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Chapter 11

Atrial HSP levels are associated with persistent AF, AF recurrence after Maze surgery and post-operative AF

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In preparation

Abstract

Background Early detection and staging of atrial fibrillation (AF) is of great importance to start optimal treatment and prevent and/or delay disease progression. Serum (bio)markers, such as heat shock proteins (HSP), may enable staging of AF and identify patients at risk for AF recurrence and development of post-operative AF (PoAF).

Objective This study evaluates the relation between serum and atrial tissue HSP levels, the presence of AF, AF stage, AF recurrence after Maze treatment and PoAF following elective cardiothoracic surgery.

Methods Patients without a history of AF (controls in sinus rhythm (SR)) and with paroxysmal (PAF), persistent (PeAF) and longstanding persistent (LSPeAF) AF presenting for coronary artery bypass grafting and/or mitral and/or aortic valve surgery and/or first correction of a congenital heart defect, partly combined with Maze procedure (AF patients), were included. HSPB1, HSPA1, HSPB7 and HSPD1 levels were measured in serum obtained prior to intervention and at 6- and 12-months post-surgery. HSPB1, HSPA1, HSPA5, HSPD1, HSPB5 and pHSF1 levels were measured in tissue from right and, in a selection of patients, left atrial appendages obtained during the open heart surgery.

Results The study population (N=124) consisted of 64 control (in SR) and 60 AF patients. HSPB1, HSPA1, HSPB7 and HSPD1 serum levels were similar among control and AF groups with and without PoAF (control patients) or AF recurrence after Maze surgery (AF patients). HSPA5 RAA levels were significantly lower in (LSPe)AF (P<0.05) and HSPD1 RAA levels significantly higher in (Pe)AF patients (P<0.01) compared to controls. HSPA1 and HSPA5 RAA levels were significantly higher in controls who developed PoAF, compared to controls without PoAF. HSPB1 RAA levels were lower and HSPA5 LAA levels higher in Maze-treated AF patients who developed AF recurrence within 1 week after surgery compared to patients who did not.

Conclusions Atrial tissue levels of HSPA5 and HSPD1 are altered in more persistent stages of AF compared to controls in sinus rhythm. Also, atrial tissue levels of HSPA1 and HSPA5 can predict PoAF development and HSPB1 RAA and HSPA5 LAA levels can predict AF recurrence in Maze-treated AF patients. Serum HSP levels cannot discriminate AF (stage) from controls, and cannot predict PoAF or AF recurrence after treatment.

Introduction

Atrial fibrillation (AF) is the most common clinical arrhythmia with a rising prevalence due to the aging population [1] and is associated with severe complications such as thromboembolic events, heart failure, cognitive impairment and increased mortality [2, 3]. AF initially presents with short, self-terminating episodes and often progresses into long-lasting episodes which are more difficult to reverse to sinus rhythm [4]. The accelerating nature of AF is rooted in the underlying electropathology, which is defined as structural damage in atrial myocardium which underlies electrical conduction abnormalities and contractile dysfunction of cardiomyocytes. Of note, electropathology is already present at first onset of AF [5-8]. Early detection and staging of AF is essential to start an appropriate treatment. Currently, AF is diagnosed using surface electrocardiograms, practically in patients with symptomatic AF who present with palpitations or thromboembolic events, while asymptomatic and very short-lasting episodes of AF may be left undiagnosed [9].

The current pharmacological and ablative treatment strategies are not curative and aim at alleviation of AF symptoms, and controlling the heart rhythm and rate. As AF progresses and damage in the heart accumulates, reversal to sinus rhythm is especially challenging in the later stages of the disease [3]. Thus, early detection and staging of AF and subsequent selection of effective treatment of AF is of utmost importance. Accordingly, there is an urgent need to identify diagnostic biomarkers to aid patient tailored therapy in order to treat the disease and prevent its progression.

Despite the fact that biomarkers are commonly accepted as a diagnostic tool to screen or monitor patients for a variety of cardiovascular diseases and several blood-based biomarkers related to AF pathology have been identified, including brain natriuretic peptides, cancer-antigen-125, fibroblast growth factor-23 [10, 11] and -21 [12], high-sensitive cardiac troponin I [13], homocysteine [14], (a)symmetric dimetylarginine [15], interleukine-6 and matrix metalloproteinase-9/tissue inhibitor of metalloproteinase-1 ratio [16], these biomarkers have not been studied to detect AF, predict the stage of AF (paroxysmal or (longstanding) persistent AF) or predict PoAF or AF recurrence after AF therapy.

Emerging evidence indicates that heat shock proteins (HSPs) may represent a suitable biomarker to discriminate AF (stage) and predict PoAF and AF recurrence. HSPs, chaperones with an important role in safeguarding proteostasis - the homeostasis of protein expression, function and degradation in cells [17] – are upregulated during stress and disease, including early stage of AF, especially via activation of the heat shock transcription factor 1 [18]. Within the HSP family, small HSPs, especially HSPB1, are probably the most important in maintaining proteostasis in cardiomyocytes by stabilizing the contractile proteins [19-22]. However, derailment of proteostasis has been identified as a key factor underlying electropathology and AF progression [17, 23, 24]. Previously, atrial HSPB1 levels were found to be induced in atrial tissue samples of patient with paroxysmal AF, while tissue HSPB1 levels get exhausted in patients with (longstanding) persistent AF [19], indicating that low tissue HSPB1 levels are associated with AF progression. Lower HSPD1 tissue levels correlated with a higher degree of myolysis in patients with AF [25] and lower HSPA1 tissue levels correlated with post-operative AF (PoAF) in patient who underwent coronary artery bypass grafting (CABG) surgery. In contrast, serum HSPA1 levels were not predictive for PoAF in these studies [26, 27]. In the study of Hu et al. low baseline serum HSPB1 levels of patients who received ablative therapy predicted AF recurrence and patients with high baseline serum levels of HSPB1 showed an improved maintenance rate of sinus rhythm [28]. Whether serum HSP levels represent biomarkers to identify the stage of AF, PoAF and/or AF recurrence is unknown. In the current study, various members of the HSP family, including HSPA1, HSPA5, HSPB1, HSPB5, HSPB7, HSPD1 and pHSF1, were measured in serum and/or atrial tissue samples of controls and patients with paroxysmal (PAF), persistent (PeAF) and longstanding persistent (LSPeAF) AF undergoing elective cardiothoracic surgery, to identify whether HSPs associate with the stage of AF, onset of PoAF in controls and AF recurrence in AF patients within 1 week after Maze-treatment.

Methods

Study population

Patients, with or without a history of AF, scheduled for elective cardiothoracic surgery due to structural heart disease, were prospectively enrolled between December 2014 and April 2017 in the HALT & REVERSE study [29] at the department of cardiology of the Erasmus MC, Rotterdam, the Netherlands. The study population for this interim analysis includes 124 patients, >18 years, who underwent either coronary artery bypass grafting (CABG) and/or mitral valve surgery and/or aortic valve surgery and/or first correction of a congenital heart defect (mostly atrial septal defects), from whom serum and/or tissue HSP data is available. The study population is divided into a control group including patients without AF (N=64) and the study group including patients with AF (N=60): PAF (<7 days of AF), PeAF (7 days - 1 year of AF) or LSPeAF (>1 year of AF). Exclusion criteria were manifest heart failure, need for inotropic or mechanical support, medical history of heart surgery, atrial ablation procedure, pericarditis or the presence of an atrial pacemaker lead.

Clinical characteristics were obtained from the electronic patients' files. After the surgical procedure, heart rhythm was continuously monitored until hospital discharge in order to detect PoAF and AF recurrence within one week. Patients were followed for 12 months post-procedure and completion of the 12 months follow-up period was the study endpoint or earlier due to death, withdrawn informed consent, pacemaker implant, or AF recurrence.

