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# Associations of MMP-2, BAX, and Bcl-2 mRNA and Protein Expressions with Development of Atrial Fibrillation

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Data Collection B  
Statistical Analysis C  
Data Interpretation D  
Manuscript Preparation E  
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**Background:** To examine changes of mRNA and protein expressions of MMP-2, Bcl-2, and BAX in atrial fibrillation (AF) patients, and investigate the correlations among these 3 biomarkers.

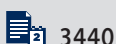
**Material/Methods:** Rheumatic heart disease patients (n=158) undergoing cardiac surgical procedures for mitral valve repair or replacement were included as the AF group (n=123), containing paroxysmal AF (n=42), persistent AF (n=36), and permanent AF (n=45). Rheumatic heart disease patients with sinus rhythm (SR) (n=35) were enrolled as the SR group (control group). Immunohistochemistry, Western blot, and real-time polymerase chain reaction (PCR) were applied to detect the protein and mRNA expression levels of MMP-2, Bcl-2, and BAX. Apoptosis was observed with light and electron microscopes and detected by TdT-mediated dUTP nick-end labeling (TUNEL).

**Results:** Compared with the SR group, the left atrial diameters (LADs), protein and mRNA expression levels of MMP-2 and BAX, apoptotic index (AI), and Bcl-2/BAX ratio were evidently increased in the 3 AF groups, but protein and mRNA expression levels of Bcl-2 decreased in the AF groups (all  $P < 0.05$ ). Correlation analysis found that MMP-2 protein expression levels was positively correlated with BAX expression, but negatively correlated with Bcl-2 expression levels.

**Conclusions:** Our study results suggest that elevated MMP-2 expression and disturbance balance of Bcl-2/BAX expressions may be associated with the development and maintenance of AF. MMP-2 may be involved in the development of AF through promoting BAX expressions and inhibiting Bcl-2.

**MeSH Keywords:** **Atrial Fibrillation • Genes, bcl-2 • Matrix Metalloproteinase 2**

**Full-text PDF:** <http://www.medscimonit.com/abstract/index/idArt/895715>



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## Background

Atrial fibrillation (AF), the most common chronic cardiac arrhythmia in clinical practice, is associated with severe morbidity and mortality across the world [1,2]. The major symptoms of AF include fainting, fatigue, heart palpitations, and chest pain, but this disease may be asymptomatic [3]. It is proposed that most of AF patients are older than age 75 years in Western countries and AF is expected to affect 6 million people by 2050 with the increasing trend of an aging population [4,5]. Evidence showed that approximately 15% of strokes are ascribed to documented AF, which suggests that AF may be a common etiologic factor for stroke [6]. Several findings have reported an association between AF and stroke, congestive heart failure, and cardiomyopathy, which poses a great challenge for the global health system [7–9]. Risk factors for development of AF include sex, age, body mass index, alcoholic consumption, total cholesterol, and hypertension [2,10–12]. Other factors involved in the occurrence of AF may include oxidative stress, ischemia, endocardial dysfunction, and inflammatory and abnormal activity of the autonomic nervous system [2,10]. Atrial structural abnormalities, including severe apoptosis, myolysis, interstitial fibrosis, and atrial enlargement, are the main risk factors for initiation and persistence of AF [13,14]. Moreover, evidence also supports that matrix metalloproteinase-2 (MMP-2) is a strong candidate for being associated with atrial structural remodeling during mitral stenosis with AF [15,16].

MMP-2, localized in cardiomyocytes, has the capability of degrading type IV collagen, along with a few other bioactive molecules, and is overexpressed in head and neck squamous cell carcinoma [17]. Evidence demonstrated that compared with patients with sinus rhythm (SR), AF patients had significantly increased MMP-2 expression level in both the right atrial appendages and the free walls [18]. As the disturbance in the homeostatic balance between cell growth and cell death plays significant role in disease cause, it is of great significance to investigate the expressions of apoptosis in diseases [19]. As numbers of the B-cell lymphoma 2 (Bcl-2) family, Bcl-2 can block cell death and Bcl-2-associated X protein (BAX) is a promoter of apoptosis [20]. It was reported that the inhabitation of Bcl-2 and related anti-apoptotic proteins shows satisfactory promise in lymphoid malignancies [21]. A study suggested that a change in the Bcl-2/BAX ratio may promote cell apoptosis following an apoptotic stimulus [13]. Moreover, evidence also demonstrated that the ablation of AF is associated with decreased serum markers for apoptosis [22,23]. However, the comparisons between AF patients and SR patients in MMP expression levels remain controversial [24]. Thus, the current study, using similar conditions, including the same experimental species and background diseases, was conducted to examine the changes in mRNA and protein expressions of MMP-2, Bcl-2, and BAX in AF patients, and to investigate the correlations among these 3 biomarkers.

## Material and Methods

### Ethical statement

The study design was reviewed and approved by the ethics committee of the Affiliated Hospital of Binzhou Medical College. All subjects had to sign informed consent in written form to undergo diagnostic and therapeutic procedure at the time of hospitalization. All procedures in this study were in compliance with the Declaration of Helsinki [25].

