Modulators of proteostasis: therapeutic targets and diagnostic markers to halt and reverse atrial fibrillation

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Chapter 10

Evaluating serum heat shock protein levels as novel biomarkers for atrial fibrillation

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Abstract

Background Staging of atrial fibrillation (AF) is essential to understand disease progression and the accompanied increase in therapy failure. Blood-based heat shock proteins (HSP) levels may enable staging of AF and the identification of patients with higher risk for AF recurrence after treatment.

Objective This study evaluates the relationship between serum HSP levels, presence of AF, AF stage and AF recurrence following electrocardioversion (ECV) or pulmonary vein isolation (PVI).

Methods To determine HSPB1, HSPA1, HSPB7 (cardiovascular HSP) and HSPD1 levels, serum samples were collected from control patients without AF and patients with paroxysmal (PAF), persistent (PeAF) and longstanding persistent (LSPeAF) AF, presenting for ECV or PVI, prior to intervention and at 3-, 6- and 12-months post-PVI.

Results The study population (N=297) consisted of 98 control and 199 AF patients admitted for ECV (N=98) or PVI (N=101). HSPB1, HSPA1, HSPB7 and HSPD1 serum levels did not differ between patients without or with PAF, PeAF or LSPeAF. Additionally, baseline HSP levels did not correlate with AF recurrence after ECV or PVI. However, in AF patients with AF recurrence, HSPB1 levels were significantly elevated post-PVI relative to baseline, compared to patients without recurrence.

Conclusions No association was observed between baseline HSP levels and the presence of AF, AF stage or AF recurrence. However, HSPB1 levels were increased in serum samples of patients with AF recurrence within one year after PVI, suggesting that HSPB1 levels may predict recurrence of AF after ablative therapy.

Introduction

Atrial fibrillation (AF) is the most common cardiac arrhythmia, with a rising prevalence due to the aging population [1]. Proper staging of AF is essential to select the optimal treatment strategies to prevent AF progression, and the accompanied risk to develop severe complications such as thromboembolic events, heart failure, cognitive impairment and increased mortality [2, 3]. At present, AF can only be diagnosed with a surface electrocardiogram when a patient already suffers from AF, presenting with palpitations or thromboembolic complications. However, diagnosis of AF may be challenging in patients with asymptomatic or very short-lasting episodes of AF [4]. In addition, treatment aimed at rhythm control, such as ablative therapy, is less successful in patients with persistent AF, compared to paroxysmal AF [3]. Hence, proper staging of AF and start of effective treatment of AF is of utmost importance. Therefore, there is an urgent need to identify diagnostic biomarkers to stage AF and guide patient tailored therapy [5].

Biomarkers are widely accepted as a diagnostic tool to screen or monitor patients for a variety of cardiovascular diseases. At present, there are no recommendations on the use of AF-specific biomarkers in the most recent guidelines [3, 6], despite that several blood-based biomarkers related to AF pathology have been identified. These biomarkers include brain natriuretic peptides, cancer-antigen-125, fibroblast growth factor-23 [7, 8] and -21 [9], highly sensitive cardiac troponin I [10], homocysteine [11], (a)symmetric dimetylarginine [12], interleukine-6 and matrix metalloproteinase-9/tissue inhibitor of metalloproteinase-1 ratio [13]. Although potential AF-related biomarkers are available, the role of these biomarkers in staging of AF (paroxysmal or (longstanding) persistent AF) or predicting AF recurrence after AF therapy has only been moderately studied.

Emerging evidence indicates that heat shock proteins (HSPs) may represent a suitable biomarker to predict AF recurrence after treatment. HSPs are chaperones that play an important role in safeguarding proteostasis, the homeostasis of protein expression, function and degradation in cells [14]. The derailment of proteostasis has been identified as a key factor underlying electropathology and AF progression [14-16]. During stress or disease, such as AF, especially, activation of the heat shock

transcription factor 1 regulates HSP transcription [17]. Within the HSP family, small HSPs, including HSPB1, are probably the most important in maintaining proteostasis in cardiomyocytes by stabilizing the contractile proteins [18-21]. Previously, atrial HSPB1 levels were found to be induced in atrial tissue samples of patients with paroxysmal AF, while tissue HSPB1 levels get exhausted in patients with (longstanding) persistent AF [18], indicating that low tissue HSP levels are associated with AF progression. The study of Hu et al. described that low baseline serum HSPB1 levels of patients who received ablative therapy predict AF recurrence and patients with high baseline serum levels of HSPB1 showed an improved maintenance rate of sinus rhythm [22]. So far, it is unknown which HSP family member(s) represent biomarkers to identify the stage of AF and recurrence after therapy. In the current study, various members of the HSP family, including HSPB1, HSPA1, HSPB7 (cardiovascular HSP) and HSPD1, were measured in serum samples of control and patients with paroxysmal atrial fibrillation (PAF), persistent atrial fibrillation (PeAF) and longstanding persistent atrial fibrillation (LSPeAF), undergoing elective electrical cardioversion (ECV) or pulmonary vein isolation (PVI), to identify whether HSPs associate with the stage of AF and recurrences after either PVI or ECV. Herein, we report that baseline HSP levels between control and AF patients are comparable. However, HSPB1 levels were increased in follow-up samples of patients with AF recurrence after PVI, suggesting that increased HSPB1 levels may predict recurrence of AF after ablative therapy.

Methods

Study population

From December 2014 till November 2016, 297 subjects >18 years with or without a history of AF were prospectively enrolled for the HALT & REVERSE study [23] at the department of cardiology in the Erasmus MC, Rotterdam, the Netherlands. The study population consists of a control group of patients without a history of AF and a study group of patients with symptomatic AF who were scheduled for electrical cardioversion (ECV) or pulmonary vein isolation (PVI).

The control group consisted of patients (N=98), who were scheduled for elective ablation of premature ventricular contractions (PVC), Wolff-Parkinson-White syndrome (WPW) or Ajmaline testing. These patients were eligible for inclusion in case of absence of structural heart disease and any atrial tachyarrhythmia. Blood serum samples were obtained 1 day prior to the scheduled intervention. Follow-up was not performed.

