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# Serum Concentrations of Interleukin-33 and its Soluble Receptor sST2 in Patients with Persistent Atrial Fibrillation

**Wael Alkhiary[1*], Mohammed Abdalaal[2,3] and Amr El-Sabbagh[4]**

[1]*Department of Clinical Pathology, Faculty of Medicine, Mansoura University, Egypt*

[2]*Department of Cardiology, Faculty of Medicine, Tanta University, Egypt*

[3]*Almoosa Specialized Hospital, Cardiology Department, KSA, Saudi Arabia*

[4]*Department of Medical Microbiology and Immunity, Faculty of Medicine, Mansoura University, Egypt*

[*]**Corresponding author:** Wael Alkhiary, Department of Clinical Pathology, Hematology Division, Hemostasis and Thrombosis Laboratory, Mansoura University, Mansoura, Egypt, Tel: +2 01113228256; Fax: +20 50 2245451; E-mail: wkhiary@yahoo.com

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## Abstract

**Objectives:** Interleukin-33 (IL-33) is a new member of the IL-1 cytokine family, which is thought to be involved in the pathogenesis of various inflammatory diseases, through its soluble receptor ST2. There is increasing evidence that inflammation is a relevant player in structural atrial remodeling that represents the main mechanism for atrial fibrillation (AF) persistence. This study was designed to investigate the state of IL-33/ST2 axis serum concentrations in patients with persistent AF.

**Design and Methods:** We investigated the concentrations of IL-33, its soluble ST2 receptors, and high-sensitivity C-reactive protein levels (hsCRP) in the sera of 92 patients with persistent atrial fibrillation, and 68 controls.

**Results:** Serum concentrations of IL-33, sST2, and hsCRP were all significantly elevated in patients with persistent AF compared to controls (P <0.0001 for all). Moreover, serum IL-33 concentrations was positively correlated with the inflammatory marker hsCRP (r=0.606, P =0.002).

**Conclusion:** These preliminary results may support the role of inflammation in AF pathogenesis and IL-33/sST2 axis may be involved in the inflammatory process in AF.

**Keywords:** Interleukin-33; Atrial fibrillation; Arrhythmia; Atrial remodeling; Cytokines; Inflammation

## Abbreviations:

ACC: American College of Cardiology; AF: Atrial Fibrillation; AHA: American Heart Association; $Ca^{2+}$: Calcium Ions; CRP: C-Reactive Protein; DBC: Diagnostic Biochem Canada; ECG: Electrocardiogram; EDTA: Ethylenediaminetetraacetic Acid; EF: Ejection Fraction; ELISA: Enzyme-Linked Immunosorbent Assay; hsCRP: high-sensitivity C-Reactive Protein; IL-1: Interlukin-1; IL-6: Interlukin-6; IL-33: Interlukin -33; IFN-γ: Interferon gamma; iNKT: invariant Natural Killer T; LA: Left Atrium; MPV: Mean Platelet Volume; mRNA: messenger-Ribonucleic Acid; NF-KB: Nuclear Factor–Kappa B; NK: Natural Killer; PAMP: Pathogen-Associated Molecular Pattern; PDGF: Platlet Derived Growth Factor; pg/Ml: Picogram/Milliliter; RDW: Red Cell Distribution Width; Th2: T-Helper 2; TF: Tissue Factor; TGF-β1: Transforming Growth Factor-beta; TNF- α: Tumor Necrosis Factor-α

## Introduction

Atrial fibrillation (AF) is the most common cardiac arrhythmia and a source of significant morbidity and mortality [1]. Persistent AF, lasting more than 7 days, may result in electro-anatomical changes in the left atrium, resulting in deposition of fibrous tissue that may increase the risk of thromboembolic complications including stroke [2]. However, the precise mechanisms that lead to the onset and persistence of AF have not completely been elucidated [3].