The HALT & REVERSE trial (MEC-2014-393)[29], approved by the institutional medical ethical committee, is executed according to the principals of the Declaration of Helsinki in accordance with the Medical Research Committee involving the Human Subjects Act. All patients gave written informed consent prior to inclusion.

Sampling

Blood samples were obtained from all patients after admission at the ward of cardiothoracic surgery (12-24 hours prior to surgery) in BD Vacutainer™ SST™ II Advance Tubes (Fisher Scientific, The

Netherlands). Serum was separated from blood by centrifugation at 2000 x g for 10 min at 4°C and subsequently frozen at -80°C until analysis. Patients visited the outpatient clinic at 6- and 12-months after the procedure to provide follow-up serum samples and to screen for the presence of (post-operative) AF.

Right atrial appendages (RAA) were obtained from all patients (both with and without AF) during cannulation for extracorporal circulation. Furthermore, amputation of the left atrial appendage (LAA) was performed after cardioplegia in a selection of AF patients. Both RAA and LAA were immediately snap frozen in liquid nitrogen and stored at -80°C until analysis.

Analysis of serum samples

HSPB1, HSPA1, HSPB7 and HSPD1 levels in serum samples were determined by ELISA measurements. HSPB1 (serum 6x diluted in 1% BSA in PBS) and HSPA1 protein (serum 2x diluted in 1% BSA in PBS) levels were measured in triplicates using human HSPB1 or HSPA1 DuoSet® ELISA kits from R&D (Cat. no. DY1580 and DY1663, respectively) according to manufacturer's instructions with minor adjustments (serum was incubated at 4°C overnight, instead of 2 hours at room temperature). HSPB7 protein levels (undiluted serum, measured singular) were determined with ELISA kits from Cusabio (CSB-EL010838HU) according to manufacturer's instructions with minor adjustments (kept incubation temperature at 20°C). HSPD1 protein levels (undiluted serum) were measured in duplicates with the HSPD1 DuoSet® ELISA kit from R&D (DYC1800) according to manufacturer's instructions.

Analysis of RAA and LAA

Part of the frozen RAA and LAA was cut in small fragments on dry ice and added to ice cold sample buffer (15% glycerol; 1% SDS; 12,5% 0.5 M Tris, pH 6.8; 2% bromophenol-blue solution and protease and phosphatase inhibitors). The tissue was homogenized using metal beads in the Qiagen Tissuelyser II for 3 min at 30 Hz, left on ice for 30 min for continued cell lysis and homogenized again for 3 min at

30 Hz. The lysates were centrifuged (20 min, 14.000 rpm at 4°C), supernatant was collected, passed through an insulin syringe, boiled for 5 min at 95°C and samples were stored at -20°C until analysis.

For Western blot analysis, equal amounts (10 µg) of protein were separated on 4–20% Criterion TGX precast gels (Bio-Rad, The Netherlands) and transferred to nitrocellulose membranes (Bio-Rad). Membranes were blocked in 5% skim milk in TBST for 1 hour at room temperature. Overnight incubation at 4°C with primary antibody (in 3% BSA in TBST) was followed by secondary antibody (in 3% BSA in TBST) incubation for 1 hour at room temperature with horseradisch peroxidase-conjugated goat-anti-rabbit or goat-anti-mouse antibodies (Dako Cytomation, Denmark), depending on the species origin of the primary antibody (Table 1). Signals were detected by the Amersham ECL prime Western blotting detection reagent (GE Healthcare Life Sciences, The Netherlands) utilizing the Amersham Imager 600 (GE Healthcare Life Sciences) and quantified by densitometry (ImageQuantTL, GE Healthcare Life Sciences). Western blot results were from quadruplicate for HSPB1, HSPA1, HSPA5 and HSPD1, duplicate for HSPB5 and triplicate measures for pHSF1. Protein amounts were expressed relative to GAPDH for whole protein lysates.

Table 1. Primary antibodies

Antibody	Cat. no	Company
Mouse anti-HSPB1	ADI-SPA-800	Enzo-Lifesciences, USA
Mouse anti-HSPA1	ADI-SPA-810	Enzo-Lifesciences, USA
Rabbit anti-HSPA5	ab21685	Abcam, UK
Rabbit anti-HSPD1	ADI-SPA-805	Enzo Life Sciences, USA
Rabbit anti-HSPB5	ADI-SPA-223	Enzo Life Sciences, Belgium
Rabbit anti-HSF1	4356	Cell Signaling Technology, USA
Rabbit anti-pHSF1	sc-30443-R	Santa-Cruz Biotechnology, USA
Mouse anti-GAPDH	10R-G109a	Fitzgerald Industries International, USA

Statistical analysis

Data were analyzed with SPSS Statistics version 26.0 for Windows (SPSS, Inc.) and GraphPad Prism version 8.0 (Graphpad Software Inc., CA). All data were tested for Gaussian distribution. Continuous normally distributed data are presented as mean ± standard deviation (SD), non-normally distributed data as median [interquartile range (IQR)], and categorical data as number (percentage). Differences in clinical characteristics and HSP levels between patients with and without AF were tested with independent-samples *t*-test, Mann-Whitney test and Chi-square test. Differences in clinical characteristics and HSP levels between patients without AF, PAF, PeAF and LSPeAF were tested with one-way analysis of variance (ANOVA), Kruskal-Wallis test, Chi-square test and Fisher's Exact test. When serum HSP levels were below detection limit of the ELISA (only for N=15 HSPD1), values at the lower limit of detection were used for statistical analysis. HSP levels are not normally distributed and are Log transformed for statistical analysis (original HSP values are presented in Tables and Figures). The difference between baseline and follow-up serum HSP levels was calculated with a repeated measures model. A two-sided *P* value of <0.05 indicates statistical significance.

Results

Study population

The entire study population consisted of 124 patients (73.4% males, age 67.7 ± 10.6 years), including a control group of 64 (51.6%) patients without AF and a study group of 60 patients with either PAF (N=15, 12.1%), PeAF (N=28, 22.6%), or LSPeAF (N=17, 13.7%). Table 2 outlines baseline characteristics of the entire study population. The parameters age, gender, BMI, hypertension, diabetes mellitus, dyslipidemia, thyroid disease and left ventricular function were similar among the control and AF group. AF patients significantly use more often statins (*P*<0.01 for PeAF, *P*<0.05 for AF total), class III antiarrhythmic drugs (*P*<0.01 for PAF, *P*<0.05 for PeAF and *P*<0.01 for AF total) and digoxin (*P*<0.001 for PeAF, LSPeAF and AF total, respectively) compared to controls. Also, 63.3% of the AF patients is

treated for AF with a Maze procedure on top of the treatment for underlying heart disease (*P*<0.001 compared to control). The type of underlying heart disease varies between control and AF group (*P*<0.001).

