

# Heat shock protein 60 antibody

## *A new marker for subsequent atrial fibrillation development*

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### ABSTRACT

**Objective:** To examine the pre- and post-operative anti-HSP60 antibodies of serum from patients in preoperative sinus rhythm.

**Methods:** We prospectively studied 45 consecutive patients admitted for elective CABG from 2004 to 2005. We randomly selected 10 patients developing AF (study sample [Group A]) and 10 postoperative patients without AF (control [Group B]). The study took place at the Department of Cardiovascular Surgery, Hacettepe University, Ankara, Turkey.

**Results:** Anti-HSP60 IgG value was  $27.76 \pm 12.69$  absorbance units (AU) in Group A preoperatively and decreased to  $13.73 \pm 5.51$  AU postoperatively. Controversially, preoperative value of anti-HSP60 IgG was  $9.94 \pm 2.92$  AU and decreased to  $6.72 \pm 1.89$  AU, postoperatively in Group B. Statistical analysis showed significant difference regarding preoperative anti-HSP60 IgG levels in Group A compared to Group B, which might be interpreted as an association between postoperative AF and preoperative levels of anti-HSP60 IgG.

**Conclusion:** We provide the first evidence demonstrating the association of pre- and post-operative circulating anti-HSP60 antibodies with postoperative AF. These results suggest that serum HSP60 antibody levels may be a marker for subsequent development of AF.

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Atrial fibrillation (AF), with an incidence reported to vary from 10-50%,<sup>1</sup> is the most common complication following coronary artery bypass grafting (CABG) and it is associated with a 2-fold increase in cardiovascular mortality<sup>4-7</sup> and morbidity.<sup>3</sup> Atrial fibrillation occurs approximately  $72 \pm 24$  hours after the operation.<sup>4,11,18,21</sup> The mortality rate in patients with AF is twice as high as that of patients in sinus rhythm.<sup>8</sup> Despite it is often regarded as a benign and self-limited condition, post-CABG AF may lead to severe complications, including systemic embolization and even significant hemodynamic instability.<sup>4,5,7</sup> In response to many stress stimuli, cardiomyocytes produce a common set of heat shock proteins (HSP). Of these proteins, heat shock protein 60 (HSP60) plays an important role in cytoprotection by preventing denaturation or by enhancing proper assembly of cellular proteins and structures.<sup>9-15</sup> In patients with dilated cardiomyopathy, a raised level of myocardial HSP60 expression could be demonstrated.<sup>12</sup> Heat shock protein 60 is also an important mediator of inflammation via stimulation of the toll-like receptor-4 (TLR 4) producing proinflammatory cytokines.<sup>16-18</sup> However, the response of HSP60 to a state of chronic fibrillating stress, such as in chronic AF, is unknown. Therefore, we examined pre-, and post-operative anti-HSP60 antibodies in serum from patients in preoperative sinus rhythm.

**Methods.** In this case-control study, pre- and post-operative blood samples were collected from 45 consecutive patients admitted to our institution for elective CABG from June 2004 to June 2005, and plasma were stored at  $-80^{\circ}\text{C}$  for subsequent analysis. Preoperatively, all patients were in sinus rhythm. In this study, we randomly selected 10 patients developing AF (study sample = Group A) and 10 without postoperative AF patients (control group = Group B). Blood samples were obtained

one day preoperatively and 3 days postoperatively. The electrocardiogram (ECG) characteristics and cardiovascular risk profile were documented. Exclusion criteria were preceding history of AF, other associated operations, active infection, history of autoimmune diseases, and use of immunosuppressive drugs.

The Ethics Committee of Hacettepe University Hospital approved the study, and informed consent was obtained from all patients. Serum total Anti-Hsp60 levels were measured with a commercial enzyme-linked immunosorbent assay (ELISA) kit (StressXpress™, Stressgen Biotechnologies, Victoria, BC, Canada); 100 ml of each 1:1000 (v/v) diluted serum samples and prepared standards were applied to each well pre-coated with recombinant human Hsp60 (hHsp60). The plate was sealed and incubated on an orbital shaker at room temperature (20°C-25°C) for 2 hours. After washing 4 times with a buffer, the captured anti-human Hsp60 antibodies were detected by adding 100 ml hydrogen peroxidase conjugated goat polyclonal antibody specific for human IgG, IgA and IgM molecules (Anti-Human GAM-HRP conjugate). The plate was sealed and incubated on an orbital shaker at room temperature for one hour. After washing 4 times with a buffer, 100 ml of tetramethylbenzidine substrate was added and allowed to react for 15 minutes. We added 100 ml acid stop solution to terminate the reaction, and the intensity of the color was measured in a TECAN microplate ELISA reader (TECAN, Crailsheim, Germany) at 450 nm. The concentration of anti-Hsp60 antibody was determined from standard dose-response curve using Easy WinRMP V6.0a software (TECAN). The sensitivity of the assay was 2.88 ng/ml. All operations were performed by means of mild hypothermic cardiopulmonary bypass with antegrade and retrograde blood cardioplegia. Intraoperative features and postoperative complications

were noted. Postoperatively, heart rate and rhythm were continuously monitored for the first 48 hours. Patients were subjected to daily morning 12-lead ECGs. Postoperative AF for this study was defined as the characteristic arrhythmia lasting for ≥15 minutes, occurring within first 3 days postoperatively.

**Statistical analyses.** Differences between the 2 groups were tested with an unpaired *t* test for continuous variables and a  $\chi^2$  test for categorical variables. Heat shock protein 60 antibodies were not normally distributed and the HSP60 antibody levels were compared between the 2 groups with Mann-Whitney U test. The probability value of <0.05 was considered significant. We used non-parametric tests. Mann-Whitney U test was used to compare the 2 groups and Wilcoxon test was used for group comparisons (postoperative values versus preoperative values).