There is increasing evidence that inflammation is a relevant player in structural atrial remodeling that represents the main mechanism for AF persistence [4]. Cardiac inflammatory disorders such as myocarditis, pericarditis, and cardiac surgery frequently are accompanied by atrial fibrillation [5]. Inflammation has been shown to be involved both the first occurrence [6] and in the risk of recurrence of AF [7]. In addition, inflammation plays an important role in postoperative atrial fibrillation, as shown by intracardiac expression of specific markers [8]. Activated inflammatory cells such as neutrophils, lymphocytes, monocytes resident macrophages, and activated platelets are all important players in this picture. Sustained atrial fibrillation would result in the release of proinflammatory cytokines, such as tumor necrosis factor (TNF)-a, interleukin (IL)-6, IL-8, Transforming growth factor-beta (TGF-β1) and platelet-derived growth factor (PDGF), which are related to cardiovascular disease and tissue injury [3].

Interleukin (IL)-33 is a new member of the IL-1 superfamily of cytokines that is expressed by mainly stromal cells, such as epithelial and endothelial cells, and its expression is upregulated following pro-inflammatory stimulation [9,10]. Unlike the other IL-1 family members IL-33 primarily induces T helper 2 (Th2) immune responses in a number of immune cell types [11].

IL-33 was identified as the ligand for the orphan receptor, ST2 (IL-1RL1). ST2 molecule is a member of the IL-1 receptor family [12] that exists in two forms: a transmembrane full-length form (ST2L) and a soluble, secreted form (sST2) due to differential splicing of ST2 mRNA [13]. Whereas ST2L exerts pro-inflammatory effects of IL-33, soluble ST2 has been implicated as a decoy receptor for IL-33, to attenuate Th2 inflammatory responses [14]. In normal conditions, the serum concentration of soluble ST2 is below the detectable level, but elevated level of ST2 has been reported in patients with autoimmune diseases [15], asthma [16], idiopathic pulmonary fibrosis [17], myocardial infarction and heart failure [18].

IL-33 has been shown to participate in several pathological processes including promoting type 2 T helper cell-associated autoimmune diseases [19]. In contrast, IL-33 has been also found to have protective effects in cardiovascular diseases [20,21]. IL-33 is expressed in the nucleus of human adult cardiac fibroblasts and myocytes and released during necrosis [9]. Proinflammatory cytokines as TNF-α, IFN-γ and IL-1β increase IL-33 in these cells, and IL-33 mRNA levels correlated with TNF-α and IFN-γ mRNA expression in human myocardial tissue [22]. Consequently, cardiac over-expression of IL-33 has been recently linked to heart fibrogenesis. We therefore thought to investigate the state and the relevance of serum IL-33 and soluble ST2 in persistent AF, associated with inflammation and structural remodeling.

## Materials and Methods

### Subject recruitment

This study population included 92 patients with persistent AF diagnosed at Almoosa Specialized Hospital, and Mansoura Specialized Internal Medicine Hospital between January 2012 and April 2014. For controls, 68 subjects were recruited, of matched age and sex, with sinus rhythm and no history of AF, as confirmed in a routine physical examination. Both patients and controls provided written informed consent. All procedures performed in the study were in accordance with the ethical standards of the institutional research committee."

### Exclusion criteria

Exclusion criteria were; congenital heart disease, acute coronary syndrome, cardiomyopathy, concomitant valvular heart disease, previous cardiac surgery, evidence of clinical heart failure, renal insufficiency, thyroid dysfunction, chronic inflammatory diseases, autoimmune diseases, sepsis, malignancy, liver diseases, hematological diseases, or bronchial asthma.

### Case definitions

Persistent AF was diagnosed According to the American College of Cardiology (ACC), American Heart Association (AHA), and the European Society of Cardiology (ESC) recommendations [23], based on the duration of disease, AF was categorized as persistent AF (recurrent episodes that last more than 7 days). Patients with persistent AF had blood collected for IL33/sST2 assessment when the sinus heart rate was measured.