The study group included patients presenting for ECV (N=98) or PVI (N=101) for either symptomatic PAF (less than 7 days of AF), PeAF (having AF between 7 days and 1 year) or LSPeAF (more than 1 year of AF). Exclusion criteria included paced atrial rhythms, cancer, inflammation and rheumatic diseases. Blood samples were obtained 1 day prior to the scheduled intervention. PVI was unsuccessful in two patients.

Patients who underwent PVI visited the outpatient clinic at 3-, 6- and 12-months after the procedure to provide follow-up serum samples and to screen for AF recurrences. Due to rescheduling of patients to different hospitals, follow-up serum samples were not available for some patients in both the AF recurrence and no recurrence group. In addition, AF recurrence data is available via recordings during inbetween visits due to AF complaints of the patients. AF recurrence was defined as an AF episode documented on either a 12-lead surface ECG or Holter monitor recordings. In patients undergoing ECV, for each individual patient, only 3-, 6- and 12-months post-procedural follow-up telephone consultations were scheduled. The study endpoint was the completion of the 12- months follow-up period or earlier due to withdrawn informed consent, pacemaker implant or AF recurrence. Clinical characteristics were obtained from the electronic patients' files.

All patients signed written informed consent prior to inclusion. This sub-study is part of the HALT & REVERSE trial (MEC-2014-393) and is approved by the institutional medical ethical committee. The study is carried out according to the principals of the Declaration of Helsinki in accordance with the Medical Research Committee involving the Human Subjects Act.

HSP measurement in serum samples

Immediately after blood sample collection, serum was harvested from blood in BD Vacutainer™ SST™ II Advance Tubes (Fisher Scientific, Bleiswijk, the Netherlands) by centrifugation at 2000 x g for 10 min at 4°C and frozen in -80°C until analysis of HSPB1, HSPA1, HSPB7 and HSPD1 levels. For measurement of serum HSPB1 levels, samples were diluted six times and for HSPA1 levels samples were diluted twice in 1% BSA in PBS. The amount of HSPB1 and HSPA1 protein was detected in triplicates using human HSPB1 or HSPA1 DuoSet® ELISA kits from R&D (Cat. no. DY1580 and DY1663, respectively, Minneapolis, MN, USA) according to manufacturer's instructions with minor adjustments (serum was incubated at 4°C overnight, instead of 2 h at room temperature). Undiluted serum was used to measure HSPB7 protein (singular) with ELISA kits from Cusabio (CSB-EL010838HU, Houston, TX, USA) according to manufacturer's instructions with minor adjustments (kept incubation temperature at 20°C). Undiluted serum was used to measure HSPD1 protein in duplicate with the HSPD1 DuoSet® ELISA kit from R&D (DYC1800) according to manufacturer's instructions.

Statistical analysis

Data were analyzed with SPSS Statistics version 26.0 for Windows (SPSS, Inc., Chicago, IL, USA) and GraphPad Prism version 8.0 (Graphpad Software Inc., San Diego, CA, USA). 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 and Chi-square test. Differences in clinical characteristics and HSP levels between patients without a history of AF, PAF, PeAF and LSPeAF were tested with one-way analysis of variance (ANOVA), Kruskal-Wallis, Chi-square and Fisher's Exact test. When serum HSP levels were below detection limit of the ELISA (only for N=7 HSPB7 and N=41 HSPD1 samples), 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). Uni- and multivariate linear regression was used to correlate baseline serum HSP levels with clinical parameters, and bivariate spearman correlation was used to correlate AF recurrence with clinical parameters. The difference between baseline and follow-up serum HSP levels was calculated with a repeated measures model. To relate HSP levels over time with AF recurrence and analyze the sensitivity of the results, sensitivity analysis was performed using joint modeling. Hereto, the occurrence of the first AF recurrence (endpoint) in relation to the standardized ('Z') value of log2(HSP) was modeled, while using all measurements up to and including the moment of the first AF recurrence in endpoint event cases, and all measurements in those who remained event-free. A two-sided *P* value of <0.05 indicates statistical significance.

Results

Study population

The entire study population consisted of 297 patients (67% males, age 56.7 ± 13.4 years), including a control group of 98 patients without AF and a study group of 199 patients with either PAF (N=86, 29%), PeAF (N=108, 36.4%), or LSPeAF (N=5, 1.7%) AF. Table 1 outlines baseline characteristics of the entire study population.

Patients with AF were older (*P*<0.001), more often male (*P*<0.001), had a higher BMI (*P*<0.001), had more often hypertension (*P*<0.001), diabetes mellitus (*P*<0.01) and dyslipidemia (*P*<0.05), compared to patients without AF (Table 1).

Clinical parameters did not differ between patients with paroxysmal and persistent AF, except for an impaired LVF (*P*<0.001) and larger left atrial volume (*P*<0.01) in the persistent AF patients.

Baseline HSP levels related to clinical stage of AF

To study the relation between baseline HSP levels and the stage of AF, HSPB1, HSPA1, HSPB7 and HSPD1 levels were determined in serum samples of PAF, PeAF and LSPeAF patients and compared to controls.