Table 2. Clinical characteristics of the study population AF

	Control	PAF	PeAF	LSPeAF	AF total
N (%)	64	15	28	17	60
Age (years), mean ± SD	66.7 ± 11	68.7 ± 14.7	67.1 ± 8.7	71.8 ± 5.8	68.9 ± 10
Gender, male, N (%)	49 (76.6)	9 (60)	19 (67.9)	14 (82.4)	42 (70)
BMI (kg/m2), mean ± SD	28 ± 4.1	25.4 ± 4.1	27.5 ± 4.8	28.4 ± 3.6	27.2 ± 4.4
Hypertension, yes, N (%)	41 (64.1)	11 (73.3)	16 (57.1)	10 (58.8)	37 (61.7)
Diabetes mellitus, yes, N (%)	18 (28.1)	2 (13.3)	4 (14.3)	6 (35.3)	12 (20)
Dyslipidemia, yes, N (%)	26 (40.6)	9 (60)	6 (21.4)	4 (23.5)	19 (31.7)
Thyroid disease, yes, N (%)	4 (6.3)	3 (20)	2 (7.1)	1 (5.9)	6 (10)
Left ventricular function (LVF)					
Normal, N (%)	49 (76.6)	12 (80)	16 (57.1)	10 (58.8)	38 (63.3)
Mild impairment, N (%)	14 (21.9)	2 (13.3)	6 (21.4)	5 (29.4)	13 (21.7)
Moderate impairment, N (%)	1 (1.6)	1 (6.7)	5 (17.9)	2 (11.8)	8 (13.3)
Severe impairment, N (%)	0 (0)	0 (0)	1 (3.6)	0 (0)	1 (1.7)
Drugs, yes, N (%)					
Drugs total	57 (89.1)	14 (93.3)	27 (96.4)	17 (100)	58 (96.7)
ACE. ARB. AT2 antagonist	42 (65.6)	8 (53.3)	17 (60.7)	15 (88.2)	40 (66.7)
Statin	47 (73.4)	9 (60)	9 (32.1)**	13 (76.5)	31 (51.7)*
Antiarrhythmic drugs (AAD) total†	46 (71.9)	13 (86.7)	26 (92.9)*	15 (88.2)	54 (90)
Class I AAD	1 (1.6)	2 (13.3)	1 (3.6)	0 (0)	3 (5)
Class II AAD	43 (67.2)	9 (60)	20 (71.4)	15 (88.2)	44 (73.3)
Class III AAD	0 (0)	4 (26.7)**	4 (14.3)*	1 (5.9)	9 (15)**
Class IV AAD	3 (4.7)	0 (0)	1 (3.6)	1 (5.9)	2 (3.3)
Digoxin	0 (0)	1 (6.7)	8 (28.6)***	5 (29.4)***	14 (23.3)***
Maze on top of procedure, N (%)	0 (0)	9 (60)***	19 (67.9)***	10 (58.8)***	38 (63.3)***
Underlying heart disease			**		***
CAD, N (%)	37 (57.8)	3 (20)	2 (7.1)	4 (23.5)	9 (15)
AVD, N (%)	6 (9.4)	4 (26.7)	5 (17.9)	3 (17.6)	12 (20)
MVD, N (%)	5 (7.8)	3 (20)	11 (39.3)	5 (29.4)	19 (31.7)
CHD, N (%)	4 (6.3)	0 (0)	5 (17.9)	1 (5.9)	6 (10)
CAD + AVD, N (%)	8 (12.5)	3 (20)	3 (10.7)	2 (11.8)	8 (13.3)
CAD + MVD, N (%)	4 (6.3)	2 (13.3)	2 (7.1)	1 (5.9)	5 (8.3)
TVR, N (%)	0 (0)	0 (0)	0 (0)	1 (5.9)	1 (1.7)

*P<0.05, **P<0.01 and ***P<0.001 compared to control. CAD = coronary artery disease, AVD = aortic valve disease, MVD = mitral valve disease, CHD = congenital heart disease and TVR = tricuspid valve regurgitation
†Patients could use more than one type of AAD; therefore, the sum of all classes is not equal to total

HSP levels in atrial tissue and serum related to clinical stage of AF

Both atrial tissue and serum HSP levels were evaluated in relation to the clinical stage of AF. Atrial tissue (RAA and LAA) levels of HSPB1, HSPA1, HSPB5 and pHSF1 are similar between control and AF subgroups (Figure 1 and Table 3). HSPA5 RAA levels are significantly lower in LSPeAF and in the total AF group (both $P<0.05$), compared to control. HSPD1 RAA levels are significantly higher in PeAF and in the total AF group compared to control (both $P<0.01$). In addition, LAA HSPD1 levels are significantly higher in PeAF compared to PAF ($P<0.05$). RAA and LAA levels are significantly positively correlated for HSPB1, HSPA1, HSPD1 and HSPB5, while this wat not the case for HSPA5 and pHSF1, the latest following an inverse line (Figure 2).

Table 3. Concentration of HSPs in tissue of control and AF patients

	Control	PAF	PeAF	LSPeAF	All AF patients
RAA, N[$]	42	11	20	13	44
HSPB1	0.566	0.556	0.457	0.373	0.482
	[0.372 – 0.775]	[0.493 – 0.833]	[0.324 – 0.648]	[0.292 – 0.553]	[0.328 – 0.592]
HSPA1	0.625	0.530	0.581	0.563	0.548
	[0.438 – 0.784]	[0.284 – 0.782]	[0.433 – 1.171]	[0.435 – 0.815]	[0.421 – 0.801]
HSPA5	0.648	0.423	0.555	0.335*	0.451*
	[0.471 – 0.853]	[0.302 – 0.565]	[0.380 – 0.891]	[0.296 – 0.573]	[0.323 – 0.665]
HSPD1	0.471	0.582	0.770**	0.632	0.645**
	[0.380 – 0.787]	[0.508 – 0.704]	[0.576 – 1.014]	[0.461 – 0.962]	[0.498 – 0.934]
HSPB5	0.607	0.771	0.715	0.718	0.745
	[0.427 – 0.745]	[0.586 – 1.045]	[0.572 – 0.937]	[0.481 – 0.848]	[0.569 – 0.874]
pHSF1	0.929	1.009	0.944	1.124	1.009
	[0.794 – 1.210]	0.923 – 1.447]	[0.632 – 1.203]	[0.884 – 1.368]	[0.768 – 1.304]
LAA, N[$]		7	14	9	30
HSPB1	-	0.606	0.517	0.527	0.529
		[0.446 – 0.837]	[0.382 – 0.871]	[0.426 – 0.750]	[0.425 – 0.781]
HSPA1	-	0.578	0.698	1.069	0.712
		[0.417 – 0.736]	[0.405 – 0.946]	[0.712 – 1.370]	[0.424 – 0.957]
HSPA5	-	0.570	0.421	0.469	0.471
		[0.407 – 0.626]	[0.264 – 0.728]	[0.378 – 0.781]	[0.364 – 0.643]
HSPD1	-	0.489	0.949[#]	0.858	0.864
		[0.399 – 1.044]	[0.649 – 1.156]	[0.611 – 1.123]	[0.605 – 1.121]
HSPB5	-	0.548	0.914	0.754	0.735
		[0.213 – 0.735]	[0.612 – 1.201]	[0.567 – 0.913]	[0.548 – 1.011]
pHSF1	-	0.549	0.725	0.674	0.640
		[0.481 – 1.253]	[0.458 – 0.873]	[0.455 – 1.314]	[0.496 – 1.011]

*$P<0.05$ and **$P<0.01$ compared to control, [#]$P<0.05$ compared to PAF

[$]Number of tissue samples measured. Results are shown as median [IQR]