### Clinical examination

All patients and controls were thoroughly examined to determine general and cardiac health. All provided detailed medical history, underwent physical examination, 12-lead electrocardiography (ECG), and transthoracic echocardiography, then blood sampling was done. Each subject had at least one ECG compatible with AF. The control group was recruited from subjects who admitted to the outpatient clinic and had normal findings at physical examination, ECG, laboratory analysis and transthoracic echocardiography.

The following clinical and demographic parameters were recorded; age, sex, body mass index, hypertension (known hypertension treated with anti-hypertensive drugs, two or more blood pressure recordings greater than 140/90 mm Hg), diabetes mellitus (known diabetes treated with diet or drugs or both; or either a fasting serum glucose of more than 126 mg/d l), hypercholesterolemia (know n treated hypercholesterolemia or fasting serum cholesterol concentrations higher than 200 mg/dl). Current cigarette smoking was defined as active smoking within the past 12 months. Cardiac medications taken at study entry were recorded.

### Echocardiography

Echocardiogram was used to evaluate myocardial ischemia, ejection fraction and cardiac structural change (atrial enlargement or left ventricular hypertrophy). In all patients and controls, each subject was examined using a Vivid 7 echocardiography device (General Electric, Waukesha, WI, USA) equipped with a middle range frequency (3–8 MHz) broad- band transducer. Two-dimensional, M-mode, and subsequent transthoracic. Patients were examined in left decubitis position. Assessments were done according to American Echocardiography Association criteria, obtaining parasternal long axis, short axis, apical four chamber and two chamber images [24]. Ejection fraction (EF) was estimated by the modified Simpson's method by measuring left ventricle end-diastolic volumes and end-systolic volumes in apical four-chamber images. Left atrium (LA) diameter was measured using parasternal long axis images.

### Blood sampling and laboratory investigations

Fasting venous blood was drawn from the antecubital vein with minimal tourniquet pressure into both EDTA- vacutainer for complete blood count (CBC) and serum separator tubes. Samples for CBC were carried out within one hour, using Hematology Analyzer (Cell-Dyn 1700, and Cell-Dyn 1800 Hematology Analyzers, Abbott). Serum Samples from patients and controls were allowed to clot for 30 minutes (min) before centrifugation (4°C; 3,000 g for 15 min) and stored at −80°C until use.

Serum IL-33 and sST2 concentrations were measured with specific human enzyme-linked immunosorbent assays (ELISAs); (RayBio® Human IL-33 ELISA Kit, and RayBio® Human sST2 ELISA Kit, RayBio ® Inc., Norcross, GA, USA), according to the manufacturer's protocols. Minimum detection limit was 2 pg/mL for both of IL-33 and sST2 kits. Serum high sensitivity C-reactive protein (hs-CRP) was assessed by ELISA high sensivity CRP kit (DBC, Diagnostic Biochem Canada, London, Canada), according to manufacturer's instructions, with sensitivity of (10 ng/mL). When a sample's value fell outside the reference range for the enzyme-linked immunosorbent assay kit, the sample was reanalyzed at a higher dilution.

### Statistical analysis

Variables were tested for normal distribution using the Kolmogorov-Smirnov test. Continuous variables were expressed as mean ± SD or as median, interquartile range according their

distribution. Categorical variables were summarized as counts and percentages and were compared by the chi-square or by Fisher exact test as appropriate. Continuous variables were compared using Student's t-test when normally distributed and by Mann-Whitney-U test when not normally distributed. Spearman correlation was used to determine the correlation between concentrations of IL-33 and sST2, hsCRP, or cardiovascular risk factors or concentrations of sST2 and hsCRP, or cardiovascular risk factors. A value of $p<0.05$ (two-tailed) was considered statistically significant. All statistical analyses were performed with the statistical software package SPSS version 18.0 (SPSS, Inc., Chicago, Illinois).

