



# The Relationship of Serum Heat Shock Protein 70 Antibody Levels with the Inflammatory Factors and Serum Uric Acid Levels in Hemodialysis Patients

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## Abstract

**Background:** Heat shock proteins are extracellular inflammatory intermediaries and intracellular cytoprotective molecules. Despite many studies on intracellular HSP70, the clinical association between inflammatory biomarkers and extracellular HSP70 antibody (anti-HSP70) levels is not well-studied.

**Objectives:** The aim of this study was to investigate whether raised serum anti-HSP70 in hemodialysis (HD) patients are related to levels of serum inflammatory markers and uric acid, as the key players, in the pathogenesis of the disease.

**Methods:** This cross-sectional study was carried out from January 2018 to July 2018, on patients referred by the nephrologists from the central outpatient dialysis center of a governmental university-affiliated hospital, in Ahvaz, Iran. Ninety HD patients enrolled based on the inclusion and exclusion criteria. Blood samples were collected directly from the arteriovenous fistula before a routine HD session. The circulating levels of anti-HSP70, highly sensitive C-reactive protein (hs-CRP), Interleukin-6 (IL-6), and endotoxin were measured by Enzyme-linked immunosorbent assay (ELISA).

**Results:** The univariate regression analysis revealed a significant association between serum anti-HSP70 level and diabetes mellitus, hypertension, hemodialysis vintage, uric acid, hs-CRP, and IL-6 ( $P < 0.05$ ). In a multiple regression model, after adjusting for confounders, the association between circulating anti-HSP70 and uric acid ( $B = 16.92$ ,  $P = 0.001$ ), hs-CRP ( $B = 11.77$ ,  $P = 0.002$ ), IL-6 ( $B = 2.87$ ,  $P = 0.002$ ), endotoxin ( $B = 0.14$ ,  $P = 0.005$ ), and hemodialysis vintage ( $B = 43.76$ ,  $P = 0.002$ ) was significant.

**Conclusions:** Our findings suggest that the development of the excessive systemic inflammatory response and uric acid contribute to a higher serum anti-HSP70 leading to cardiovascular disease.

**Keywords:** Endotoxins, Anti-HSP70 Heat-Shock Proteins, Inflammation, Interleukin-6, Renal Replacement Therapy, Uric Acid

## 1. Background

Chronic kidney disease is one of the prevalent chronic diseases that mostly leads to end-stage renal disease (ESRD) needing hemodialysis (HD). The major cause of mortality in HD patients is cardiovascular diseases (CVDs) (1). Inflammation and oxidative stress are among the contributing factors to the pathogenesis of CVDs (2). Among HD patients inflammatory factors, including C-reactive protein (CRP) and interleukin-6 (IL-6) increase with on the duration of dialysis, known as dialysis vintage (3). Progression of the disease in HD patients, including increased cell death, oxidative stress, and the inflammation begins to release heat shock proteins (HSPs) to the circulation (4). It has been shown that serum or extracellular HSPs and their antibod-

ies are independent predictors of CVD risk (5). HSPs are extracellular inflammatory intermediaries and intracellular cytoprotective molecules. Recently, it has been reported that inducible extracellular HSP70 is up-regulated during oxidative stress and binds to surface receptors, which stimulate the release of pro-inflammatory cytokines such as TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 (6). Higher levels of serum HSP70 are associated with different disease states (7). Only a few studies measured the levels of serum HSP70 in patients with renal disease that showed a higher amount of it and its antibodies in ESRD patients, particularly who are undergoing hemodialysis (8-10) However, the role of serum HSP70 in HD patients has not been explained in detail so far.

The serum uric acid level, which is related to inflammation in ESRD patients (11), is another independent risk

factor for cardiovascular diseases (12). Previous in vivo and in vitro studies stated that the higher level of uric acid might induce the expression of hepatic inflammatory molecules by stimulating the pro-inflammatory NF- $\kappa$ B signaling cascade (13). Because inflammation has an essential role in releasing the HSPs to the circulation, we hypothesized that the serum level of uric acid might correlate with the serum anti-HSP70 level and the rise of it, which contribute to more pronounced inflammatory effects. However, two studies demonstrated a positive correlation between serum HSP70 and serum uric acid levels in patients with pre-eclampsia (6, 14).

## 2. Objectives

Although higher levels of inflammatory markers, uric acid, and HSP70 or its antibody have been shown previously in HD patients, there is no study regarding the independent relationship of anti-HSPs, uric acid, and systemic inflammation. Furthermore, the relationship between these factors discovers a new marker for its pathogenesis in HD patients in the future. Therefore, this research investigated the association between serum levels of heat shock protein 70 antibody and uric acid and systemic inflammatory markers in HD patients.