Table 1. Clinical characteristics of the study population

	Control	PAF	PeAF	LSPeAF	All AF patients
N (%)	98 (33)	86 (29)	108 (36.4)	5 (1.7)	199 (67)
Group, N (%)					
Control	98 (100)	-	-	-	-
Electro cardioversion (ECV)	-	12 (14)	83 (76.9)	3 (60)	98 (49)
Pulmonary vein isolation (PVI)	-	74 (86)	25 (23.1)	2 (40)	101 (51)
Age (years), mean ± SD	48.2 ± 15.3	61.3 ± 9.5***	60.8 ± 10.6***	57.5 ± 9	60.9 ± 10.1***
Gender, male, N (%)	51 (52)	64 (74.4)**	81 (75)**	4 (80)	149 (74.9)***
BMI (kg/m^2), mean ± SD	25.1 ± 3.7	27.2 ± 3.8*	28.8 ± 5.4***	30.4 ± 7.4	28.2 ± 4.9***
Hypertension, yes, N (%)	23 (23.5)	43 (50)***	51 (47.2)**	3 (60)	97 (48.7)***
Diabetes mellitus, yes, N (%)	5 (5.1)	10 (11.6)	15 (13.9)	1 (20)	26 (13.1)*
Dyslipidemia, yes, N (%)	16 (16.3)	25 (29.1)	33 (30.6)	3 (60)	61 (30.7)**
Thyroid disease, yes, N (%)	2 (2)	4 (4.7)	8 (7.4)	1 (20)	13 (6.5)
Left ventricular function (LVF), N (%)			*/###		
Normal	61 (79.2)	73 (84.9)	60 (58.3)	3 (60)	136 (70.1)
Mild impairment	10 (13)	9 (10.5)	29 (28.2)	2 (40)	40 (20.6)
Moderate impairment	3 (3.9)	3 (3.5)	10 (9.7)	0 (0)	13 (6.7)
Severe impairment	3 (3.9)	1 (1.2)	4 (3.9)	0 (0)	5 (2.9)
Missing†	*21*	*0*	*5*	*0*	*5*
Left atrial volume index (ml/m2), median [IQR]	27.9 [21.2-39.7]	38.6* [29.3-48.4]	47**/## [35.7-60.5]	43.1 [25.9-73.6]	41.1** [31.8 – 54]
Drugs, yes, N (%)					
Drugs total	52 (53.6)	84 (97.7)***	104 (96.3)***	5 (100)	193 (97)***
ACE. ARB. AT2 antagonist	26 (26.8)	40 (46.5)*	48 (44.9)*	3 (60)	91 (46)**
Statin	17 (17.5)	32 (37.2)*	37 (34.3)*	4 (80)*	73 (36.7)***
Antiarrhythmic drugs (AAD) total‡	43 (44.3)	79 (91.9)***	103 (95.4)***	5 (100)	187 (94)***
Class I AAD	5 (5.2)	31 (36.0)***	14 (13)###	1 (20)	46 (23.1)***
Class II AAD	31 (32)	36 (41.9)	55 (50.9)*	1 (20)	92 (46.2)*
Class III AAD	6 (6.2)	42 (48.8)	55 (50.9)	2 (40)	99 (49.7)***
Class IV AAD	3 (3.1)	4 (4.7)	7 (6.5)	5 (100)	11 (5.5)
Digoxin	0 (0)	6 (7)*	18 (16.7)***	1 (20)	25 (12.6)***

†The percentages of LVF are the valid percentage, thus corrected for the missing values
‡Patients could use more than one type of AAD; therefore, the sum of all classes is not equal to total
*P<0.05, **P<0.01 and ***P<0.001 compared to control
##P0.01 and ###P<0.001 comparing paroxysmal AF with persistent AF

Figure 1 shows baseline concentrations of serum HSPB1, HSPA1, HSPB7 and HSPD1 of both control and AF patients; corresponding values are depicted in Supplemental Table 1. These findings and the absence of a correlation between AF stage and HSP levels after correction for potential confounders in a multivariate model (Supplemental Table 2 and 3) indicate that there are no differences in serum HSP values between the control patients and AF patients with PAF, PeAF and LSPeAF.

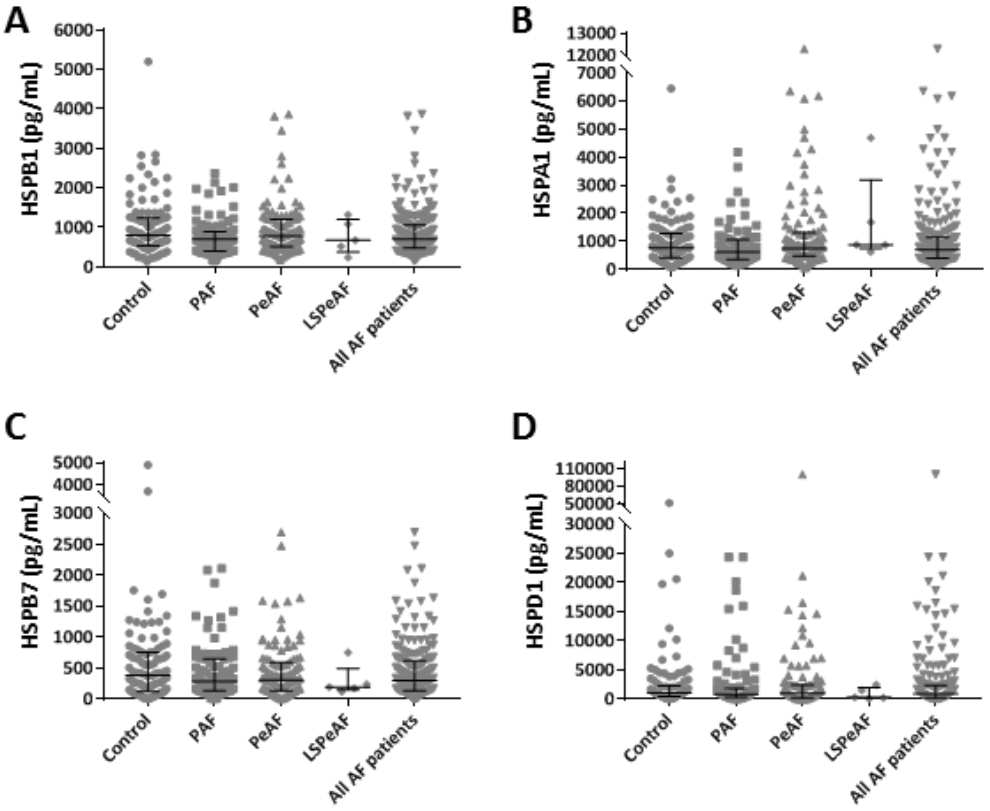

Figure 1. Baseline HSP serum levels in patients without and with (paroxysmal, persistent and longstanding persistent) AF

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.

Relation between baseline HSP levels and AF recurrence

In the total AF population, AF recurrence was significantly correlated to AF stage, age, dyslipidemia and LVF, but not related to medication usage (Supplemental Table 4).

After ECV, AF recurrence was determined within 3 months (N=52, 53.1%), 6 months (N=55, 56.1%) and one year (N=64, 65.3%) (Supplemental Table 5). Figure 2 shows the distribution of the various HSP levels in patients with AF recurrence (red) compared to the remainder of the ECV population (green). Baseline HSPB1, HSPA1, HSPB7 and HSPD1 levels did not differ between patients with or without AF recurrences within the first year after ECV (Figure 2, Supplemental Table 6).