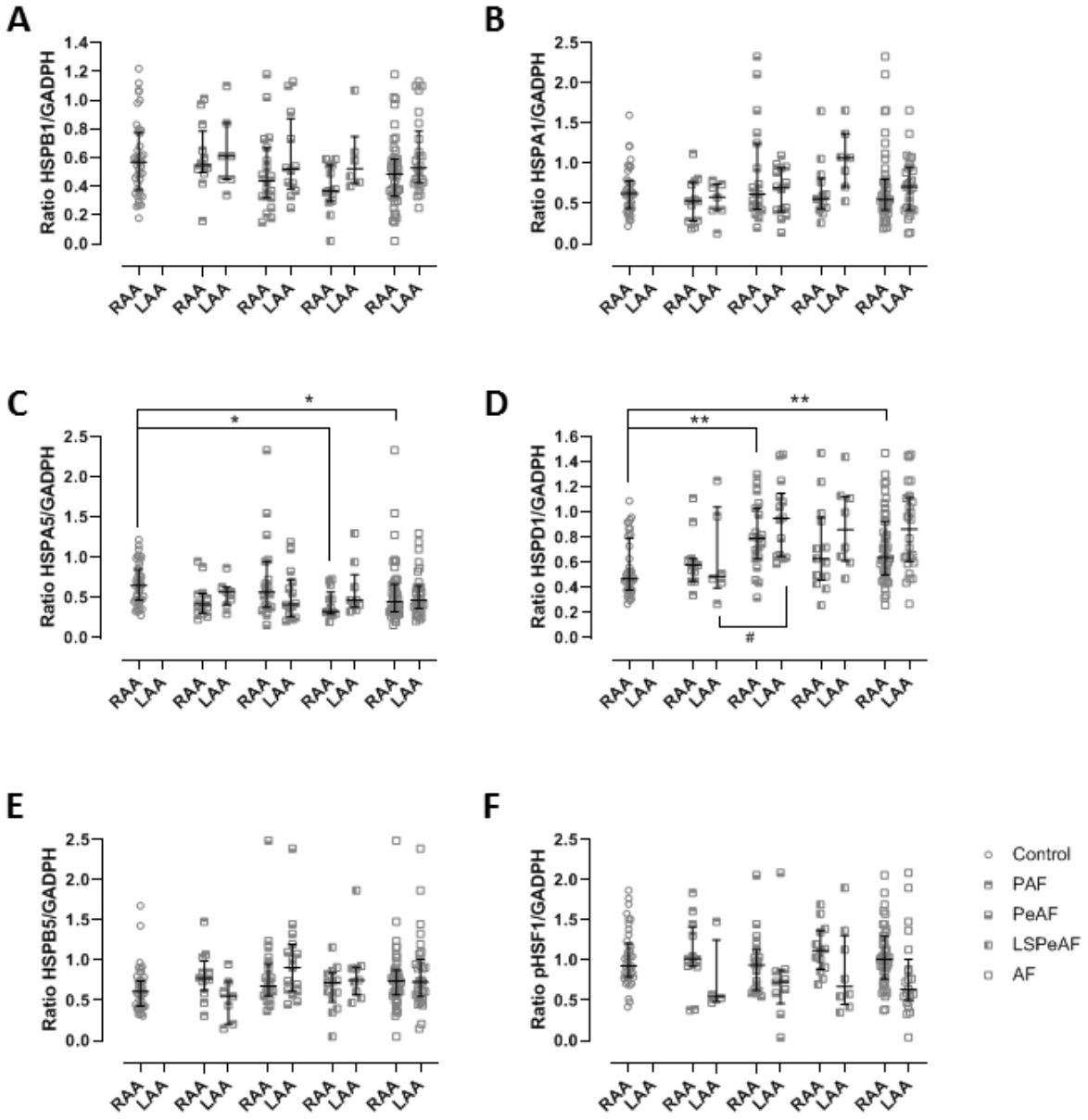

Figure 1. HSP levels in RAA and LAA tissue of patients without (control) and with (paroxysmal, persistent and longstanding persistent) AF

HSPB1 (**A**), HSPA1 (**B**), HSPA5 (**C**), HSPD1 (**D**), HSPB5 (**E**) and pHSF1 (**F**) expression levels relative to GAPDH in RAA and LAA of control, PAF, PeAF, LSPeAF and all AF patients. **P*<0.05 and ***P*<0.01 compared to control, #*P*<0.05 compared to PAF

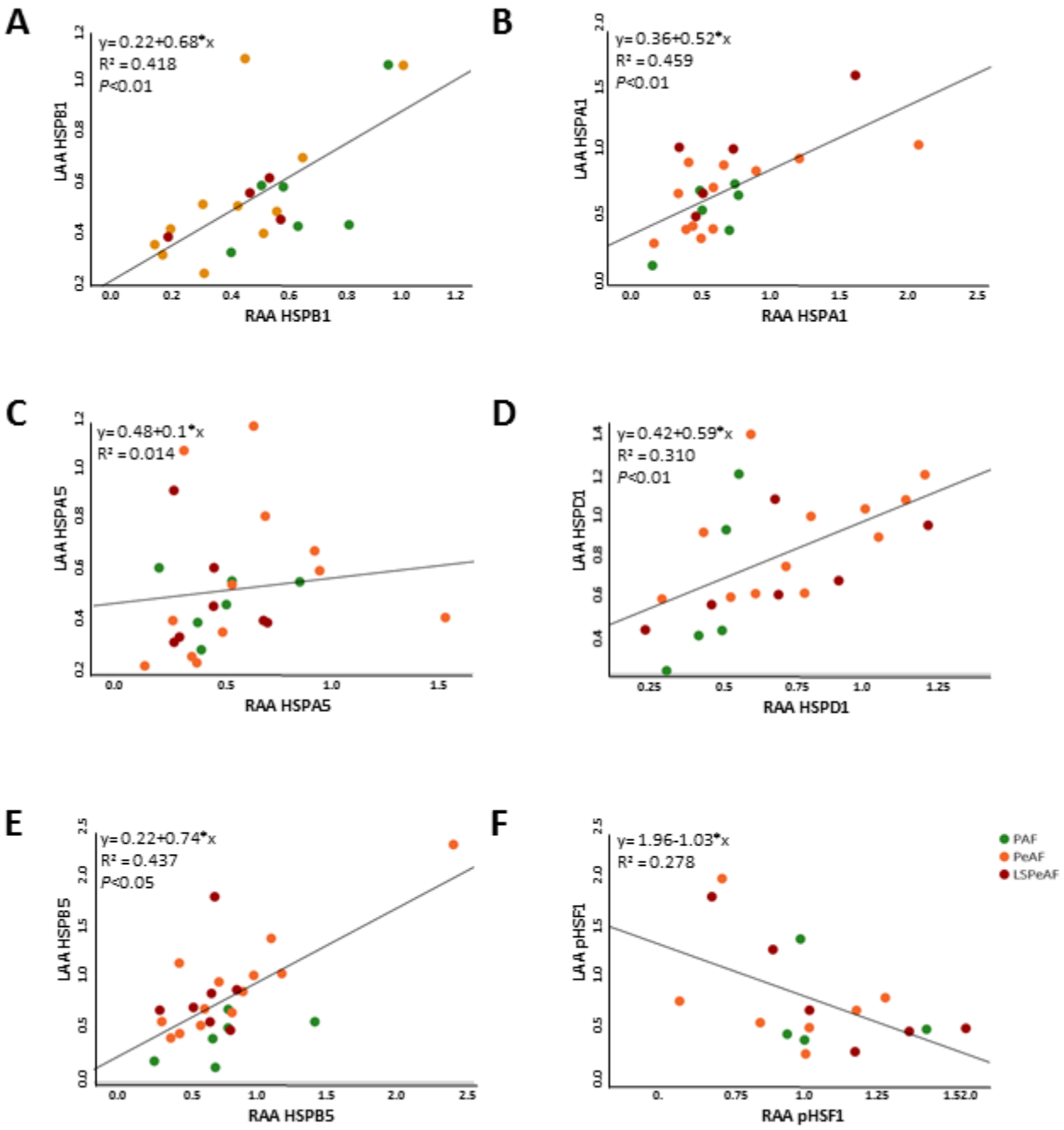

Figure 2. Correlation of HSP levels between RAA and LAA tissue

HSPB1 (**A**), HSPA1 (**B**), HSPA5 (**C**), HSPD1 (**D**), HSPB5 (**E**) and pHSF1 (**F**) expression levels relative to GAPDH in RAA and LAA.