### Subjects

This study included 158 patients with rheumatic heart disease recruited from the Affiliated Hospital of Binzhou Medical College between July 2012 and September 2014. All patients were undergoing cardiac surgical procedures for mitral valve repair or replacement. According to the consensus on the terminology and classification of AF by European Society of Cardiology (ESC) and the North American Society of Pacing and Electrophysiology (NASPE), patients in the present study were classified into 3 groups: (1) paroxysmal AF: spontaneous termination <7 d and most often <48 h; (2) persistent AF: not self-terminating and lasting >7 d or prior cardioversion; and (3) permanent AF: not terminated, terminated but relapsed, no cardioversion attempt [26]. Based on the above standards, 123 patients were grouped into the AF group containing 42 patients with paroxysmal AF, 36 patients with persistent AF, and 45 patients with permanent AF. Then 35 rheumatic heart disease patients with sinus rhythm (SR) excluded from AF was included as the SR group (control group). The exclusion criteria were: (1) patients with bacterial infection, virus infection, acute and chronic inflammatory diseases, or systemic inflammatory diseases (rheumatism, systemic lupus erythematosus); (2) patients with severe liver and renal failure; (3) patients complicated with other tumors; (4) patients with history of surgery or trauma; (5) subjects who used anti-inflammation drugs within 3 days; (6) women who are in menstruation or had used oral contraceptives; (7) patients diagnosed with acute coronary syndrome (ACS) within the last 3 months; (8) patients with New York Heart Association (NYHA) functional class III or IV; and (9) patients with combined cerebrovascular diseases or neurological disorders. The basic clinical information all each subjects were collected and recorded, including age, sex, height, weight, complicating diseases, and medication usage.

### Sample collection

A left atrial appendage (LAA) sample (100 mg) was collected from each subject before cardiopulmonary bypass (CPB) with an open chest. Each sample was divided into 2 parts after the bleeding, necrotic, and adipose tissues were removed,

**Table 1.** The primers, length of the PCR products and annealing temperature in Real-time PCR.

Gene	Primers	Product length (bp)	Annealing temperature (°C)
β-actin	F: 5'-AAGGACCTGTATGCCAACACA-3'	152	57
	R: 5'-ATCCACACAGAATACTTGCGTT-3'		
MMP-2	F: 5'-TCAAGTTCCCGGCGATG-3'	225	70
	R: 5'-AGTTGGCCACATCTGGGTTG-3'		
Bcl-2	F: 5'-CACAAAGAGGCCAAGGCTACCT-3'	158	58
	R: 5'-CAGGAAAGCAGGAAGTCTCAA-3'		
BAX	F: 5'-ATTGAGAAACGATTTGCCTACA-3'	187	59
	R: 5'-GGGAAATGGCTTATTCTCCTTTGCTT-3'		

MMP-2 – matrix metalloproteinase-2; Bcl-2 – B-cell lymphoma-2; BAX – Bcl-2-associated X protein; PCR – polymerase chain reaction; F – forward; R – reverse.

and washed with normal saline to remove blood. Samples in one part were fixed in 4% paraformaldehyde for 12 h, paraffin-embedded, and serially sectioned (4-μm). Samples in another part were immediately soaked in liquid nitrogen and stored in -80°C for further use.

### Echocardiography

An American GE-Vivid E-9 color Doppler ultrasonic system equipped with a 2.5 MHz transducer (GE Healthcare, Fairfield, USA) was applied for echocardiography by sonographers. Subjects were arranged in supine position with a parasternal long axis view. Prior to echocardiography, the acoustic beam was adjusted to perpendicular to interventricular septum and left ventricular posterior wall. After the ideal image was obtained, M-mode echocardiography was applied to measure left atrial diameter (LAD), left ventricular end-diastolic diameter (LVED), and mitral valve area (MVA).

### Immunohistochemistry

Streptavidin-peroxidase (SP) method was used for immunohistochemistry. Rat anti-human monoclonal antibodies for MMP-2, Bcl-2, and BAX were obtained from ZSGB-BIO, Beijing, Corp., China. The diluted antibodies were added with specific antigens. After treatment with biotin-conjugated secondary antibody (ZSGB-BIO, Beijing, Corp., China), antigen-antibody complexes were added with 3, 3'-diaminobenzidine (DAB) reagent. Positive reaction mainly presented in endochylema and produced brown-yellow granules. The immunohistochemistry results were observed with a light microscope and digital photographs were taken under the same light conditions.

### Real-time polymerase chain reaction (PCR)

Total RNA was isolated from 100 mg LAA using Trizol (Invitrogen Life Technologies, Carlsbad, CA, USA) according

to the manufacturer's recommendations. Then the total RNA (2 μg) was transcribed into cDNA using reverse transcriptase (Bio-Rad, Hercules, CA, USA). Primers used were from TaKaRa Biotechnology (Dalian, China). Then the cDNA were diluted 5 times. PCR was performed in a reaction system (25 μl) containing 2 μl of cDNA, 12.5 μl of SybrGreen qPCR Master Mix (Applied Biosystems, Carlsbad, CA, USA), 0.5 μl of forward primer (10 μM), 0.5 μl of reverse primer, and 9.5 μl of ddH<sub>2</sub>O. PCR reaction conditions were: initial denaturation at 95°C for 2 min, then total 40 cycles of denaturation at 95°C for 10 s, annealing at 56°C for 30 s, and a final extension at 72°C for 40 s. After PCR was done, the mRNA was calculated by a comparative threshold cycle (Ct) method using the formula 2<sup>-ΔCt</sup>. PCR products of MMP-2, BAX, Bcl-2, and β-actin (internal reference) (5-μl) were electrophoresed in 2% agarose gel with ethidium bromide and the gel images were obtained using a BIO-RAD gel imaging system (Table 1).

### Western blot

Total protein content was isolated and detected using Bicinchoninic acid (BCA) protein assay kit according to the instructions from manufacture (Solarbio, Beijing). An amount of 50 μg total protein per sample was run on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel, and the proteins on gels were transferred to a nitrocellulose filter (NC filter). Then the reaction was blocked with 5% skim milk powder/TBS buffer at room temperature. Samples were added with primary antibodies (1:2000) for MMP-2, BAX, Bcl-2, and β-actin for overnight reaction at 4°C. After repeated washing, the samples were incubated for 1 h with the addition of secondary antibody goat anti-rabbit (1:2000) (Abcam Inc., Cambridge, MA, USA) conjugated with horseradish peroxidase (HRP). Using DAB as selenium organic reagent, hybridization signals were captured on a Bio-Rad imaging system.