AF recurrences after PVI occurred within 3 months (N=34, 34%), 6 months (N=47, 47%) and within 1 year (N= 59, 58%) (Supplemental Table 7). For all time points, AF episodes were more often observed

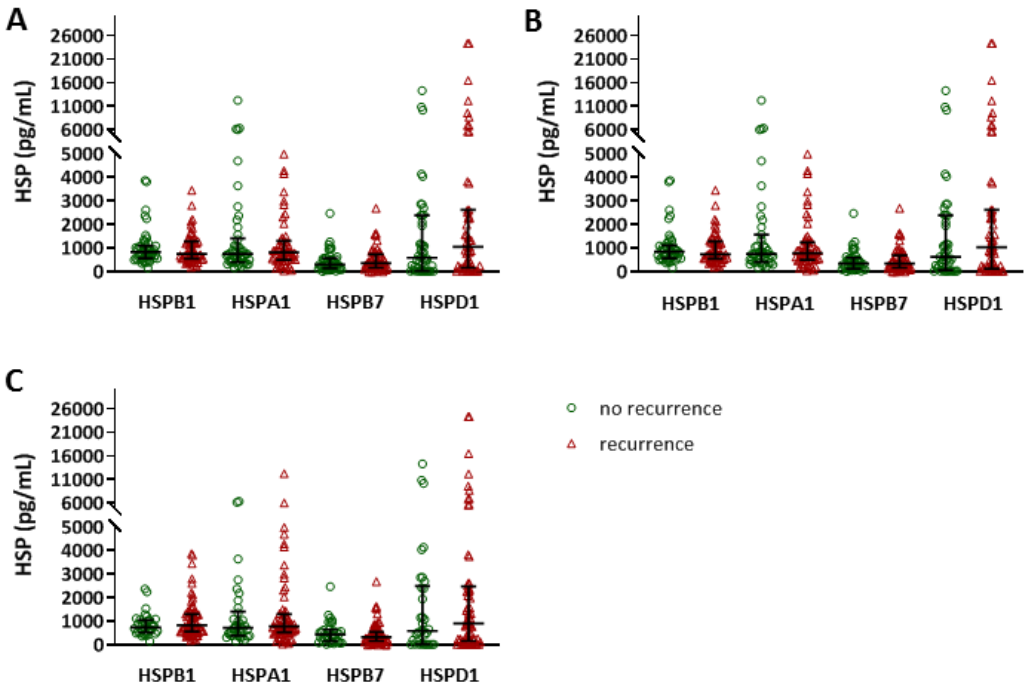

Figure 2. No differences in baseline HSPB1, HSPA1, HSPB7 or HSPD1 serum levels between patients with and without AF recurrence after ECV

HSPB1, HSPA1, HSPB7 and HSPD1 serum levels (pg/mL) at baseline comparing patients with and without AF recurrence within 3 months (**A**), 6 months (**B**) and 1 year (**C**) after ECV treatment.

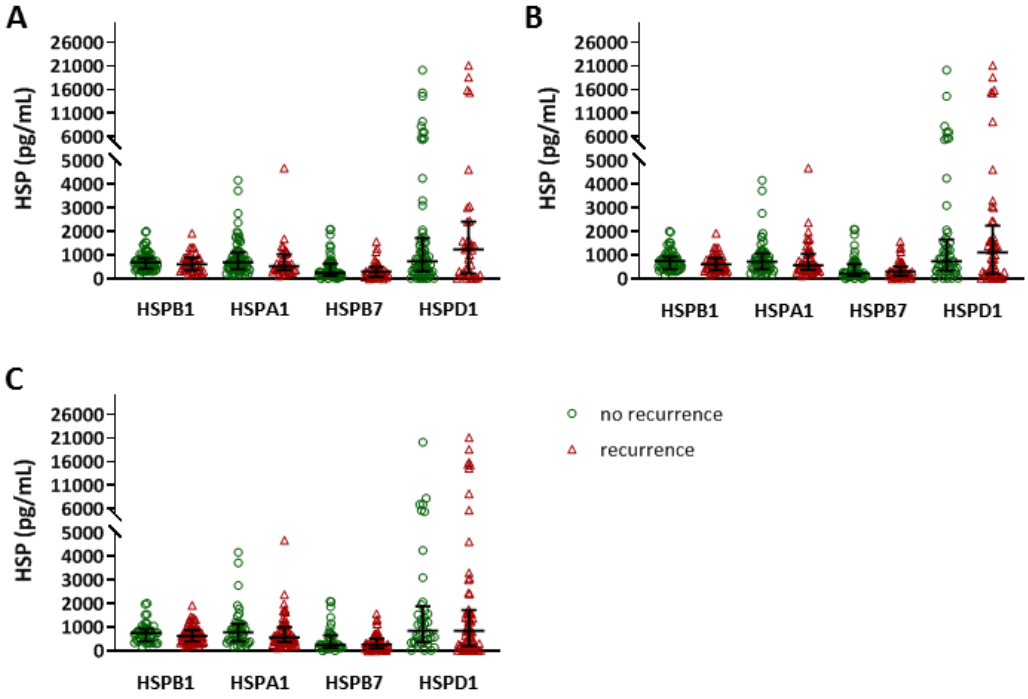

Figure 3. No differences in baseline HSPB1, HSPA1, HSPB7 or HSPD1 serum levels between patients with and without AF recurrence after PVI

HSPB1, HSPA1, HSPB7 and HSPD1 serum levels (pg/mL) at baseline comparing patients with and without AF recurrence within 3 months (**A**), 6 months (**B**) and 1 year (**C**) after PVI treatment.

in patients with PeAF or LSPeAF compared to patients with PAF (*P*<0.01). Baseline serum HSP levels did not discriminate between patients with and without AF recurrence, as demonstrated in Figure 3 and Supplemental Table 6. However, HSPB1 and HSPA1 levels were significantly increased at 3-, 6- and 12-months (only HSPB1) post-PVI treatment in patients with an AF recurrence within one year compared to baseline levels (Figure 4 and Supplemental Table 6). The increase in serum HSPB1 levels, and not HSPA1 levels, corrected for repeated measures, was significantly associated with AF recurrence (*P*<0.013), substantiating the role of HSPB1 in the prediction of AF recurrence after PVI.