Baseline HSPB1, HSPA1, HSPB7 and HSPD1 levels were determined in serum samples of PAF, PeAF and LSPeAF patients and compared to controls. Figure 3 shows similar baseline concentrations of serum HSPB1, HSPA1, HSPB7 and HSPD1 for control and AF patients; corresponding values are depicted in Table 4. So, the findings indicate that tissue HSPA5 and HSPD1 are associated with more persistent stages of AF and serum levels cannot be used to stage AF.

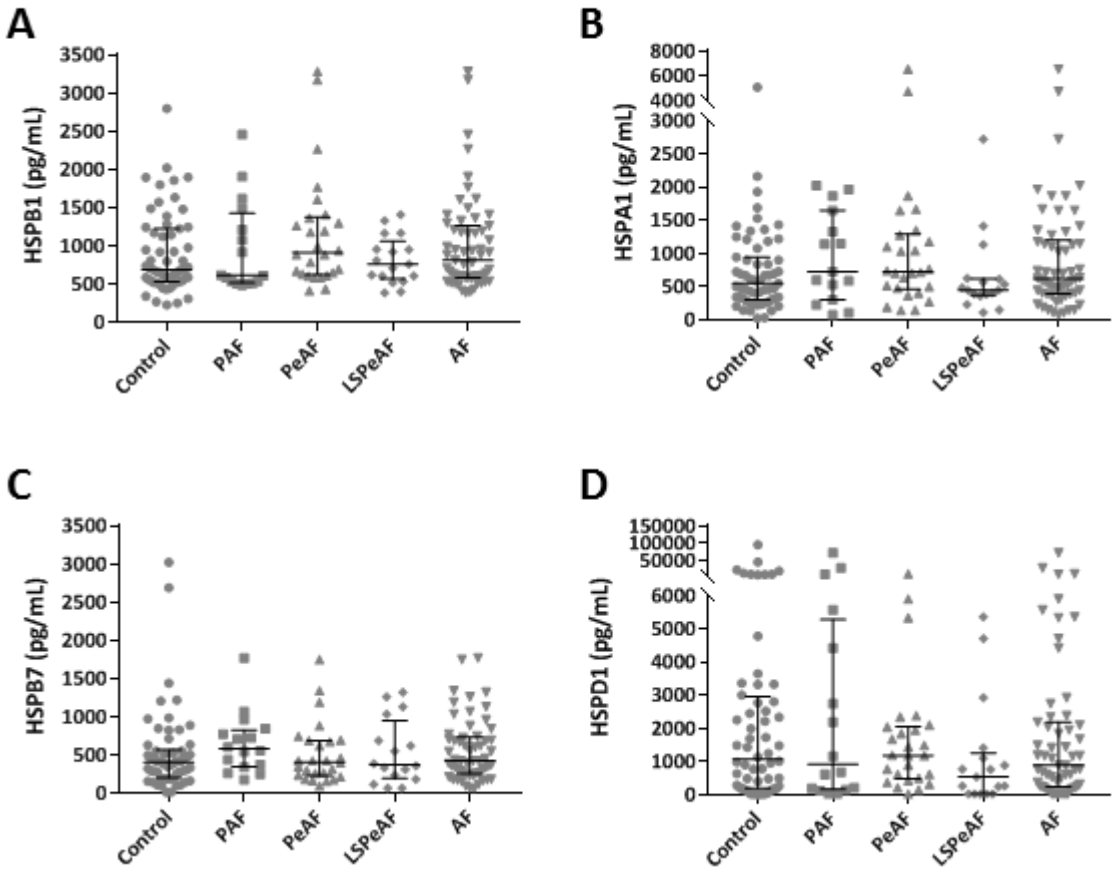

Figure 3. Baseline HSP serum levels in patients without (control) and with (paroxysmal, persistent and longstanding persistent) AF

HSPB1 (**A**), HSPA1 (**B**), HSPB7 (**C**) and HSPD1 (**D**) expression levels (pg/mL) in baseline serum of control, PAF, PeAF, LSPeAF and all AF patients.

Table 4. Concentration of HSPs in serum at baseline and after 6 and 12 months in control and AF patients

	Control	PAF	PeAF	LSPeAF	All AF patients
Baseline, N[$]	60	15	27	17	59
HSPB1	690.8	611.7	915.3	759.5	810.0
	[531.9 – 1223]	[508.8 – 1495]	[627.1 – 1360]	[567.9 – 1058]	[585.0 – 1262]
HSPA1	545.3	931.8	710.7	445.7	615.9
	[299.8 – 941.5]	[277.1 – 1702]	[472.7 – 1276]	[370.2 – 615.9]	[386.1 – 1199]
HSPB7	405.0	601.0	394.0	370.5	421.0
	[202.3 – 571.3]	[374.0 – 844.0]	[234.0 – 688.0]	[194.5 – 947.8]	[255.0 – 729.3]
HSPD1	1004	678.0	1184	530.0	883.0
	[182.0 – 2800]	[139.0 – 5571]	[499.0 – 2098]	[20.0 – 1253]	[238.0 – 2188]
6M, N[$]	32	8	14	11	33
HSPB1	774.0	753.0	789.0	695.0	766.0
	[577.5 – 980.3]	[358.3 – 930.3]	[532.5 – 987.8]	[663.0 – 1206]	[515.0 – 1019]
HSPA1	917.0	658.5	821.5	850.0	794.0
	[530.0 – 1260]	[401.5 – 972.5]	[675.0 – 1656]	[446.0 – 1433]	[641.0 – 1123]
12M, N[$]	23	3	5	6	14
HSPB1	665.0	660.0	733.0	741.5	696.5
	[585.0 – 922.0]	[N/A]	[648.5 – 1188]	[500.8 – 1425]	[542.8 – 1225]
HSPA1	735.0	697.0	1094	847.5	879.5
	[512.0 – 1038.0]	[N/A]	[507.0 – 1124]	[610.5 – 1355]	[610.5 – 1130]

[$]Number of serum samples measured. Results are shown as median [IQR]

Relating HSP levels in atrial tissue and serum to PoAF in control patients

In the control group, 29 (45.3%) of the patients developed PoAF, compared to 35 (54.7%) who did not. Figure 4 displays RAA tissue HSP levels, serum HSP levels at baseline and the levels of HSPB1 and HSPA1 in follow-up serum samples comparing no PoAF to PoAF in controls; corresponding values are depicted in Table 5. HSPA1 and HSPA5 RAA levels are significantly higher (both $P<0.05$) in patients who developed PoAF compared to patients without PoAF. HSPB1, HSPD1, HSPB5 and pHSF1 RAA levels were similar between PoAF and no PoAF.

Baseline serum HSPB1, HSPA1, HSPB7 and HSPD1 levels were similar between PoAF and no PoAF in controls. In addition, HSPB1 and HSPA1 levels at 6 and 12 months after surgery, relative to baseline levels and corrected for repeated measures, were comparable between the PoAF and no PoAF groups. These findings suggest that tissue HSPA1 and HSPA5 associate with PoAF whereas serum levels do not.