## Results

### Study population characteristics

The flow-chart illustrating the design of this study is demonstrated in Figure 1. Demographic data of the entire study participants, controls and patients with persistent AF, respectively are shown in Table 1. There were no differences in regard to cardiovascular risk factors; gender, age, diabetes, hypertension, body mass index, and smoking between controls and patients groups.

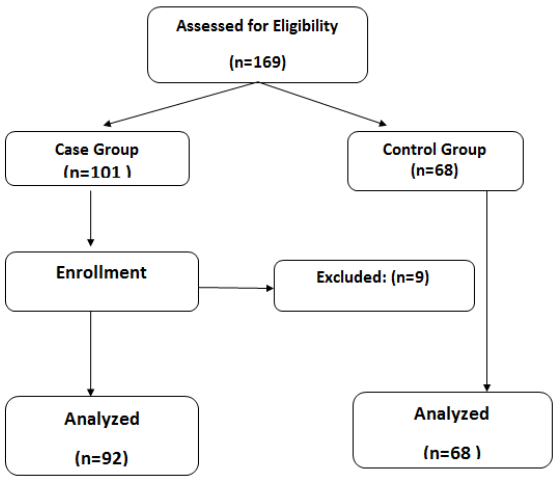


**Figure 1:** Flow chart for the enrolment of patients and controls of the study.

### Serum concentrations of sST2 and IL-33

As demonstrated in Table 2, sST2 and IL-33 concentrations were significantly higher in patients with persistent AF ($p<0.0001$ for both). In addition, the inflammatory markers used in this study (hsCRP, and RDW) showed significant increase in AF patients ($p<0.0001$, $p=0.0001$, respectively).

Serum concentrations of sST2 and IL-33 (Figures 2 and 3) in relation to hsCRP (as an inflammatory marker), hematological parameters, and Cardiovascular Risk Factors Serum concentrations of sST2 and IL-33 showed a significant positive correlation in AF patient group ($r=0.836$, $p<0.0001$) (Figure 4). hsCRP concentrations were also measured in sera of patients with AF to investigate whether IL-33 and/or sST2 may reflects inflammatory status of the disease. IL-33 concentrations correlated positively only with hsCRP in AF patients ($r=0.606$; $p=0.002$) (Table 3 and Figure 5), but were not associated with other parameters or cardiovascular risk factors. No significant correlation was observed between sST2 and hsCRP concentrations, or between sST2 and other parameters.

| | AF (n=92) | Control (n=68) | p value |
|---|---|---|---|
| Age (years) | 60.2 ± 6.4 | 55.81 ± 5.6 | 0.154 |
| Males (n;%) | 52 (56.52) | 40 (58.82) | 0.872 |
| Body Mass Index (kg/m$^2$) | 27.1 ± 2.18 | 26.53 ± 2.46 | 0.124 |
| Hypertension (n;%) | 30(32.61) | 23(33.82) | 1.00 |
| Diabetes Mellitus (n;%) | 28 (30.43) | 20(29.41) | 1.00 |
| Smoking (n;%) | 40(43.48) | 35(51.47) | 0.34 |
| ECG | AF | SR | - |
| Left Atrial Size (cm) | 4.1 ± 1 | 3.4 ± 2 | 0.004* |
| LVIDD (cm) | 5.2 ± 2 | 5.1 ± 1 | 0.706 |
| LVISD (cm) | 3.6 ± 1 | 3.6 ± 1 | 1.0 |
| EF (%) | 64 ± 11 | 67 ± 10 | 0.078 |

ECG: Electrocardiogram; LVIDD: Left Ventricular Internal Diastolic Dimension; LVISD: Left Ventricular Internal Systolic Dimension; EF: Ejection Fraction; NS: Non-significant. **p* significant if <0.05.

**Table 1:** Demographic and clinical characteristics of the study groups.

## Discussion

Although there is mounting clinical evidence to support the influence of inflammation in the pathogenesis of AF, limited controversial studies have investigated the correlation between inflammatory cytokines and AF [25-28].