**Results.** The mean age in Group A was 62.1 ± 7.9 years and 62.3 ± 7.5 years in Group B, where 8/10 (80%) patients were male in Group A and 6/10 (60%) patients were male in Group B. There was no significant difference between the 2 groups regarding demographic data, which are summarized in **Table 1**. The intraoperative and postoperative data were respectively summarized in **Table 2**. None resulted in a significant difference between the 2 groups. Anti-HSP60 IgG value was 27.76 ± 12.69 absorbance units (AU) in Group A preoperatively and decreased to 13.73 ± 5.51 AU postoperatively. In contrast, preoperative value of anti-HSP60 IgG was 9.94 ± 2.92 AU and decreased to 6.72 ± 1.89 AU postoperatively in Group B (**Table 3**). Mann-Whitney U test showed significant difference regarding preoperative anti-HSP60 IgG levels in Group A compared to Group B ( $p < 0.002$ ), which might be interpreted as a strong association between postoperative AF and preoperative levels of anti-HSP60 IgG.

**Table 1** - Patients' demographic data.

Demographic data	Atrial fibrillation (n=10)	No atrial fibrillation (n=10)	P value*
Age	62.1 ± 7.9	62.3 ± 7.5	0.9547
Male/female	8/2	6/4	0.3553
Diabetes (%)	1 (10)	4 (40)	0.1346
Hypertension (%)	2 (20)	3 (30)	0.627
Chronic obstructive arterial disease (%)	1 (10)	0 (0)	0.3306
Smoker (%)	6 (60)	4 (40)	0.3979
Cholesterol, mg/dL	208.4 ± 39.9	205.7 ± 32.6	0.8704
Preoperative myocardial infarction (%)	5 (50)	3 (30)	0.388
Ejection fraction	44.7 ± 8.7	46.4 ± 7.6	0.6499

\* $p < 0.05$  was considered significant

**Discussion.** Postoperative AF is a common problem in cardiac surgery. Recent reports from several groups demonstrate the influence of inflammation on the development of AF. In our study, we provide the first evidence demonstrating the association of pre- and post-operative circulating anti-HSP60 antibodies with postoperative AF. These results suggest that serum HSP60 antibody levels may be a marker for subsequent development of AF. Alternatively, our findings may highlight their role in the pathogenesis of postoperative AF. The majority of proteins for ATP production (such as F1-ATPase) are synthesized in the cytoplasm and transported to the mitochondrion.<sup>19</sup> Once they are imported in an unfolded state, HSP60 and HSP10 were responsible for correct folding and translocation of the mitochondrial proteins.<sup>20</sup> Heat shock protein 60 is a large donut-shaped protein with a central cavity that is essential for the folding of a huge spectrum of proteins in the mitochondrial matrix. It functions in conjunction with a coprotein, HSP10, which enhances its ability to eject proteins during the ATPase cycle.<sup>21</sup> In AF, a state of increased atrial activity, there is a need for increased ATP requirements and subsequently higher protein turnover. High levels of unfolded protein are the

primary initiator of the HSP synthesis.<sup>20</sup> The presence of high HSP60/HSP10 levels in mitochondria seems to reflect high activity (such as ATP synthesis).<sup>22</sup> The chronic fibrillating stress leads to an adaptive response of the heart cells. Heat shock protein 60 mRNA and protein increased during stimulation.<sup>23</sup> One mechanism to cope with the high energy and protein metabolism might be the upregulation of HSP60.<sup>24</sup> This could also be a mechanism that the failing myocardium uses to adapt to increased hemodynamic stress.<sup>11</sup> Schaeffer et al demonstrated elevated levels of HSP60 in atrial specimens in patients with AF.<sup>25</sup> The novel aspect of our study was to detect increased preoperative serum levels of anti-HSP60.

The study would have had to build a large prospective controlled trial including CABG patients with preoperative sinus rhythm and screening of the postoperative rhythm (atrial fibrillation or not). It would have allowed to calculate sensitivity, specificity and predictive value of anti-HSP60 to discriminate between patients with and without postoperative AF. Thus, we are continuing this case-control study with a large number of patients.

**Table 2** - Intraoperative and postoperative data.

Data	Atrial fibrillation	No atrial fibrillation	P value*
Cardiopulmonary bypass (minutes)	67.9 ± 14.5	65.6 ± 13.2	0.7154
Cross clamp (minutes)	36.2 ± 7.4	34.4 ± 6.8	0.5808
Flow, ml/min	4305 ± 397.8	4284 ± 307.2	0.8964
Distal anastomosis (number)	2.7 ± 0.8	2.5 ± 0.7	0.5679
Duration of ventilation, (hours)	7.6 ± 2.2	7.2 ± 1.6	0.6556
Inotropic support (%)	3 (30)	3 (30)	1.0000
Chest infection (%)	0 (0)	1 (10)	0.3306
Renal dysfunction (%)	0 (0)	1 (10)	0.3306
Intensive care unit stay (day)	2.9 ± 1.8	2.7 ± 1.0	0.7704

\**p*<0.05 was considered significant.

**Table 3** - Pre- and post-operative anti-heat shock protein 60 immunoglobulin G levels.

Anti-HSP60 IgG levels	Atrial fibrillation	No atrial fibrillation
Preoperative (AU)	27.76 ± 12.69	9.94 ± 2.92
Postoperative (AU)	13.73 ± 5.51	6.72 ± 1.89

AU - absorbance units

In conclusion, with these preliminary results we report for the first time a novel association between HSP60 antibodies and the occurrence of postoperative AF. This finding lends support to the possible role of inflammation and cross-reactive autoimmunity in the development of AF.

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