**Table 2.** The comparisons on baseline characteristics between AF group and SR group.

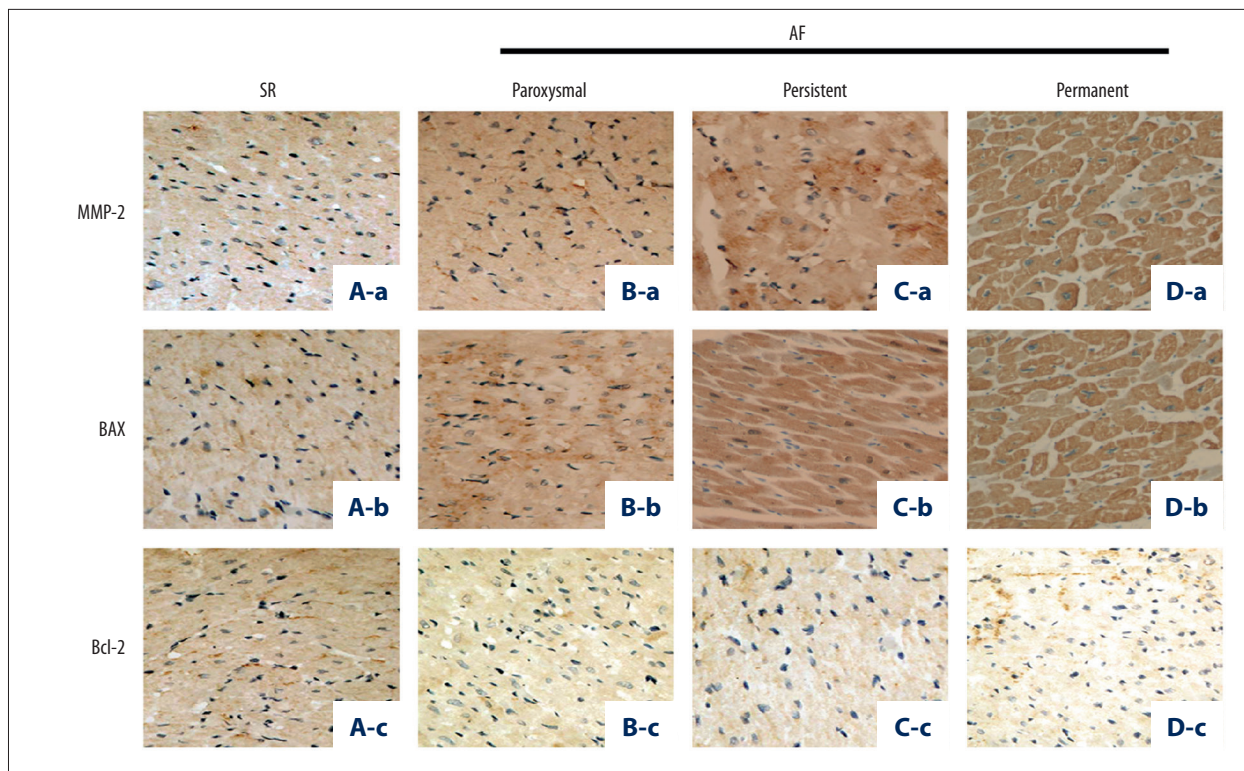
Variables	AF group			SR group (n=35)	F/ $\chi^2$	P
	Paroxysmal AF (n=42)	Persistent AF (n=36)	Permanent AF (n=45)			
Age ( $\bar{x}\pm s$ , years)	69.5 $\pm$ 9.7	68.8 $\pm$ 8.5	70.4 $\pm$ 9.5	65.2 $\pm$ 10.2	2.163	0.095
Sex (M/F)	28/14	25/11	31/14	24/11	0.082	0.994
BMI ( $\bar{x}\pm s$ , kg/m <sup>2</sup> )	26.8 $\pm$ 4.2	25.9 $\pm$ 4.5	27.1 $\pm$ 4.3	26.5 $\pm$ 4.7	0.522	0.663
Duration of AF (years)	4.6 $\pm$ 2.4	4.8 $\pm$ 2.1	5.1 $\pm$ 1.8		0.621	0.539
Smoking history (n, %)	18 (42.9)	21 (58.3)	28 (62.2)	16 (45.7)	4.409	0.221
Medication usage (n, %)					5.947	0.981
AECI	3 (7.1)	4 (11.1)	5 (11.1)	4 (11.4)		
ARB	10 (23.8)	8 (22.2)	8 (17.8)	5 (14.3)		
$\beta$ -blocker	8 (19.0)	9 (25.0)	14 (31.1)	7 (20.0)		
Calcium antagonist	12 (28.6)	7 (19.4)	11 (24.4)	10 (28.6)		
Diuretics	5 (11.9)	3 (8.3)	4 (8.9)	5 (14.3)		
Digitalis	4 (9.5)	5 (13.9)	3 (6.7)	4 (11.4)		
Laboratory examination indexes						
Cr ( $\bar{x}\pm s$ , umol/L)	80.1 $\pm$ 15.1	80.2 $\pm$ 14.9	80.0 $\pm$ 14.5	80.2 $\pm$ 15.2	0.002	>0.99
ALT ( $\bar{x}\pm s$ , U/L)	22.4 $\pm$ 6.1	23.2 $\pm$ 5.8	22.8 $\pm$ 6.4	20.6 $\pm$ 5.4	1.326	0.268
SBP (mmHg)	132.5 $\pm$ 12.4	133.1 $\pm$ 11.6	131.8 $\pm$ 13.2	133.2 $\pm$ 12.1	0.110	0.954
DBP (mmHg)	77.6 $\pm$ 5.3	78.1 $\pm$ 6.2	78.5 $\pm$ 4.7	79.4 $\pm$ 6.1	0.709	0.548
LVEF ( $\bar{x}\pm s\pm s$ , %)	62.2 $\pm$ 4.8	60.3 $\pm$ 5.6	59.6 $\pm$ 5.3	60.1 $\pm$ 6.8	1.707	0.168
Echocardiography indexes						
LAD (mm)	44.6 $\pm$ 7.2* <sup>#</sup>	48.4 $\pm$ 6.7*	51.9 $\pm$ 6.9*	40.6 $\pm$ 6.4	18.480	<0.0001
LVED (mm)	46.7 $\pm$ 6.9	47.1 $\pm$ 7.2	48.3 $\pm$ 7.1	45.2 $\pm$ 6.9	1.299	0.277
MVA (cm <sup>2</sup> )	1.02 $\pm$ 0.16	1.04 $\pm$ 0.18	1.01 $\pm$ 0.19	0.96 $\pm$ 0.12	1.490	0.220