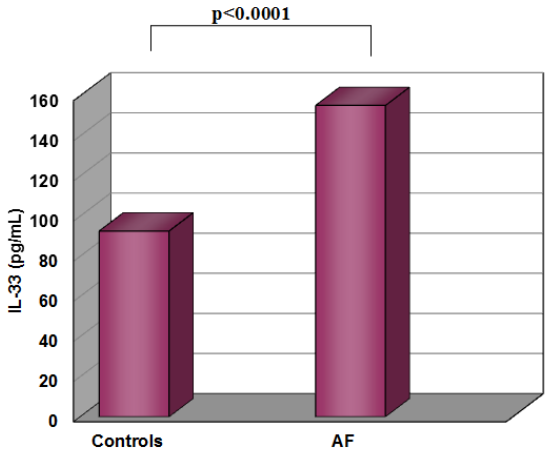


**Figure 2:** IL-33 serum concentrations in controls and persistent AF patients.

IL-33 is a new member of the IL-1 superfamily of cytokines that is expressed by mainly stromal cells, such as epithelial and endothelia l

cells, and its expression is upregulated following pro-inflammatory stimulation. Increasing evidence has shown that IL-33 and its receptor ST2 contribute to the pathogenesis of various autoimmune, allergic, some fibrotic disease and cardiovascular disease [29].

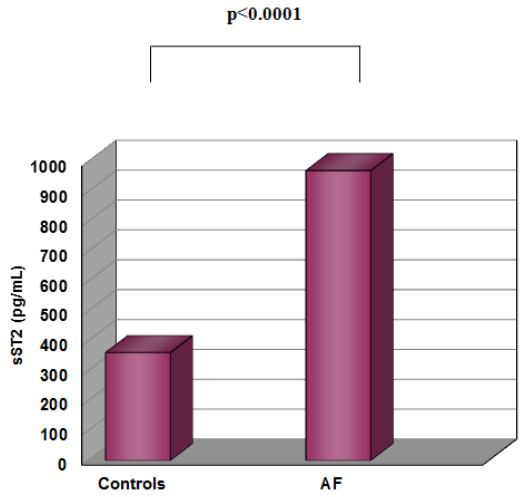


**Figure 3:** Soluble ST2 serum concentrations in controls and persistent AF patients.

| | AF (n=92) | Control (n=68) | p value |
|---|---|---|---|
| FBG (mg/dl) | 128.5 ± 10 | 126.6 ± 7 | 0.182 |
| HbA1C (%) | 7.5 (5.9–8.8) | 7.2 (5.2–8.7) | 0.073 |
| T.Cholesterol (mg/dl) | 211 ± 15 | 207 ± 12 | 0.072 |
| Creatinine (mg/dl) | 0.77 ± 0.17 | 0.75 ± 0.14 | 0.430 |
| Hb (g/dL) | 14.3 ± 0.9 | 14.1 ± 0.8 | 0.147 |
| Hct (%) | 43.3 ± 4.1 | 42.7± 4.3 | 0.372 |
| RDW (%) | 13.6 (11.5–15.2) | 12.8 (11.2–14.6) | 0.0001* |
| MCV (fL) | 86.9 ± 5.3 | 85.4 ± 4.6 | 0.063 |
| WBC (103/µL) | 8.13 ± 2.19 | 7.75 ± 2.3 | 0.290 |
| NLR | 2.20 (0.8–3.9) | 2.17 (0.5–4.2) | 0.062 |
| Platelets count (103/µL) | 253.4 ± 31.8 | 247.4 ± 36.3 | 0.302 |
| MPV (fL) | 8.31 ± 1.12 | 7.99 ± 1.39 | 0.109 |
| hsCRP (mg/L) | 1.88 ± 0.22 | 0.45 ± 0.13 | <0.0001* |
| IL-33 (pg/ml) | 155.2 (118.2–172.5) | 92.6 (76.4–121.8) | <0.0001* |
| ST2 (pg/ml) | 970.5 ± 98.6 | 361.4 ± 44.8 | <0.0001* |

