-2962.

3. Haïssaguerre M, Jaïs P, Shah DC, Takahashi A, Hocini M, Quiniou G, Garrigue S, Le Mouroux A, Le Métayer P, ClémentyJ. Spontaneous initiation of atrial fibrillation by ectopic beatsoriginating in the pulmonary veins. N Engl J Med 1998;339(10):659–666.

4. de Groot NM,Houben RP,Smeets JL,et al. de Groot NM,Houben RP,Smeets JL,Boersma E, Schotten U,Schalij MJ,Crijns H,Allessie MA. Electropathological substrate of longstandingpersistent atrial fibrillation in patients with structural heart disease: epicardial breakthrough.Circulation 2010;122(17):1674–1682.

5. Ganesan AN, Shipp NJ, Brooks AG, Kuklik P, Lau DH, Lim HS, Sullivan T, Roberts-Thomson KC, Sanders P. Long-term outcomes of catheter ablation of atrial fibrillation: a systematicreview and meta-analysis. J Am Heart Assoc 2013;2(2):e004549.

6. Bisleri G, Rosati F, Bontempi L, Curnis A, Muneretto C. Hybrid approach for the treatment oflong-standing persistent atrial fibrillation: electrophysiological findings and clinical results. EurJ Cardiothorac Surg 2013;44(5):919-23.

7. Marrouche NF, Wilber D, Hindricks G, Jais P, Akoum N, Marchlinski F, Kholmovski E, Burgon N, Hu N, Mont L, Deneke T, Duytschaever M, Neumann T, Mansour M, Mahnkopf C, Herweg B, Daoud E, Wissner E, Bansmann P, Brachmann J. Association of atrial tissuefibrosis identified by delayed enhancement MRI and atrial fibrillation catheter ablation: theDECAAF study. JAMA 2014;311:498–506.

8. Pump A, Di Biase L, Price J, Mohanty P, Bai R, Santangeli P, Mohanty S, Trivedi C, Yan RX, Horton R, Sanchez JE, Zagrodzky J, Bailey S, Gallinghouse GJ, Burkhardt JD, Natale A. Efficacy of catheter ablation in nonparoxysmal atrial fibrillation patients with severe enlargedleft atrium and its impact on left atrial structural remodeling. J CardiovascElectrophysiol2013;24(11):1224-31.

9. Bortone A, Boveda S, Pasquié JL, Pujadas-Berthault P, Marijon E, Appetiti A, Albenque JP. Sinus rhythm restoration by catheter ablation in patients with long-lasting atrial fibrillation andcongestive heart failure: impact of the left ventricular ejection fraction improvement on theimplantablecardioverter defibrillator insertion indication. Europace2009;11(8):1018-23.

10. Landstrom AP, Dobrev D, Wehrens XHT. Calcium Signaling and Cardiac Arrhythmias. Circ Res. 2017;120:1969-1993.

11. Dobrev D ,Wehrens XH. Calcium-mediated cellular triggered activity in atrial fibrillation. J Physiol 2017;595(12):4001-4008.

12. Hove-Madsen L, Llach A, Bayes-Genís A, Roura S, Rodriguez Font E, Arís A, Cinca J. Atrial fibrillation is associated with increased spontaneous calcium release from the sarcoplasmic reticulum in human atrial myocytes. Circulation 2004;110(11):1358-63.

13. Kontaraki JE, Parthenakis FI, Nyktari EG, Patrianakos AP, Vardas PE. Myocardial gene expression alterations in peripheral blood mononuclear cells of patients with idiopathic dilated cardiomyopathy. Eur J Heart Fail 2010;12(6):541-8.

14. Davia K, Bernobich E, Ranu HK, del Monte F, Terracciano CM, MacLeod KT et al. SERCA2A over expression decreases the incidence of aftercontractions in adult rabbit ventricular myocytes. J Mol Cell Cardiol 2001;33: 1005–15.

15. King JH, Zhang Y, Lei M, Grace AA, Huang CL, Fraser JA. Atrial arrhythmia ,triggering events and conduction abnormalities in isolatedmurine RyR2-P2328S hearts. ActaPhysiol (Oxf) 2013;207:308–323.

16. Heijman J, Algalarrondo V, Voigt N, Melka J, Wehrens XH, Dobrev D, Nattel S. The value of basic research insights into atrial fibrillation mechanisms as a guide to therapeutic innovation: a critical analysis. Cardiovasc Res 2016;109:467–479.