AF – atrial fibrillation; SR – sinus rhythm; M – male; F – female; AECI – angiotensin converting enzyme inhibitor; ARB – angiotensin receptor blocker; Cr – creatinine; ALT – alanine aminotransferase; SBP – systolic blood pressure; DBP – diastolic blood pressure; LVEF – left ventricular ejection fraction; LAD – left atrial diameter; LVED – left ventricular end-diastolic diameter; MVA – mitral valve area; \* compared with SR group,  $P < 0.05$ ; # compared with permanent AF group,  $P < 0.05$ .

**TdT-mediated dUTP nick-end labeling (TUNEL)**

Tissue sections were deparaffinized and rehydrated to water. For antigen retrieval, sections were added with 20  $\mu$ g/mL of proteinase k (Roche, Cat No.745723) at 37°C for 30 min and washed in 0.01 mol/L of phosphate buffer solution (PBS) (pH7.4) for 3 times  $\times$  5 min. The specific reaction were terminated by 20% bovine calf serum and 3% bovine serum proteins at 37°C for 15 min. Subsequently, the sections were incubated in a wet box at 37°C for 1 h with 50  $\mu$ l of reaction solution containing 5  $\mu$ l of TdT and 45  $\mu$ l of fluorescein conjugated nucleotide mixed buffer, and washed with 0.01 mol/L of PBS (pH7.4) 3 times  $\times$  5 min. Subsequently, endogenous peroxidase activity was blocked by 0.3% hydrogen peroxide solution (H<sub>2</sub>O<sub>2</sub>) at room temperature and sections were rinsed 3 times in PBS. The specific responses

were blocked by again adding 20% bovine calf serum, 3% bovine serum proteins, and 1% blocking agent at 37°C for 15 min. Immunological reaction was amplified at wet box at 37°C for 30 min with 20 l of POD (HRP conjugated anti-fluorescein antibody; 1:2). We used 0.01 mol/L of PBS (pH7.4) to wash the sections for 3 times  $\times$  5 min. Staining was performed using DAB-H<sub>2</sub>O<sub>2</sub> and was observed using a microscope. After the nucleuses were colored dark brown, the sections were washed under running water, followed by hematoxylin staining, ethanol dehydration, and vitrification by dimethylbenzene, and were mounted by neutral resins. Five consecutive non-overlapping  $\times$ 400 high-power fields were selected. The average count of total cardiomyocyte and apoptotic cardiomyocyte cell numbers per high-power field was calculated from these 5 fields. Apoptotic index (AI)=number of apoptotic cardiomyocyte/total cardiomyocyte  $\times$  100%.



**Figure 1.** The immunohistochemistry results on MMP-2, Bcl-2, and BAX between SR group and AF groups (**Aa–Ac**, positive staining of MMP-2 (**A-a**), Bcl-2 (**A-b**) and BAX (**A-c**) on SR group; **Ba–Bc**, positive staining of MMP-2 (**B-a**), Bcl-2 (**B-b**) and BAX (**B-c**) on paroxysmal AF group; **Ca–Cc**, positive staining of MMP-2 (**C-a**), Bcl-2 (**C-b**) and BAX (**C-c**) on persistent AF group; **Da–Dc**, positive staining of MMP-2 (**D-a**), Bcl-2 (**D-b**) and BAX (**D-c**) on permanent AF group; SR, sinus rhythm; AF, atrial fibrillation; MMP-2, matrix metalloproteinase-2; Bcl-2, B-cell lymphoma 2; BAX, Bcl-2-associated X protein).

### Statistical analysis

All data were saved in an Excel database. SPSS 21.0 version was used for the data analysis. Quantitative data are presented with percentage and tested by chi-square test. Test of normality and homogeneity test of variance were applied for continuous data. Mean comparisons between 2 groups on continuous data were conducted using the *t* test and comparisons among groups were conducted with univariate analysis of variance. The correlations between MMP-2 protein expression level, Bcl-2 expression level, and BAX expression level were conducted using Pearson correlation analysis.  $P < 0.05$  was regarded as statistical significance.

## Results

### Clinical data

The clinical data, such as age, body mass index (BMI), medication usage, and laboratory examination indexes, between AF groups and SR group showed no significant differences (all  $P > 0.05$ ). AF groups had increased LAD compared with SR

group (all  $P < 0.05$ ). More specifically, the permanent AF group had a larger LAD than the paroxysmal AF group and persistent AF group (both  $P < 0.05$ ), but the LVED, left ventricular ejection fraction (LVEF), and MVA among the 3 groups showed no differences (all  $P > 0.05$ ) (Table 2).