FBG means fasting blood glucose; Hb hemoglobin; Hct hematocrit; MCV corpuscular volume; WBC white blood cell count; MPV mean platelet volume; NLR neutrophil/lymphocyte ratio; RDW red cell distribution width; WBC white blood cell count. * Significant

**Table 2:** Laboratory results of the studied groups.

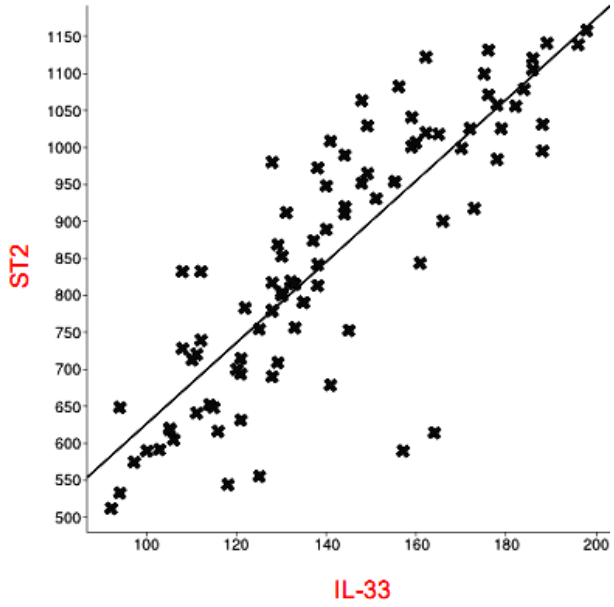


**Figure 4:** Correlation between IL-33 and soluble ST2 serum concentrations.

| | AF | | | |
|---|---|---|---|---|
| | IL-33 | | sST2 | |
| | r | p value | r | p value |
| hsCRP | 0.606 | 0.002* | 0.228 | 0.119 |
| RDW | 0.103 | 0.284 | 0.187 | 0.092 |
| NLR | 0.158 | 0.427 | 0.022 | 0.956 |
| MPV | 0.054 | 0.771 | 0.130 | 0.811 |
| LA Size | 0.219 | 0.136 | 0.014 | 0.980 |
| EF (%) | 0. 323 | 0.094 | 0.174 | 0.23 |

hsCRP: High-sensitivity CRP; RDW: Red Cell Distribution Width; NLR: Neutrophil/Lymphocyte Ratio; MPV: Mean Platelet Volume; LA: Left Atrium; EF: Ejection Fraction. * Significant

**Table 3:** Correlation between laboratory data and serum IL-33 or sSt2 concentrations.

However, no information yet is available on the serum levels and the roles of IL-33 and sST2 in AF. In this study, we found that serum IL-33, sST2, and hsCRP concentrations were all elevated in patients with persistent AF compared to those in the controls. Moreover, serum IL-33 concentration was positively correlated with the inflammatory marker hsCRP.

IL-33 is proven to be associated with an array of inflammatory cytokines/mediators. At the mRNA level, IL-33 is expressed in many organs [9] in humans and mice. However, at the protein level, IL- 33 is mainly and constitutively expressed in epithelial and endothelial cells [29]. Immune cells, macrophages and dendritic cells also produce

IL-33 after adequate stimulation [30]. Pathogen-associated molecular pattern (PAMP) molecules and also cytokines; TNF- α, IL-1 and IFN-γ stimulate the production of IL-33 in macrophages [31,32]. On the other hand, some proinflammatory cytokines such as TNF-α and IL-6 are also potent inducers of soluble ST2 [33], which block the effects of IL-33.