17. Lefort CT, Kim M. Human T lymphocyte isolation, culture and analysis of migration in vitro. J Vis Exp. 2010;(40). Pii: 2017.

18. Trotta MC, Salerno M, Brigida AL, Monda V, Messina A, Fiore C, Avola R, Bernardini R, Sessa F, Marsala G, Zanghì GN, Messina G, D'Amico M, Di Filippo C. Inhibition of aldose-reductase-2 by a benzofuroxane derivative bf-5m increases the expression of kcne1,kcnq1 in high glucose cultured H9c2 cardiac cells and sudden cardiac death. Oncotarget 2017;9(25):17257-17269.

19. Lin YK, Chen YC, Chen YA, Yeh YH, Chen SA, Chen YJ. B-Type Natriuretic PeptideModulates Pulmonary Vein Arrhythmogenesis: A Novel Potential Contributor to the Genesis ofAtrial Tachyarrhythmia in Heart Failure. J Cardiovasc Electrophysiol 2016;27(12):1462-1471.

20. Zhang Y, Chen A, Song L, Li M, Chen Y, He B. Association Between Baseline NatriureticPeptides and Atrial Fibrillation Recurrence After Catheter Ablation.IntHeart J 2016;57(2):183-9.

21. Sardu C, Santulli G, Santamaria M,Barbieri M,Sacra C, Paolisso P, D'Amico F, Testa N, Caporaso I, Paolisso G, Marfella R, Rizzo MR. Effects of Alpha Lipoic Acid on Multiple Cytokines and Biomarkers and Recurrence of Atrial Fibrillation Within 1 Year of Catheter Ablation. Am J Cardiol 2017;119(9):1382-1386.

22. Calkins H, Kuck KH, Cappato R, Brugada J,Camm AJ, Chen SA, Crijns HJ, Damiano RJ, Davies DW, Di Marco J,et al. 2012 HRS/EHRA/ECAS Expert Consensus Statement on Catheter and Surgical

Ablation of Atrial Fibrillation: recommendations for patient selection, procedural techniques, patient management and follow-up, definitions, endpoints, and research trial design. Europace. 2012;14:528–606.

23. Pump A,Di Biase L,Price J,Mohanty P,Bai R,Santangeli P,Mohanty S,Trivedi C,Yan RX, Horton R,Sanchez JE,Zagrodzky J,Bailey S,Gallinghouse GJ,Burkhardt JD,Natale A.Efficacy of catheter ablation in nonparoxysmal atrial fibrillation patients with severe enlargedleft atrium and its impact on left atrial structural remodeling. J CardiovascElectrophysiol2013;24(11):1224-31.

24. Psaty BM,Manolio TA,Kuller LH,Kronmal RA,Cushman M,Fried LP,White R,FurbergCD,RautaharjuPM.Incidence of and risk factors for atrial fibrillation in older adults.Circulation 1997;96:2455–61.

25.Sardu C,Santamaria M,Paolisso G,Marfella R. microRNA expression changes after atrial fibrillation catheter ablation. Pharmacogenomics 2015;16(16):1863-77.

26. Dell' Era G,Rondano E,Franchi E,Marino PN; Novara Atrial Fibrillation (NAIF) Study Group. Atrial asynchrony and function before and after electrical cardioversion for persistent atrial fibrillation. Eur J Echocardiogr 2010;11(7):577-83).

27. Zhou X,Otsuji Y,Yoshifuku S,Yuasa T,Zhang H,Takasaki K,Matsukida K,Kisanuki A, Minagoe S,TeiC.Impact of atrial fibrillation on tricuspid and mitral annular dilatation andvalvular regurgitation. Circ J 2002;66:913– 6.

28. Gertz ZM,Raina A,Saghy L,Zado ES,Callans DJ,Marchlinski FE,Keane MG,SilvestryFE.Evidence of atrial functionalmitral regurgitation due to atrial fibrillation: reversal withArrhythmiacontrol. J Am CollCardiol 2011;58:1474–81

29. Pellman J and Sheikh F. Atrial Fibrillation: Mechanisms,Therapeutics,and Future Directions.ComprPhysiol 2015; 5(2): 649–665.

30. Sardu C,Santamaria M,Rizzo MR,Barbieri M,di Marino M,Paolisso G,Santulli G,Marfella R. Telemonitoring in heart failure patients treated by cardiac resynchronisation therapy with defibrillator (CRT-D): the TELECART Study. Int J Clin Pract 2016;70(7):569-76.