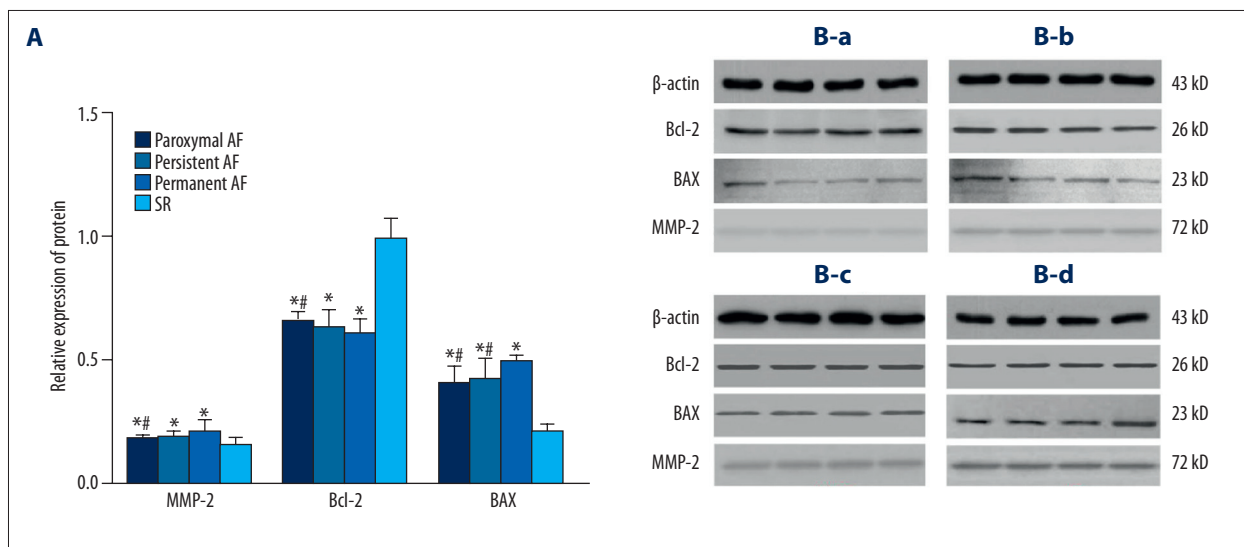
### Comparison of immunohistochemistry staining between AF and SR group

Positive reaction mainly occurred in endochylema with brown-yellow granules. Regarding the expression of MMP-2 and BAX, the endochylema observed in the SR group were well-distributed and presented with sparse light-brown granules. The staining results in AF groups were presented with an increased trend in the color of brown-yellow granules in the paroxysmal AF, persistent AF, and permanent AF groups. As for the expression of Bcl-2, the SR group had more widespread light-brown granules than in AF groups. In contrast to the expression of MMP-2 and BAX, the permanent AF group had the lightest brown granules while the paroxysmal AF group had the darkest granules (Figure 1).

**Table 3.** The comparisons on MMP-2, Bcl-2 and BAX mRNA between AF group and SR group ( $\bar{x}\pm s$ ).

	AF group			SR group	F	P
	Paroxysmal AF	Persistent AF	Permanent AF			
MMP-2	1.186±0.245*	1.199±0.236*	1.207±0.274*	0.988±0.184	0.680	0.002
Bcl-2	3.045±0.348*,&#	2.945±0.372*#	2.675±0.214*	3.256±0.278	24.98	<0.0001
BAX	1.418±0.148*,&#&	1.564±0.189*#	1.875±0.248*	1.256±0.217	67.7	<0.0001

AF – atrial fibrillation; SR – sinus rhythm; MMP-2 – matrix metalloproteinase-2; Bcl-2 – B-cell lymphoma-2; BAX – Bcl-2-associated X protein; \* compared with SR group,  $P<0.05$ ; # compared with permanent AF group,  $P<0.05$ ; & compared with persistent AF,  $P<0.05$ .