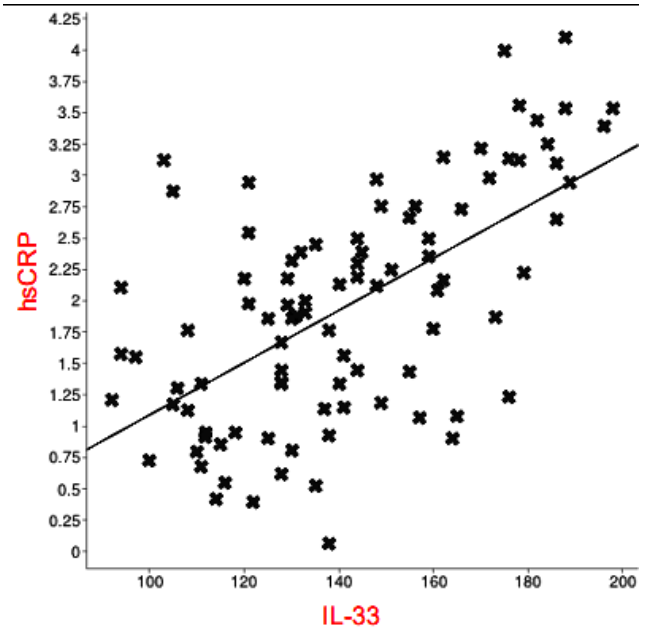


**Figure 5:** Correlation between serum concentrations of IL-33 and hsCRP.

IL-33 binds to its receptor complex, which comprises ST2L and sST2 on eosinophils, basophils, mast cells, natural killer (NK) cells, Th2 lymphocytes, and invariant natural killer T (iNKT) cells [34]. IL-33 enhances adhesion and CD11b expression in human eosinophils and basophils [35]. IL- 33 induces the production of IL-6, IL-1 β, TNF-α, monocyte chemotactic protein-1 (MCP-1), and prostaglandin D2 production in bone marrow-derived mast cells [36]. It also increases the production of IL-5 and IL-13 by polarized Th2 cells [10] and interferon-γ (IFN-γ) production by both iNKT and NK cells [37].

Leukocyte activation is considered an important inflammatory pathway underlying AF [3,8,26-38]. These cytokines and chemokines (IL-6, IL-1 β, TNF-α, MCP-1, IFN-γ) orchestrate leukocyte trafficking and activation and have been assessed as potential mediators in the establishment and perpetuation of AF, its recurrence and AF-related thrombosis [8,38].

Until now, it is not known definitely whether inflammation is a consequence or cause of AF or if inflammation reflects an underlying disease or AF per se [38]. So, the exact mechanism for increased serum IL-33/sST2 axis in AF may be uncertain. It may play a direct contributing role in the development or maintenance of AF, or might just be a marker of systemic inflammation. Increased circulating and local IL-33/sST2 may localize in atrial tissue, activating the complement system and inducing inflammation.

Angiotensin II is a potent promoter of fibrosis, and atrial fibrosis, a frequent finding in patients with AF, may lead to intra-atrial conduction disturbances and to persistent susceptibility to AF [39]. In the heart, IL-33 is predominantly synthesized by cardiac fibroblasts. IL-33 and ST2 may represent a cardio-protective signaling system as IL-33 was found to markedly antagonize angiotensin II-induced cardiomyocyte hypertrophy as well as hypoxia-induced apoptosis and protect mice from experimental pressure overload and myocardial infarction [20,40]. Additionally, IL-33 also attenuated ischaemia/reperfusion injuries in the diabetic myocardium in mice [41]. A cardioprotective role of this system was confirmed in ST2-deficient mice [20,40].