To relate HSP levels over time with AF recurrence as an endpoint, joint modeling was utilized. Therefore, the occurrence of first AF recurrence (endpoint) in relation to the standardized ('Z') value of log2(HSP) was modeled, while using all measurements up to and including the moment of the first AF recurrence in endpoint event cases, and all measurements in those who remained event-free. The results are depicted in Table 2 (row 'all events'). One standard deviation (SD) difference in log2 HSPB1 level was associated with a hazard ratio (HR) of 1.32 for having an AF recurrence, supporting the outcomes from the repeated measure model. Unfortunately, the joint modeling for HSPA1 did not converge, and therefore no reliable HR estimate could be obtained.

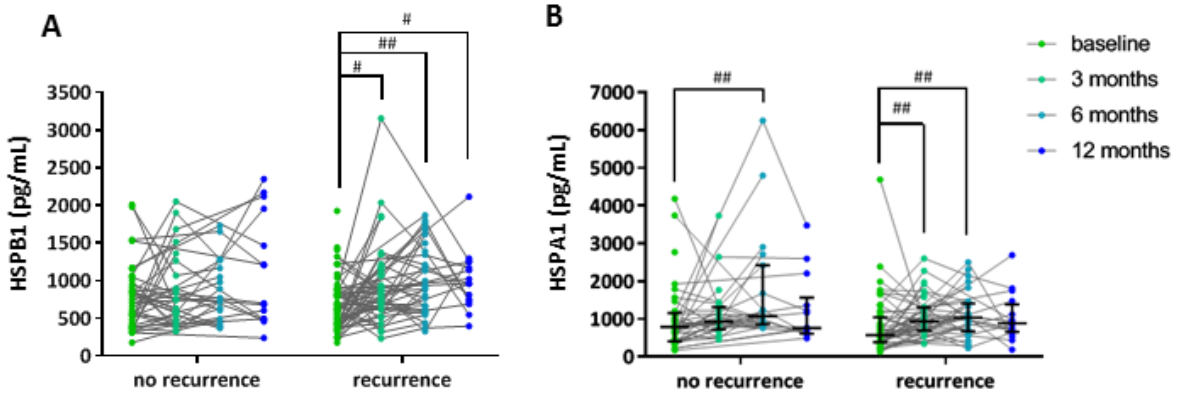

Figure 4. HSPB1 and HSPA1 levels in follow-up serum were higher compared to baseline in patients having AF recurrence

HSPB1 serum levels (pg/mL) at baseline and at 3, 6 and 12 months follow-up in patients having AF recurrence within one year compared to patients not having AF recurrence (**A**). HSPA1 serum levels (pg/mL) at baseline and at 3, 6 and 12 months follow-up in patients having AF recurrence within one year compared to patients not having AF recurrence (**B**). $^{\#}P$<0.05 and $^{\#\#}P$<0.01 compared to baseline serum HSP levels

Sensitivity analysis non-random dropout

Although an association between HSPB1 levels and the recurrence of AF after PVI was observed, sensitivity analyses were conducted to evaluate the robustness of our findings in relation to possible non-random drop out. Therefore, joint models based on the following sensitivity datasets were run: all available data (all events); patients with an AF recurrence at 3 months who also had an HSP measurement at 3 months (21 cases), in combination with patients who were free of AF recurrence at 3 months and with an HSP measurement ≥3 months (54 patients) (complete until 3m); patients with an AF recurrence at 3 or 6 months who also had an HSP measurement at the moment of the AF recurrence (29 cases), in combination with patients who were free of AF recurrence at 6 months and with an HSP measurement ≥6 months (36 patients) (complete until 6m); and patients with an AF recurrence at 3, 6 or 12 months who also had an HSP measurement at the moment of the AF recurrence (31 cases), in combination with patients who were free of AF recurrence at 12 months and with an HSP measurement at 12 months (13 patients) (complete until 12m). The findings of the sensitivity analyses are provided in Table 2. HSPB1 levels were associated with a HR of 2.03 for having an AF recurrence within 6 months post-PVI treatment. The results confirm that HSPB1 is associated with AF recurrence, and may be used as a predictor. However, HSPA1 does not seem to be associated with AF recurrence.

Table 2. Outcomes joint modeling of AF recurrence in relation to HSP levels, and sensitivity analyses

	Patients		**Samples**		**HSPB1**				**HSPA1**			
	AF	**AF-free**	**AF**	**AF-free**	**HR**	**95%CI LL**	**95%CI UL**	***P*-value**	**HR**	**95%CI LL**	**95%CI UL**	***P*-value**
All events	59	41	114	99	1.32	1.71	1.54	<0.001	model does not converge			
Complete until 3m	21	54	42	129	1.31	0.84	2.89	0.258	0.97	0.56	1.5	0.969
Complete until 6m	29	36	63	99	2.03	1.17	3.7	<0.001	1.03	0.54	1.97	0.926
Complete until 12m	31	13	70	34	model does not converge				model does not converge			

Discussion

In this study, we observed that serum HSPB1, HSPA1, HSPB7 and HSPD1 levels in control patients and patients with paroxysmal and (longstanding) persistent AF were comparable between the groups. Thus, HSPB1, HSPA1, HSPB7 or HSPD1 levels could not discriminate between the different AF stages in the total AF population. Additionally, at baseline, HSPB1 and HSPA1 serum levels were similar in patients without and with AF recurrence within one year after treatment. However, both HSPB1 and HSPA1 levels were higher at 3-, 6- and 12- months post-PVI compared to baseline levels in patients with AF recurrence within one year. After joint modeling and sensitivity analyses, only increased HSPB1 levels post-PVI remained as a predictor of AF recurrence.