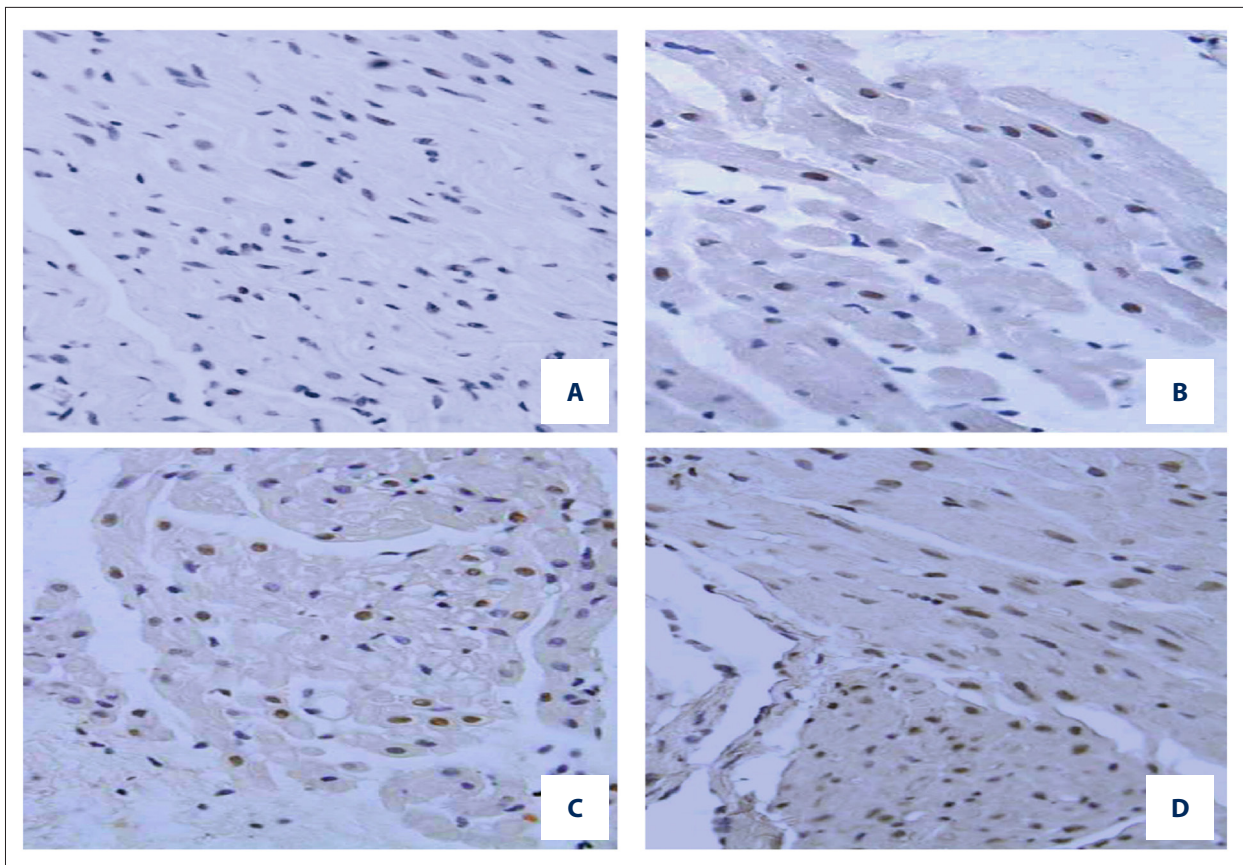
**Figure 2.** Electrophoretogram of protein expression of MMP-2, Bcl-2 and BAX between SR group and AF groups (A, protein expression of MMP-2, Bcl-2 and BAX in SR and AF group; B-a, protein expression of MMP-2, Bcl-2 and BAX in SR group; B-b, protein expression of MMP-2, Bcl-2 and BAX in paroxysmal group; B-c, protein expression of MMP-2, Bcl-2 and BAX in persistent AF group; B-d, protein expression of MMP-2, Bcl-2 and BAX in permanent AF group; SR, sinus rhythm; AF, atrial fibrillation; MMP-2, matrix metalloproteinase-2; Bcl-2, B-cell lymphoma 2; BAX, Bcl-2-associated X protein.).

**Comparisons of MMP-2, Bcl-2, and BAX mRNA between AF and SR groups ( $\bar{x}\pm s$ )**

Our results show that the mRNA levels of MMP-2 and BAX were significantly increased, whereas the levels of Bcl-2 decreased in the AF groups compared with the SR group (all  $P<0.05$ ). Specifically, no statistical significance was found on MMP-2 mRNA expression level among the 3 AF groups (all  $P>0.05$ ). The permanent AF group had higher BAX mRNA expression level than the persistent AF group and paroxysmal AF group (both  $P<0.05$ ). The persistent AF group had elevated BAX mRNA expression level compared with the paroxysmal AF group ( $P<0.05$ ). Among the 3 AF groups, the permanent AF group had the lowest Bcl-2 mRNA expression level ( $P<0.05$ ). The expression level of Bcl-2 mRNA between the paroxysmal AF group and persistent AF group showed no statistical significance ( $P>0.05$ ) (Table 3).

**Comparisons of MMP-2, Bcl-2, and BAX expression levels between AF and SR groups ( $\bar{x}\pm s$ )**

AF groups had higher protein expression levels of MMP-2 and BAX than the SR group (both  $P<0.05$ ). Consistent with the results of MMP-2 mRNA expression level, our results found no statistically significant difference in protein expression levels among the 3 AF groups (all  $P>0.05$ ). In the 3 AF groups, the permanent AF group had higher expression levels of BAX than the other 2 groups ( $P>0.05$ ), while no significant difference was detected between the persistent AF group and the paroxysmal AF group ( $P>0.05$ ). Compared with the SR group, AF groups had remarkably decreased Bcl-2 expression levels ( $P<0.05$ ). The permanent AF group had higher Bcl-2 expression level than did the persistent AF group ( $P<0.05$ ). No significant difference was detected between the persistent AF group and the paroxysmal AF group ( $P>0.05$ ) (Figure 2).



**Figure 3.** TdT-mediated dUTP nick-end labeling (TUNEL) staining on apoptosis between SR group and AF groups (SR, sinus rhythm; AF, atrial fibrillation; **A**, SR group; **B**, paroxysmal AF group; **C**, persistent AF group; **D**, permanent AF group).

### TUNEL staining in cardiomyocyte apoptosis

The sections in the SR group were almost normal tissues and no apparent apoptotic cells were observed, while the AF groups had slight TUNEL staining in the nucleus with expanded heterochromatin volume and uniform distribution. The TUNEL staining in the SR and AF groups suggested that the AF groups had an elevated cardiomyocyte apoptosis rate compared to the SR group. Moreover, among the AF groups, our study also found that the highest cardiomyocyte apoptosis rate was observed in the permanent AF group, followed by the persistent AF group and paroxysmal AF group (Figure 3).

### AI and BAX/Bcl-2 ratio (%) between SR and AF groups

The AIs in AF groups were evidently higher than in the SR group ( $P<0.05$ ). The highest and lowest AIs observed in the AF groups were in the permanent AF group and paroxysmal AF group, respectively (all  $P<0.05$ ) (Table 4). AF groups had elevated BAX/Bcl-2 ratio (%) compared with the SR group (all  $P<0.05$ ). In AF groups, the permanent AF group had the highest BAX/Bcl-2 ratio (%) among the 3 AF groups ( $P<0.05$ ), while the comparison between paroxysmal AF group and

persistent AF group showed no statistically significant difference ( $P>0.05$ ) (Table 4).