C-reactive protein (CRP), a biomarker of inflammation, may represent a strong and independent marker of risk of adverse cardiovascular events [38]. However, clinical data on the relationship of CRP levels and clinical presentation/duration of AF are inconsistent [25,26,42,43]. Serum levels of hs-CRP have been noted to be higher among patients with AF compared with controls in sinus rhythm [44-49]. hs-CRP has also been shown to be predictive of subsequent development of AF among a large cohort of patients in sinus rhythm at baseline. In addition, patients with persistent AF have higher hs-CRP levels than patients with paroxysmal AF, and both groups have higher levels than controls without AF [42]. Further, a longer duration of AF is associated with higher hs-CRP levels and larger left atrial dimensions, supporting a link between the burden of AF, inflammation, and structural remodeling. hs-CRP has been consistently and significantly predictive of early AF relapse after successful cardioversion [38,42,50].

In our study, although elevated hsCRP concentrations were revealed in persistent AF patients, there were no significant correlations with any clinical parameter. In the presence of $Ca^{2+}$ ions, CRP binds to phosphatidylcholine leading to the generation of long-chain acylcarnitines and lysophosphatidly cholines. These can contribute to cellular membrane dysfunction by affecting transmembrane ion transport [51].

Recently, some hematological parameters have emerged as inflammatory biomarker in various diseases, especially in cardiovascular research area. In this respect, many studies have shown that red cell distribution width (RDW), a laboratory measure of the variability of red blood cell sizes, is a strong predictor of adverse outcomes in patients with coronary artery disease [52], heart failure [53,54], and stroke [55]. The increase of RDW levels are correlated with inflammatory markers and are accepted as a sign of ineffective erythropoiesis which occurs in critically ill patients [56]. In this study, RDW levels were significantly higher in persistent AF patients when compared to controls. However, RDW levels did not correlate with any of the concentrations for hsCRP, IL-33, or sST2.

The present study is the first to demonstrate that IL-33 and sST2 concentrations are higher among persistent AF patients. These findings may support the hypothesis that inflammation contributes to the etiology of AF. Although the precise mechanism for the increased circulating markers is uncertain, the results might reflect active participation of local/systemic inflammatory responses during AF. It could be hypothesized that serum IL-33 may work as a new and easy-to-detect biomarker for the diagnosis of the inflammatory process in persistent AF. Nevertheless, it is still unclear whether raised IL-33 levels represent the cause or rather a consequence of the progression of AF-related heart disease. Moreover, our study is limited by its relatively small size, so further large-scale studies are required to better characterize the role of this cytokine through- out the course of a chronic and progressive disease. Further, we decided to investigate

IL33/sST2 axis in persistent AF with proven complex, and long course, however, more universal studies are warranted to address the situation of this new inflammatory axis in paroxysmal AF, recurrence, and the response to therapeutic procedures. Of importance, the effect of cardioversion and catheter ablation on serum levels of these inflammatory biomarkers should be investigated in further studies.

Finally, IL33/sST2 axis should be assessed in relation to the prothrombotic states of AF. It is well-known that tissue factor (TF) is the primary trigger of blood coagulation. TF expression induced by local inflammation is involved in the pathogenesis of thrombosis in patients with nonvalvular atrial fibrillation [57]. Recently, IL-33 was found to increase the production of TF, in human endothelial cells via ST2/NFkB-pathway, but independent on IL-1. IL-33 also increases cell surface TF activity, and this increase of TF can affect coagulation capacity of human blood [58,59]. Thus, further studies are required to address the clinical implications of the inflammatory IL33/sST2 axis in the prediction of AF-related thromboembolism. Indeed, inflammatory pathways could be also considered as therapeutic targets in an effort to reduce the clinical consequences of thromboembolism and improve outcomes in AF [60].

In conclusion, the results of the study show that serum concentrations of IL-33 and sST2 are significantly elevated in patients with persistent AF, compared with control subjects. Serum IL-33 concentration was significantly associated with hsCRP concentration. These findings suggest that IL-33 may play an important role in the pathomechanism of persistent AF. The potential for using novel agents that can influence the inflammatory processes in AF may represent a shift in the thinking of this common arrhythmia, from an electrical to a more structural emphasis.

## Acknowledgment

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