Heat shock proteins are not biomarkers to differentiate the stage of atrial fibrillation

There is a great need for biomarkers to stage AF and to improve the selection of the proper treatment for patients. Several (AF-related) serum biomarkers are routinely measured in clinical practice, such as natriuretic peptide, troponin I, troponin T, creatinine and C-reactive protein [24-26], but lack specificity for AF. Our findings indicate no role for HSPB1, HSPA1, HSPB7 or HSPD1 as a biomarker for the presence or staging of AF. Our findings are in contrast to findings observed in other studies, as reports revealed a correlation between serum HSP levels and AF. Hu et al. revealed an association between baseline serum HSPB1 levels and AF. In that study, serum HSPB1 levels were reduced in paroxysmal AF and (longstanding) persistent AF patients, compared to controls in normal sinus rhythm [22]. Additionally, low serum HSPB1 associated with larger left atrial diameter, low atrial voltage (indication of fibrosis [27]), and non-pulmonary ectopies. In addition, serum HSPB1 could predict sinus rhythm maintenance and low serum HSPB1 correlated with a higher AF recurrence rate [22]. Our study could not confirm these findings. On the contrary, we observed an increase in follow-up serum HSPB1 and HSPA1 levels in patients who developed AF recurrence post-PVI. In addition, the increased HSPB1 levels significantly correlate with AF recurrence. Despite this interesting observation, due to the current study design we

cannot determine whether the increase in serum HSP levels is correlated to the duration of the AF episodes and whether this increase started prior or post AF recurrence.

Serum HSPA1 levels were similar in control and AF patients in our study and that of others [22, 28]. In line with our study, no correlation between clinical or echocardiographic variables and serum HSPA1 levels was found and increased serum HSPA1 levels at 6 months post-PVI correlated with AF recurrence, substantiating our findings [28].

In patients who underwent coronary artery bypass grafting (CABG), baseline serum HSPA1 levels were not predictive for post-operative AF [29, 30]. While no association between serum HSPD1 level and occurrence of AF was found by Maan et al. [31], Cao et al. [32] described that patients undergoing mitral valve replacement with AF had higher plasma HSPD1 levels than patients in sinus rhythm. Higher plasma HSPD1 levels were also predictive for early (<7days) post-operative AF [32]. Although not the protein itself, pre- and post-operative circulating anti-HSPD1 antibodies are associated with post-operative AF in patients undergoing CABG [33]. In our study, serum HSPD1, as well as HSPB7, did not discriminate between the stage of AF or AF recurrence. The findings indicate that for baseline serum HSPB1, HSPA1, HSPB7 and HSPD1 levels, no clear consensus exists for their use as a biomarker in AF.

Limitations and future directions: HSPs in relation to degree of electropathology

The current cross-sectional analysis provides information whether HSP levels in serum samples of AF patients differ from controls in sinus rhythm. To elucidate whether HSP levels predict AF onset, detect early AF or detect progression of AF, a longitudinal study with repeated blood sampling for HSP measurements and AF testing in subjects with normal sinus rhythm is required.

Our control serum HSPB1 and HSPA1 range is lower compared to the ranges described in other reports, which can be explained by differences in the analytic assays used [22, 29, 30, 34] and due to differences in disease status of the various patient groups. The absence of difference in serum HSP levels between control and AF patients may be attributed to the clinical nature of the control group.

It is generally accepted that AF is a multifactorial disease and predisposing conditions, e.g diabetes mellitus, hypertension and higher age, were omnipresent in all our study groups, including the control group. It might be possible that the clinical variables we studied were too common and we might need to search for more AF specific parameters, such as markers of structural damage that are found to underlie atrial electrical conduction disorders, i.e. electropathology [35]. It has been suggested that the clinical classification of AF, based on ECG measurements, as presented in the guidelines, is inaccurate for AF staging because it is not related to the degree of atrial electropathology as measured by high-resolution epicardial mapping [36]. Additionally, undiagnosed, silent and/or very short lasting AF episodes might have been overlooked in control patients and during follow-up of ECV or PVI patients by using ECG measurements. As previous studies revealed exhaustion of HSP levels in atrial tissue of persistent AF patients [18], in future research projects it is recommended to investigate human HSP levels in both serum and atrial tissue and their relation to parameters related to AF-induced electrical and/or structural remodeling and also structural remodeling-induced (post-operative) AF [37]. Thus, a lack of correlation between HSPs and AF stage in the current study does not negate a role for HSPs as potential biomarker in AF. It is conceivable that serum HSP levels do correlate with the degree of electropathology. Future studies with continuous monitoring of electrical parameters and HSP levels should elucidate such an association.

Conclusion

Serum HSPB1, HSPA1, HSPB7 and HSPD1 levels did not differentiate between AF stages and controls in sinus rhythm. Moreover, AF recurrence after ECV or PVI was not associated with baseline HSP levels. However, HSPB1 levels were increased during follow-up in patients with AF recurrence after ablative therapy and may be used as predictors. Future research directed at elucidation of an association between HSP levels and the degree of electropathology is recommended.

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Supplemental Tables

Supplemental Table 1. **Concentration of HSPs in serum at baseline of patients without and with (paroxysmal, persistent or longstanding persistent) AF**

	Control	PAF	PeAF	LSPeAF	All AF patients
N	98	86	108	5	199
HSPB1, median [IQR]	807.9 [538.5-1240]	705.5 [406.5-898]	783.5 [519.8-1203]	679 [382-1206]	708 [479-1056]
HSPA1, median [IQR]	758.7 [420.6-1287]	624.5 [359.8-1058]	750.5 [472.5-1306]	876 [751-3186]	701 [406-1144]
HSPB7, median [IQR]	381 [120.8-752.8]	286 [131-646]	301 [135-585]	189 [144-490]	293 [131-615]
HSPD1, median [IQR]	1020 [438.5-2236]	775 [271.3-1804]	927 [165-2423]	246 [107.5-1941]	851 [219.7-2358]

Supplemental Table 2. Univariate linear regression associations between clinical parameters and HSP levels in baseline serum

	HSPB1 St β	HSPA1 St β	HSPB7 St β	HSPD1 St β
AF	-0.087	0.016	-0.029	-0.079
Stage of AF	-0.048	0.066	-0.030	-0.084
Age (years)	-0.058	-0.028	0.080	-0.117*
Gender	0.077	0.012	0.089	0.130*
BMI (kg/m^2)	0.073	0.057	0.039	-0.098
Hypertension	-0.033	-0.053	0.027	-0.107
Diabetes mellitus	-0.009	0.022	0.167**	-0.098
Dyslipidemia	-0.043	0.042	0.076	-0.047
Thyroid disease	0.025	0.046	0.039	-0.007
Left ventricular function (LVF)	0.061	0.072	0.116	0.059
ACE. ARB. AT2 antagonist	-0.061	-0.058	0.060	-0.146*
Statin	-0.030	0.076	0.053	-0.108
Class I AAD	-0.14*	-0.097	0.042	0.058
Class II AAD	0.031	-0.005	0.127*	0.002
Class III AAD	0.023	0.027	-0.110	-0.078
Class IV AAD	-0.080	-0.025	0.023	0.068
Digoxin	0.003	0.017	-0.013	-0.016
Recurrence within one year	0.046	-0.051	-0.102	0.003