### Correlation between MMP-2, Bcl-2 and BAX

Our analysis demonstrated negative correlations between MMP-2 protein expression levels and Bcl-2 protein expression levels (paroxysmal AF group:  $r=-0.892$ ,  $P<0.01$ ; persistent AF group:  $r=-0.927$ ,  $P<0.01$ ; permanent AF group:  $r=-0.853$ ,  $P<0.01$ ) and positive correlations between MMP-2 protein expression levels and BAX protein expression levels (paroxysmal AF group:  $r=0.911$ ,  $P<0.01$ ; persistent AF group:  $r=0.945$ ,  $P<0.01$ ; permanent AF group:  $r=0.876$ ,  $P<0.01$ ) (Figure 4A–4C) among the 3 AF groups.

### Discussion

The present study was conducted to investigate the expression levels of MMP-2, Bcl-2, and BAX in AF patients and SR patients with the application of immunohistochemistry, Western blot, RT-PCR, and TUNEL because no agreement has been reached so far regarding to the expressions of MMP-2, Bcl-2, and BAX

**Table 4.** The comparisons on apoptotic index and Bcl-2/BAX ratio between AF group and SR group (%) ( $\bar{x}\pm s$ ).

Groups	n	AI (%)	F	P	BAX/BCL-2 (%)	F	P
AF group							
Paroxysmal AF group	42	8.45±2.34*,&			62.722±14.327*,&		
Persistent AF group	36	12.6±5.85*,&	154.1	<0.0001	69.413±21.220*,&	135.4	<0.0001
Permanent AF group	45	16.3±6.34*			82.656±11.580*		
SR group	35	3.47±0.52			20.914±2.741		

AF – atrial fibrillation; SR – sinus rhythm; AI – apoptotic index; Bcl-2 – B-cell lymphoma-2; BAX – Bcl-2-associated X protein;  
\* compared with SR group,  $P<0.05$ ; # compared with permanent AF group,  $P<0.05$ ; & compared with persistent AF group,  $P<0.05$ .

in AF patients and in apoptosis. Our results demonstrated that AF patients had notably increased MMP-2 and BAX expression levels, but decreased Bcl-2 levels, compared with SR patients. Correlation analysis also defined a positive correlation between MMP-2 and BAX, and a negative correlation between MMP-2 and Bcl-2. In the current study, investigations on mRNA and protein expression for MMP-2, Bcl-2, and BAX were integrated to conduct a comprehensive study, which is a unique characteristic of our study. Moreover, the AF patients included in the current study were further sub-grouped into paroxysmal AF patients, persistent AF patients, and permanent AF patients, which contributes to the abundant and comprehensive results in our study and can be considered as an advantage over other studies. Therefore, our study may serve as an important reference for future studies into the possible mechanisms of AF.

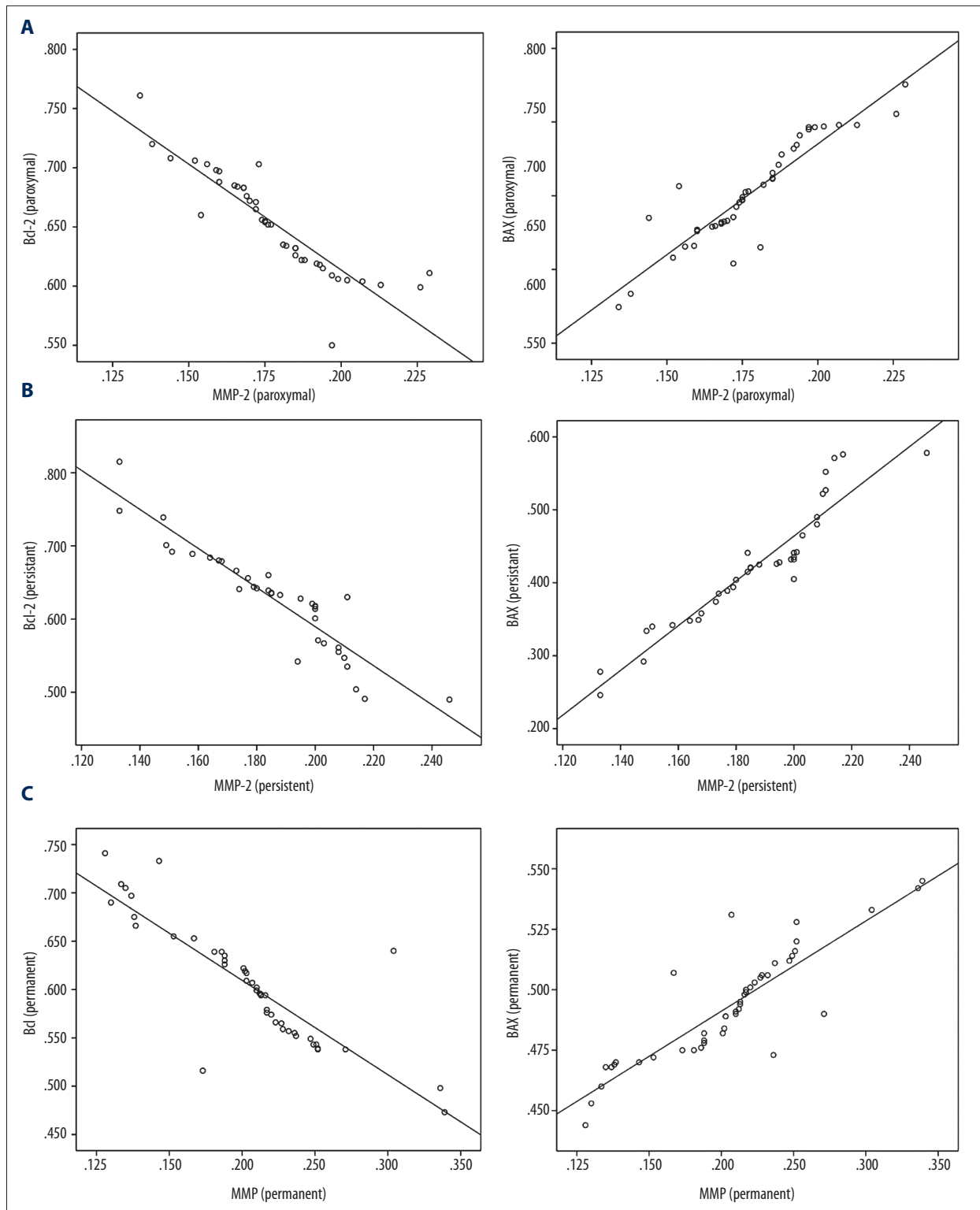
Our results showed that AF patients had enlarged LAD compared with SR patients, which was supported by other studies suggesting left atrial (LA) enlargement is an independent risk factor for AF [27,28]. Indeed, fibrosis and extracellular matrix remodeling are significantly implicated in left atrial enlargement, and increased left atrial volume is a strong predictor of postoperative AF [29,30]. Therefore, left atrial fibrosis and enlarged LAD could contribute to the pathogenesis of AF [31].