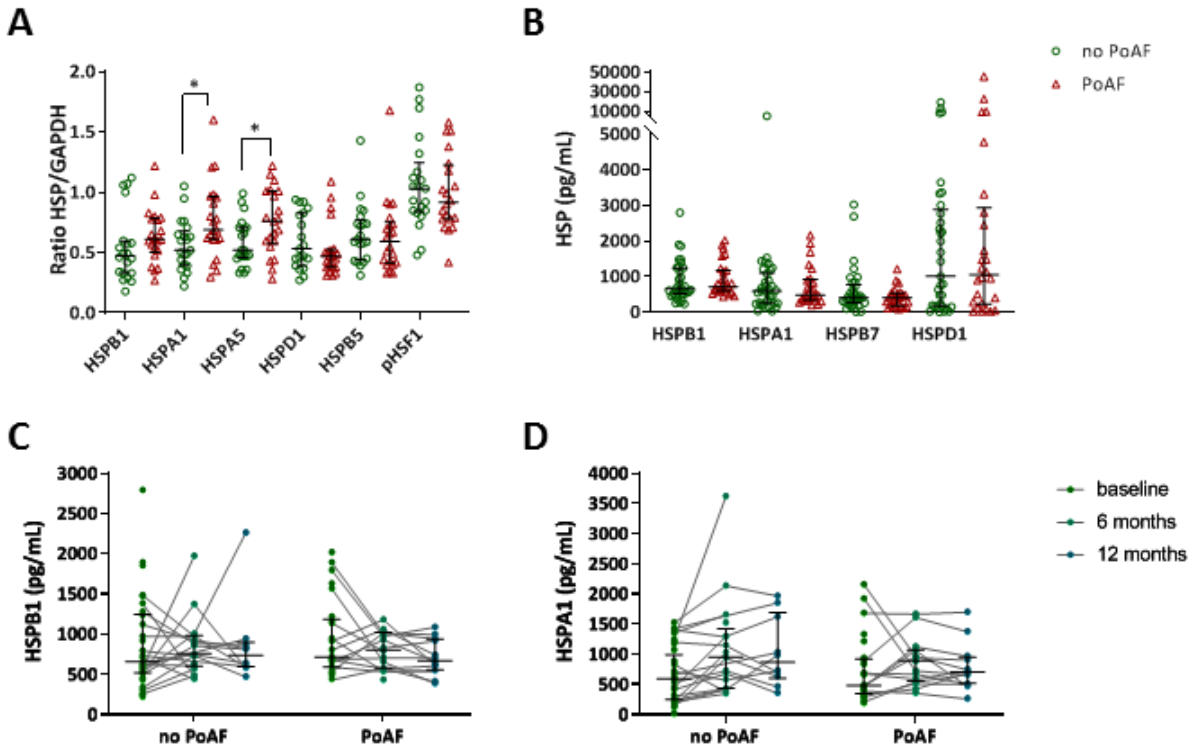

Figure 4. Tissue HSP levels and serum HSP levels of control patients with and without PoAF

(**A**) HSPB1, HSPA1, HSPA5, HSPD1, HSPB5 and pHSF1 expression levels relative to GAPDH in RAA of control patients, comparing no PoAF with PoAF. (**B**) HSPB1, HSPA1, HSPB7 and HSPD1 baseline serum levels (pg/mL) of control patients, comparing no PoAF with PoAF. (**C**) HSPB1 serum levels (pg/mL) at baseline and at 6 and 12 months follow-up of control patients, comparing no PoAF with PoAF. (**D**) HSPA1 serum levels (pg/mL) at baseline and at 6 and 12 months follow-up of control patients, comparing no PoAF with PoAF. **P*<0.05 compared to no PoAF

Relating HSP levels in tissue and serum to AF recurrence in AF patients treated with Maze procedure

All patients are treated for their underlying heart disease. On top of that, a selection of AF patients (N=38, 63.3%) was treated for AF with a Maze procedure during open heart surgery. We studied AF recurrences in the Maze-treated AF patients (Figure 5 and Table 6). HSPA1, HSPA5, HSPD1, HSPB5 and pHSF1 RAA levels are similar between patients with and without AF recurrence after Maze treatment.

Table 5. Concentration of HSPs in tissue and serum of control patients with or without poAF

	No PoAF	PoAF
RAA, N[$]	21	21
HSPB1	0.473 [0.332 – 0.599]	0.606 [0.497 – 0.781]
HSPA1	0.519 [0.393 – 0.679]	0.689 [0.614 – 0.964]*
HSPA5	0.523 [0.458 – 0.710]	0.756 [0.576 – 1.012]*
HSPD1	0.527 [0.389 – 0.832]	0.469 [0.378 – 0.523]
HSPB5	0.607 [0.441 – 0.771]	0.586 [0.417 – 0.756]
pHSF1	1.033 [0.838 – 1.242]	0.913 [0.784 – 1.223]
Baseline serum, N[$]	34	26
HSPB1	652.5 [512.8 – 1244]	712.2 [586.6 – 1182]
HSPA1	590.0 [245.3 – 1103]	475.9 [338.0 – 918.5]
HSPB7	401.0 [269.0 – 767.5]	409.0 [177.0 – 528.5]
HSPD1	1004 [173.5 – 2887]	1053 [224.3 – 2930]
6M serum, N[$]	17	15
HSPB1	752.0 [595.0 – 978.0]	796.0 [577.0 – 1016]
HSPA1	946.0 [433.0 – 1417]	888.0 [551.0 – 1068]
12M serum, N[$]	10	13
HSPB1	731.0 [594.8 – 893.8]	665.0 [550.0 – 934.0]
HSPA1	862.5 [595.0 – 1686]	694.0 [509.5 – 943.5]

*$P<0.05$ compared to No PoAF. [$]Number of tissue samples measured. Results are shown as median [IQR]

HSPB1 RAA levels are significantly lower ($P<0.05$) in patients who developed AF recurrence after Maze treatment compared to patients without AF recurrence (Figure 5A), while this was not the case for LAA (Figure 5B). However, HSPA5 LAA levels were significantly higher in patients who developed AF recurrence after Maze treatment compared to patients without AF recurrence (Figure 5B).

This observation suggests that HSPB1 RAA levels and HSPA5 LAA levels may have value to predict AF recurrence in patients treated with Maze surgery.

No differences in baseline serum HSPB1, HSPA1, HSPB7 and HSPD1 levels were observed between Maze-treated patients with and without AF recurrence (Figure 5C). HSPB1 and HSPA1 levels at 6 and 12 months after surgery, relative to baseline levels and corrected for repeated measures, were similar between Maze-treated patients with and without AF recurrence (Figure 5D,E).