*P<0.05 and **P<0.01

Supplemental Table 3. Multivariate linear regression associations between AF, AF stage and recurrence within one year and HSP levels in baseline serum, corrected for potential confounders

	HSPB1 St β corrected for age, gender, diabetes mellitus and Class I AAD	**HSPA1 St β** corrected for age, gender and diabetes mellitus	**HSPB7 St β** corrected for age, gender, diabetes mellitus and Class II AAD	**HSPD1 St β** corrected for age, gender, diabetes mellitus and ACE. ARB. AT2 antagonist
AF	-0.043	0.038	-0.108	0.024
Stage of AF	-0.009	0.097	-0.104	0.013
Recurrence within one year	0.035	-0.063	-0.151*	0.031

*P<0.05

Supplemental Table 4. Bivariate spearman correlation between clinical parameters and recurrence within 3 months, 6 months and one year

	Recurrence within 3M	**Recurrence within 6M**	**Recurrence within 1Y**
Stage of AF	0.212**	0.276**	0.190**
Age (years)	0.199**	0.144*	0.164*
Gender	0.086	0.088	0.065
BMI (kg/m^2)	0.050	0.086	0.022
Hypertension	0.174*	0.067	0.092
Diabetes mellitus	0.057	-0.009	0.048
Dyslipidemia	0.152*	0.087	0.156*
Thyroid disease	0.081	-0.068	-0.069
Left ventricular function	0.123	0.214**	0.173*
ACE. ARB. AT2 antagonist	0.097	0.077	0.048
Statin	0.136	0.079	0.103
Class I AAD	-0.024	-0.111	-0.077
Class II AAD	0.072	-0.011	0.023
Class III AAD	-0.031	-0.041	-0.040
Class IV AAD	0.008	0.099	0.059
Digoxin	-0.029	-0.044	-0.042

*P<0.05 and **P<0.01 compared to no recurrence

Supplemental Table 5. Clinical characteristics and serum HSP levels for subjects without AF and with (paroxysmal, persistent and longstanding persistent) AF in the ECV group

	Control	**PAF**	**PeAF**	**LSPeAF**	**All AF Patients**
N	98	12	83	3	98
Age (years), mean ± SD	48.2 ± 15.3	62.2 ± 10.8	60.9 ± 11.2	60.3 ± 8.2	61 ± 11
Gender, male, N (%)	51 (52)	10 (83.3)	63 (75.9)	3 (100)	76 (77.6)
BMI (kg/m2), mean ± SD	25.1 ± 3.7	28.7 ± 3.0	29 ± 5.8	25.8 ± 1.6	28.9 ± 5.4
Hypertension, yes, N (%)	23 (23.5)	9 (75)	37 (44.6)	1 (33.3)	47 (48)
Diabetes mellitus, yes, N (%)	5 (5.1)	1 (8.3)	12 (14.5)	0 (0)	13 (13.3)
Dyslipidemia, yes, N (%)	16 (16.3)	5 (41.7)	23 (27.7)	1 (33.3)	29 (29.6)
Thyroid disease, yes, N (%)	2 (2)	0 (0)	7 (8.4)	1 (33.3)	8 (8.2)
Left ventricular function (LVF), N (%)					
Normal	61 (79.2)	10 (83.3)	40 (51.3)	2 (66.7)	52 (55.9)
Mild impairment	10 (13)	0 (0)	25 (32.1)	1 (33.3)	26 (28)
Moderate impairment	3 (3.9)	1 (8.3)	9 (11.5)	0 (0)	10 (10.8)
Severe impairment	3 (3.9)	1 (8.3)	4 (5.1)	0 (0)	5 (5.4)
Missing[†]	*21*	*0*	*5*	*0*	*5*
Left atrial volume index (ml/m2), median [IQR]	27.9 [21.2-39.7]	46 [29.5-54]	47.5 [37.5-60.8]	52 [N/A]	47 [35-60]
Cumulative recurrence, yes, N (%)					
Within 3 months	-	6 (50)	43 (51.8)	3 (100)	52 (53.1)
Within 6 months	-	7 (58.3)	45 (54.2)	3 (100)	55 (56.1)
Within 1 year	-	7 (58.3)	54 (65.1)	3 (100)	64 (65.3)
Baseline HSP serum levels, N	98	12	83	3	98
HSPB1, median [IQR]	807.9 [538.5-1240]	766 [609.5-1655]	843 [561-1236]	522 [N/A]	795.5 [560-1235]
HSPA1, median [IQR]	758.7 [420.6-1287]	317 [229-1127]	787 [510-1366]	873 [N/A]	774 [490-1333]
HSPB7, median [IQR]	381 [120.8-752.8]	388 [196.5-1178]	335 [150-615]	171 [N/A]	345 [164-635.8]
HSPD1, median [IQR]	1020 [438.5-2236]	1713 [55.3-9843]	882 [132-2382]	246 [N/A]	849 [124-2445]

[†]The percentages of LVF are the valid percentage, thus corrected for the missing values.
Statistical testing performed on cumulative recurrence and baseline HSP serum levels: Students T test on log transformed values (No AF vs All AF patients), Anova with Bonferroni corrections on the log transformed values (No AF vs PAF or No AF vs PeAF or No AF vs LSPeAF)