We examined the mRNA and protein expression levels of MMP-2, Bcl-2, and BAX between AF patients and SR patients. Our results demonstrated that AF patients had elevated MMP-2 and BAX expressions, compared with SR patients. Although the details remain unclear, the persistence of AF is considered as a result of atrial remodeling, which could in turn contribute to the initiation and development of AF [14]. LAD enlargement triggers the activation of the renin aldosterone angiotensin system (RAAS) and subsequently lead to increase in matrix metalloproteinases, which result in atrial remodeling and fibrosis, with loss of atrial muscle mass [32]. In addition, interstitial fibrosis may induce local intra-atrial conduction block and increase atrial susceptibility to AF, as well as forming stable local

sources for AF induction [33,34]. Consistent with our results, evidence showed that MMP-2 and TIMP-2 were significantly involved in the pathogenesis of arrhythmogenic atrial remodeling [16]. It is likely that apoptosis appeared in the early stage of AF and was associated tissue remodeling [35]. Accordingly, our study examined the AI in patients with AF and suggested that AF patients showed higher AI than SR patients as well as an increased trend of AI among paroxysmal AF, persistent AF, and permanent AF patients. Bcl-2 and BAX are hallmarks of cell death and play a pathophysiological role in the protection or acceleration of apoptosis [36]. Bcl-2 is a member of the Bcl-2 family, belonging to the anti-apoptotic group, whereas BAX was highlighted for its pro-apoptotic function [37,38]. It was suggested that the mitochondrial destabilization, a major determinant of the apoptosis cascade, was related to the adenosine triphosphate (ATP) depletion, while the decreased ATP levels promotes the transfer of the BAX to the mitochondria [39,40]. In agreement with our results, a study identified an impaired balance between BAX and Bcl-2, with increased levels of BAX and lower levels of Bcl-2 with ageing and in AF, which determined that the expression of BAX and bcl-2 were correlated with the frequency of apoptosis in left atria organization with ageing and in AF [33].

Our results demonstrated that BAX/Bcl-2 ratio was significantly increased in AF patients compared with SR patients. The ratio between anti- and pro-apoptotic proteins is considered as a key point for tissue homeostasis due to its significances in cells sensitivity to induce cell apoptosis [41]. Our correlation analysis found a positive correlation between MMP-2 and BAX but a negative correlation between MMP-2 and Bcl-2. Brunelle JK et al. reported that the trigger of mitochondrial apoptosis requires the involvement of the Bcl-2 family, including anti-apoptotic Bcl-2 and anti-apoptotic BAX, which contribute to the myocardial injury and fibrosis deposition [42,43]. Moreover, studies demonstrated that MMP-2 was involved in the atrial structural remodeling and proved it is a significant mediator of ventricular remodeling and systolic dysfunction [44,45]. Therefore, we proposed a hypothesis that MMP-2 may be





**Figure 4.** The correlation between MMP-2 and Bcl-2, BAX in AF groups (A, paroxysmal AF group; B, persistent AF group; C, permanent AF group; AF, atrial fibrillation; MMP-2, matrix metalloproteinase-2; Bcl-2, B-cell lymphoma 2; BAX, Bcl-2-associated X protein).

associated with the expression of Bcl-2 and BAX. More specifically, our results implied the MMP-2 was positively correlated with BAX and negatively correlated with Bcl-2.

The limitations of current study should be considered when interpreting our results. Firstly, although 123 AF patients were included in the current study, the sample sizes in sub-groups of AF patients were rather small. Moreover, the possible mechanism among the interaction of MMP-2, BAX, and Bcl-2 was not discussed in the current study, and requires further investigation.

## Conclusions

In summary, our study demonstrated that elevated expression level of MMP-2 and disturbed balance between Bcl-2 and BAX expression levels may be a possible mechanism for

atrial remodeling and atrial fibrosis in the occurrence and persistence of AF. Although the end point for the experiments in the current study was February 2015, our study is the first to hypothesize the interplay among MMP-2, BAX, and Bcl-2 in AF progression. However, due to the limitations mentioned above, our investigation was planned to carry on to the next step to explore the exact mechanism among the interaction among these 3 proteins.

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## Competing interests

We declare that we have no conflicts of interest.

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