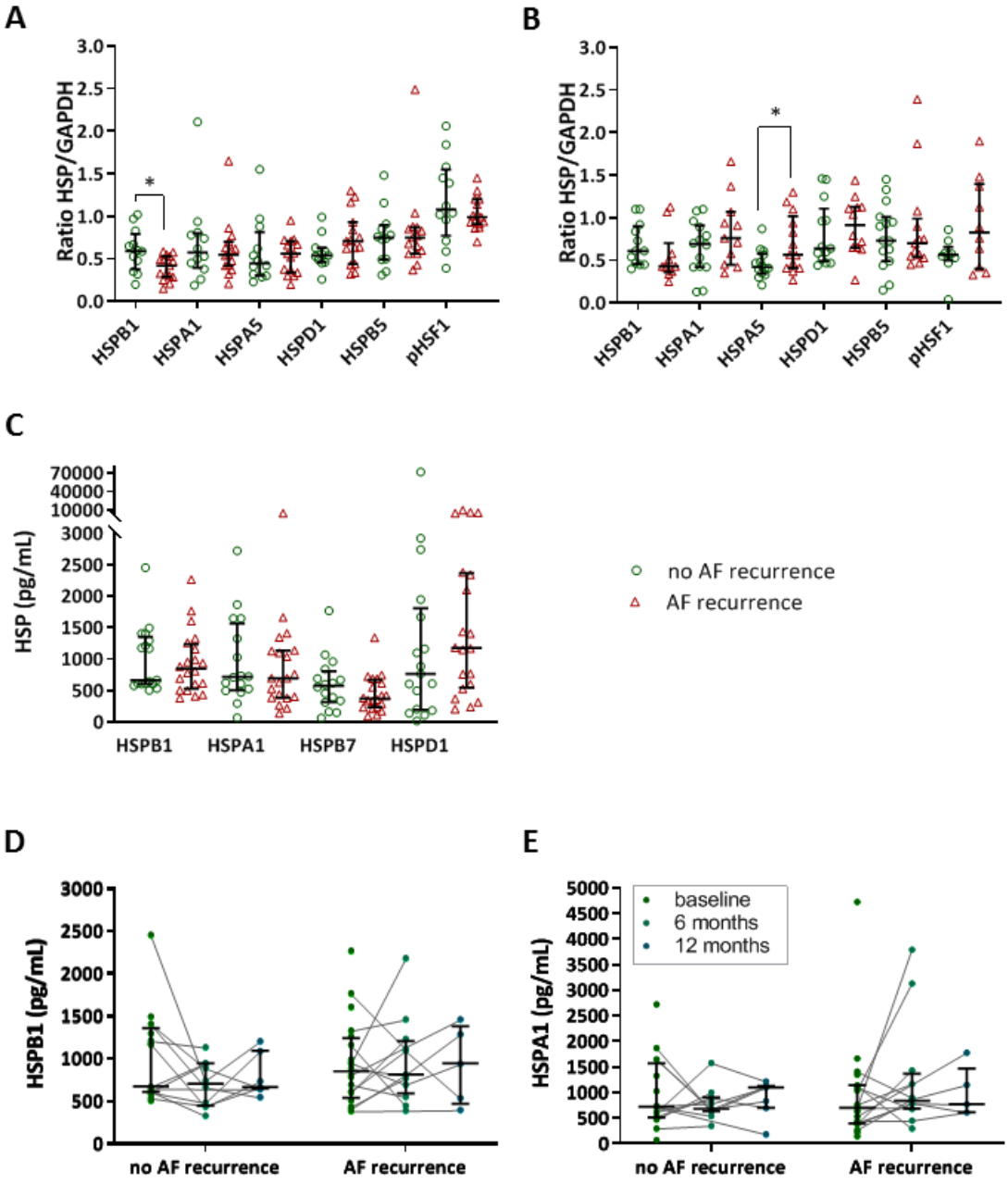

Figure 5. Tissue HSP levels and serum HSP levels of Maze-treated AF patients with and without AF recurrence

(**A**) HSPB1, HSPA1, HSPA5, HSPD1, HSPB5 and pHSF1 expression levels relative to GAPDH in RAA of Maze-treated AF patients, comparing no AF recurrence with AF recurrence. (**B**) HSPB1, HSPA1, HSPA5, HSPD1, HSPB5 and pHSF1 expression levels relative to GAPDH in LAA of Maze-treated AF patients, comparing no AF recurrence with AF recurrence. (**C**) HSPB1, HSPA1, HSPB7 and HSPD1 baseline serum levels (pg/mL) of Maze-treated AF patients, comparing no AF recurrence with AF recurrence. (**D**) HSPB1 serum levels (pg/mL) at baseline and at 6 and 12 months follow-up of Maze-treated AF patients, comparing no AF recurrence with AF recurrence. (**E**) HSPA1 serum levels (pg/mL) at baseline and at 6 and 12 months follow-up of Maze-treated AF patients, comparing no AF recurrence with AF recurrence. **P*<0.05 compared to no AF recurrence.

Table 6. Concentration of HSPs in tissue and serum of AF patients treated with MAZE surgery with and without AF recurrence

	No AF recurrence	AF recurrence
RAA, N[$]	12	15
HSPB1	0.599 [0.383 – 0.792]	0.421 [0.288 – 0.534]*
HSPA1	0.577 [0.396 – 0.801]	0.545 [0.419 – 0.702]
HSPA5	0.451 [0.303 – 0.814]	0.565 [0.343 – 0.711]
HSPD1	0.543 [0.465 – 0.629]	0.713 [0.437 – 0.934]
HSPB5	0.747 [0.495 – 0.896]	0.752 [0.563 – 0.866]
pHSF1	1.082 [0.774 – 1.550]	0.988 [0.913 – 1.198]
LAA, N[$]	14	13
HSPB1	0.609 [0.457 – 0.899]	0.434 [0.363 – 0.704]
HSPA1	0.691 [0.418 – 0.910]	0.760 [0.451 – 1.069]
HSPA5	0.421 [0.344 – 0.581]	0.573 [0.411 – 1.016]*
HSPD1	0.636 [0.491 – 1.113]	0.916 [0.650 – 1.126]
HSPB5	0.735 [0.491 – 1.011]	0.702 [0.537 – 0.986]
pHSF1	0.573 [0.496 – 0.658]	0.827 [0.404 – 1.400]
Baseline serum, N[$]	17	20
HSPB1	668.0 [606.8 – 1352]	846.4 [532.7 – 1237]
HSPA1	717.3 [506.1 – 1566]	697.1 [391.7 – 1138]
HSPB7	578.5 [320.8 – 806.0]	370.5 [234.3 – 669.5]
HSPD1	765.0 [194.0 – 1809]	1178 [548.5 – 2370]
6M serum, N[$]	11	12
HSPB1	740.0 [446.0 – 945.0]	808.0 [586.0 – 1202]
HSPA1	675.0 [629.0 – 899.0]	829.5 [679.5 – 1366]
12M serum, N[$]	7	5
HSPB1	660.0 [640.0 – 1088]	938.0 [465.5 – 1374]
HSPA1	1094 [697.0 – 1123]	767.0 [609.0 – 1463]

*$P<0.05$ compared to No AF Recurrence.
[$]Number of tissue samples measured. Results are shown as median [IQR]

Discussion

In this study, we observed that atrial tissue (RAA and LAA) levels of HSPB1, HSPA1, HSPB5 and pHSF1 were similar in control patients and patients with paroxysmal and (longstanding) persistent AF. However, HSPA5 RAA levels were significantly lower in (LSPe)AF and HSPD1 RAA levels were significantly higher in (Pe)AF compared to controls, indicating that tissue HSPA5 and HSPD1 are associated with more persistent stages of AF. Serum HSPB1, HSPA1, HSPB7 and HSPD1 levels were not associated with AF (stage). In control patients, HSPA1 and HSPA5 RAA levels were significantly higher

in patients who developed PoAF compared to patients who did not. However, neither baseline HSPB1, HSPA1, HSPB7 and HSPD1 serum levels nor HSPB1 and HSPA1 levels at 6 and 12 months after surgery could discriminate between no PoAF and PoAF. In Maze-treated AF patients, HSPB1 RAA levels were lower and HSPA5 LAA levels higher in the patients who developed AF recurrence short after surgery compared to patients who did not, suggesting that HSPB1 RAA levels and HSPA5 LAA levels may predict AF recurrence in patients treated with Maze surgery. Here, baseline HSPB1, HSPA1, HSPB7 and HSPD1 serum levels and HSPB1 and HSPA1 levels in follow-up serum were similar between patients with and without AF recurrence.

In conclusion, tissue levels of HSPA5 and HSPD1 may have value to discriminate persistent AF from controls, HSPA1 and HSPA5 RAA levels may indicate PoAF in control patients and HSPB1 RAA levels and HSPA5 LAA levels may indicate AF recurrence in patients with AF.