Supplemental Table 6. Serum HSP concentrations after one year follow-up

	No recurrence	Recurrence
ECV patients		
Baseline, N	34	64
HSPB1, median [IQR]	737 [523.8-1059]	832.5 [569.5-1314]
HSPA1, median [IQR]	727.5 [391-1409]	787 [533.8-1306]
HSPB7, median [IQR]	441.5 [153.5-648.8]	330.5 [164-553.3]
HSPD1, median [IQR]	587 [32-2491]	907.5 [167-2482]
PVI patients		
Baseline, N	41	59
HSPB1, median [IQR]	753 [398.5-925]	620 [394-862]
HSPA1, median [IQR]	787 [406-1150]	566 [384-1040]
HSPB7, median [IQR]	247 [131-663]	280 [112-522]
HSPD1, median [IQR]	854.5 [357-1888]	850 [206-1756]
3 months follow-up, N	26	40
HSPB1, median [IQR]	633 [445.5-1119]	862.5 [668-1147]$^{\#}$
HSPA1, median [IQR]	922 [721.8-1312]	939 [693-1302]$^{\#\#}$
6 months follow-up, N	19	32
HSPB1, median [IQR]	771 [607-1270]	930 [598.5-1378]$^{\#\#}$
HSPA1, median [IQR]	1079 [858-2415]$^{\#\#}$	1034 [675.3-1410]$^{*,\#\#}$
12 months follow-up, N	15	16
HSPB1, median [IQR]	700 [489-1957]	961.5 [730-1232]$^{\#}$
HSPA1, median [IQR]	756.5 [489-1957]	883.5 [656.8-1385]

$^{*}P<0.05$ compared to no recurrence, $^{\#}P<0.05$ and $^{\#\#}P<0.01$ compared to baseline serum
Statistical testing performed: Students T test on log transformed values (No recurrence vs Recurrence), Anova with Bonferroni corrections on the log transformed values (baseline vs follow-up serum samples for HSPB1 and HSPA1 within the group of patients with or without recurrence)

Supplemental Table 7. Clinical characteristics and serum HSP levels for subjects without AF and with (paroxysmal, persistent and longstanding persistent) AF in the PVI group

	Control	PAF	PeAF	LSPeAF	All AF Patients
N	98	74	25	3	102
Age (years), mean ± SD	48.2 ± 15.3	61.2 ± 9.4	60.5 ± 8.3	53.3 ± 11.3	60.8 ± 9.1
Gender, male, N (%)	51 (52)	54 (73)	18 (72)	1 (50)	73 (72.3)
BMI (kg/m2), mean ± SD	25.1 ± 3.7	27 ± 3.9	28.1 ± 3.9	37.3 ± 7.5	27.4 ± 4.2
Hypertension, yes, N (%)	23 (23.5)	34 (45.9)	14 (56)	2 (100)	50 (49.5)
Diabetes mellitus, yes, N (%)	5 (5.1)	9 (12.2)	3 (12)	1 (50)	13 (12.9)
Dyslipidemia, yes, N (%)	16 (16.3)	20 (27)	10 (40)	2 (100)	32 (31.7)
Thyroid disease, yes, N (%)	2 (2)	4 (5.4)	1 (4)	0 (0)	5 (5)
Left ventricular function (LVF), N (%)					
Normal	61 (79.2)	63 (85.1)	20 (80)	1 (50)	84 (83.2)
Mild impairment	10 (13)	9 (12.2)	4 (16)	1 (50)	14 (13.9)
Moderate impairment	3 (3.9)	2 (2.7)	1 (4)	0 (0)	3 (3)
Severe impairment	3 (3.9)	0 (0)	0 (0)	0 (0)	0 (0)
Missing[†]	*21*	*0*	*0*	*0*	*0*
Left atrial volume index (ml/m2), median [IQR]	27.9 [21.2-39.7]	38.5 [29.1-47.6]	39.5 [29.3-60]	43.1 [N/A]	38.7 [29.8-49.5]
Cumulative recurrence, yes, N (%)					
Within 3 months	-	19 (25.7)	13 (54.2)¥¥	2 (100)¥¥	34 (34)
Within 6 months	-	29 (39.2)	16 (66.7)¥¥	2 (100)¥¥	47 (47)
Within 1 year	-	37 (50)	20 (83.3)¥¥	2 (100)¥¥	59 (58)
Baseline HSP serum levels, N	98	74	25	2	101
HSPB1, median [IQR]	807.9 [538.5-1240]	678.5* [393-869]	610 [405-877]	1205 [N/A]	659** [400.5-891.5]
HSPA1, median [IQR]	758.7 [420.6-1287]	641 [396-1043]	529 [347-951]	3186 [N/A]	636 [385.5-1058]
HSPB7, median [IQR]	381 [120.8-752.8]	276 [122.5-645]	224 [122-564.5]	211 [N/A]	248 [126-573]
HSPD1, median [IQR]	1020 [438.5-2236]	739.5 [293.3-645]	1394 [483.3-5066]	1305 [N/A]	851 [297.3-1744]
3 months FU, N		50	15	2	67
HSPB1, median [IQR]	-	823# [563.8-1194]	784 [627-1115]	617.5 [N/A]	811# [569-1157]
HSPA1, median [IQR]	-	939### [721-1323]	755 [541-1229]	1091 [N/A]	930.5### [711-1308]
6 months FU, N		37	14	1	52
HSPB1, median [IQR]	-	806# [601.5-1271]	1138# [810.8-1659]	440 [N/A]	907## [606.3-1282]
HSPA1, median [IQR]	-	1065### [822.5-1427]	923 [660.8-1593]	1052 [N/A]	1059### [791.5-1434]
12 months FU, N		22	8	1	31
HSPB1, median [IQR]	-	961.5## [590.3-1339]	841 [470-1240]	1021 [N/A]	959## [605-1261]
HSPA1, median [IQR]	-	924 [615.5-1415]	739 [637.3-916.5]	1748 [N/A]	804# [634.5-1385]

[†]The percentages of LVF are the valid percentage, thus corrected for the missing values.

*P<0.05 and **P<0.01 compared to control, ¥¥P<0.01 PeAF and LSPeAF compared to PAF, #P<0.05, ##P<0.01 and ###P<0.001 compared to baseline

Statistical testing performed on cumulative recurrence and baseline HSP serum levels: Students T test on log transformed values (No AF vs All AF patients), Anova with Bonferroni corrections on the log transformed values (No AF vs PAF or No AF vs PeAF or No AF vs LSPeAF or baseline vs follow-up (FU) serum samples for HSPB1 and HSPA1 within the group of patients with PAF, PeAF and All AF patients)