HSPs as a potential biomarker in AF (stage)

There is a great need for biomarkers to detect and stage AF to subsequently start patient-tailored therapy. Despite that several (AF-related) serum biomarkers are routinely measured in clinical practice, such as natriuretic peptide, troponin I, or troponin T, creatinine and C-reactive protein [30-32], these lack specificity for AF. Similar to our previous study in control patients and AF patients treated with electrocardioversion and pulmonary vein isolation, no role for baseline serum HSPB1, HSPA1, HSPB7 or HSPD1 as a biomarker for the presence or staging of AF in patients with underlying heart diseases was found in the current study. In other studies, serum HSPA1 levels were also found to be similar in control and AF patients [28, 33]. Yet, for HSPB1, our findings are in contrast to findings in the report of Hu et al. [28]. Baseline serum HSPB1 levels were found to associate with AF, serum HSPB1 levels were reduced in paroxysmal AF and (longstanding) persistent AF patients compared to controls in normal sinus rhythm [28]. For HSPD1, no association between serum HSPD1 level and occurrence of AF was found by Maan et al. [34]. However, for prevalent AF, Cao et al. [35] described that patients undergoing mitral valve replacement with AF had higher plasma HSPD1 levels compared

to patients in sinus rhythm. No previous studies, except for our recent study [36], investigated serum HSPB7 as a biomarker to stage AF.

While we found an increase in follow-up serum HSPB1 levels in patients who developed AF recurrence post-PVI previously [36] and in another study increased serum HSPA1 levels at 6 months post-PVI correlated with AF recurrence [33], no such increase in HSPs in follow-up serum was observed in the current study. Thus, for patients undergoing elective cardiothoracic surgery due to underlying heart diseases, serum HSPs cannot discriminate PAF, PeAF or LSPeAF from controls.

On the contrary, tissue levels of HSPA5 and HSPD1 may have value to indicate more persistent stages of AF. Lower RAA levels of the ER chaperone-protein HSPA5 correlates with (LSPe)AF, and HSPA5 is increased in RAA of control patients with PoAF and in LAA of AF patients with AF recurrence, indicating a role for ER stress to contribute to AF onset. Previously, Wiersma et al. found a role for ER stress to induce HSPA5 expression to compensate for ER stress and at the same time activation of downstream excessive autophagic protein degradation which drives AF promotion in experimental model systems of AF [24]. Higher levels of HSPA5, as observed in patients with PoAF and AF recurrence after Maze surgery, may indicate ER stress and subsequent activation HSPA5 expression. In LSPeAF patients, low levels of HSPA5 may indicate exhaustion of ER-stress chaperone expression and therefore loss of protection against ER stress. For the mitochondrial stress marker HSPD1, the higher levels in RAA of (Pe)AF patients compared to controls is in line with previous findings showing mitochondrial stress to drive AF [37] and is also confirmed by previous reports. The mitochondrial HSPs, HSPD1 and HSPE1, were more than doubled in the atrial myocardium of patients with persistent AF compared to control patients who underwent CABG and/or valve surgery [38, 39]. The higher HSPD1 levels in patients with AF were confirmed in the study of Cao et al. [35]. In that same study, HSPB1, HSPA1 and HSF1 tissue levels were lower in patients with AF, compared to patients in sinus rhythm [35]. Other studies found HSPB1 levels to be increased in PAF compared to patients in sinus rhythm and PeAF [19, 25], while no such increase was found for HSPA1. Although our findings for HSPB1, HSPA1, HSPA5, HSPB5 and pHSF1

are not reaching consensus with other reports, HSPD1 tissue levels are indicative for more persistent stages of AF in combination with underlying heart diseases.

HSPs as marker for PoAF

Baseline serum HSPB1, HSPA1, HSPB7 and HSPD1 and follow-up serum HSPB1 and HSPA1 levels in control patients were not predictive for PoAF. For HSPA1, this is in line with other studies. In patients who underwent CABG, baseline serum HSPA1 levels were also not predictive for post-operative AF [27, 40]. Another study, including 45 patients undergoing CABG surgery, showed that higher pre- as well as post-operative circulating HSPA1 associated with PoAF [41]. Higher pre- and post-operative circulating anti-HSPD1 antibodies also associated with PoAF in patients who underwent CABG [42]. Cao et al. also demonstrated for the protein itself, that higher plasma HSPD1 levels were predictive for early (<7days) PoAF [35].

Our findings of increased HSPA1 RAA levels in control patients with PoAF are not coherent with previous reports showing that low preoperative tissue HSPA1 levels correlated significantly with high incidence of PoAF after cardiac surgery [26, 27]. Still, the ER stress marker HSPA5 may help to predict PoAF in patients undergoing cardiac surgery.

HSP as marker in AF recurrence

Serum HSP levels in the current study could not predict AF recurrence in patients who underwent elective cardiothoracic surgery. Baseline serum HSPB1 was found to predict sinus rhythm maintenance and low serum HSPB1 correlated with a higher AF recurrence rate after ablative therapy in the study of Hu et al. [28]. While we observed an increase in follow-up serum HSPB1 levels in patients who developed AF recurrence post-PVI in our previous study [36] and Kornej et al. found increased serum HSPA1 levels at 6 months post-PVI to be correlated with AF recurrence [33], no such association between HSPB1 or HSPA1 levels after surgery and AF recurrence was found in AF patients treated with

Maze procedure. On the contrary, tissue levels of HSP may have value to predict AF recurrence after Maze surgery in AF patients with underlying heart disease. Here we observed low levels of HSPB1 to associate with AF recurrence after Maze treatment. This is in line with previous studies showing lower atrial HSPB1 levels to associate with more structural damage (myolysis) [19]. Possibly patients with AF recurrence after Maze surgery may reveal more structural damage and therefore are more vulnerable for AF recurrence compared to patients with higher levels of HSPB1 and less structural damage. Furthermore, the higher levels of HSPA5 indicate ER stress, possibly provoked by the Maze surgery. Further research is warranted to elucidate the patho-mechanism underlying AF recurrence in these patients.

Limitations

In the current study, there is a lack of correlation between serum HSPs and AF stage, however that does not negate a role for serum HSPs as potential biomarker in AF. Since AF is a multifactorial disease and predisposing conditions for AF such as diabetes mellitus, hypertension and higher age were omnipresent in both the AF and control group, serum HSPs might need to be correlated with more AF specific parameters, such as markers of structural damage which are found to underlie atrial electrical conduction disorders, i.e. electropathology [43]. It has been suggested that the clinical classification of AF is inaccurate for AF staging because it is not related to the degree of atrial electropathology, as measured by high-resolution epicardial mapping [44]. With high-resolution epicardial mapping, it has been identified that the degree of atrial electropathology varies between right and left atrium [45]. This might explain the inverse correlation between RAA and LAA pHSF1 levels in the current study. Since tissue HSPs have been associated with structural damage [19], future studies in larger-scale prospective trials with continuous monitoring of electrical parameters and HSP levels should elucidate the role of human HSP levels in both serum and atrial tissue in relation to parameters of electropathology. It is conceivable that serum HSP levels do correlate with the degree of

electropathology. In addition, since the pathophysiology of AF is complex and multifactorial, it may be helpful to combine HSP levels with other AF-related markers, such as cell-free-circulating mitochondrial DNA, which may lift the diagnostic power of such a combination-biomarker, including improved sensitivity and specificity. Finally, the current study findings were observed in patients with underlying heart disease and may not apply to AF patients without these underlying heart diseases. More research is warranted to discriminate AF patients with underlying heart disease from patients without underlying heart disease.

Conclusions

Atrial tissue levels of HSPA5 and HSPD1 are altered in more persistent stages of AF compared to controls in sinus rhythm. Also, atrial tissue levels of HSPA1 and HSPA5 RAA levels can predict post-operative AF development and HSPB1 RAA and HSPA5 LAA levels can predict AF recurrence in Maze-treated AF patients. Serum HSP levels cannot discriminate AF (stage) from controls, and cannot predict PoAF or AF recurrence after Maze-treatment.

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