## 3. Methods

### 3.1. Study Design and Setting

This cross-sectional study was carried out from January 2018 to July 2018. The data were collected from 123 patients, referred by the nephrologists from the central outpatient dialysis center of the governmental Emam Khomeini Hospital of Ahvaz University of Medical Sciences (AJUMS), Ahvaz, Iran. According to the inclusion and exclusion criteria, the study population included 90 patients undergoing HD (56.7% men, 47.5  $\pm$  10.6 years old) for at least 3 consecutive months.

### 3.2. Sample Size and Sampling Method

The sample size in a cross-sectional design for multivariate regression analysis is calculated using the formula:

$$\left[ \frac{(2 - 2 \times \rho^2 + \epsilon)}{\epsilon} \right] (k + 1) \quad (1)$$

where  $n$  (sample size),  $\rho$  (Pearson Correlation Coefficient estimate), and  $k$  (the number of dependent variables entered the model (15)). The sample of 86 was calculated with  $\rho = 0.63$  as the Pearson coefficient of correlation between HSP70 and uric acid levels in a previous study (14), and  $k = 6$  as the number of variables entered the regression

model. The sample size was adequate for a clinical observational design. A simple random sampling technique was applied to select those study participants by using the table of random numbers and computer by the simple random sampling method.

### 3.3. Study Population

The inclusion criteria were adults aged 30 to 65 years, started hemodialysis at least three months before the start of the study and with an arteriovenous fistula. The exclusion criteria were previous kidney transplant or likely to receive a transplant, those using a central catheter for hemodialysis access, smoking, those medically diagnosed with severe infections, inflammatory disease, malignancy, chronic liver disease, having amputated limbs, pregnancy and patients using steroidal and/or nonsteroidal anti-inflammatory drugs and antibiotics, antioxidant, and/or anti-inflammatory supplements within 1 month of study commencement. Renal failure etiologies included diabetes mellitus ( $N = 34$ , 37.8%), chronic glomerulonephritis ( $N = 8$ , 8%), hypertension ( $N = 32$ , 35.6%), polycystic kidney disease ( $N = 12$ , 13.3%), and unknown ( $N = 4$ , 4%). The average arterial blood pressure of patients was 125.6/78.9 mmHg. Regarding anti-hypertensive treatments and hypoglycemic agents in the HD patients, 35% of the patients were receiving anti-hypertensive medications, including ACE-inhibitors ( $N = 22$ ), calcium channel blockers ( $N = 12$ ), or in combination ( $N = 12$ ), and 32% of patients were receiving metformin ( $N = 34$ ) and glibenclamide alone ( $N = 15$ ) or in combination ( $N = 25$ ). The study was conducted in accordance with the guidelines of the Helsinki Declaration. The Ethics Committee of Ahvaz University of Medical Sciences, Ahvaz, Iran (IR.AJUMF.REC.1395.812) approved the study protocol, and each patient gave written informed consent.

### 3.4. Laboratory Methods

Blood samples (7 mL) were drawn immediately before the HD session from each subject in the morning after 12 h fasting before the HD using the slow flow/stop pump technique. The serum was separated (15 min; 3000  $\times$  g; 4°C) and collected at -80°C. Circulating levels of serum anti-HSP70 antibodies were assessed using an enzyme-linked immunosorbent assay (ELISA) kit (ab133063; Abcam; USA) with intra- and inter-assay CVs of < 10%. The serum level of IL-6 (CK-E10140; Eastbiopharma; China), hs-CRP (CK-E11183; Eastbiopharma; China) and endotoxins (CK-E10840; Eastbiopharma; China) were measured using ELISA kits with intra-assay and inter-assay coefficients of variation of 10% and 12%, respectively. The serum concentrations of creatinine and urea were assessed using colorimetric methods by commercial kits (Pars Azemom, Tehran, Iran) with the

aid of a Selectra 2 Autoanalyzer (Vital Scientific, Spankeren, The Netherlands). The intra-assay and inter-assay CVs for the serum creatinine and urea were 5.6%, and 3.7%, respectively. The serum level of uric acid was measured by standard analytical methods (Claus technique and uricase enzymatic test, respectively) with a normal range of uric acid levels: 3.4 - 7 mg/dL for males and 2.4 - 6 mg/dL for females. The dialysis adequacy (as Kt/V) was assessed for each patient based on blood urea concentration, 24-h urine volume, urine urea concentration, 24-h dialysate drain volume, dialysate urea concentration, weight, height, and age, using a Kt/V calculator.

### 3.5. Statistical Methods

Continuous variables were reported as mean  $\pm$  SD. Moreover, categorical variables were presented as number (percentage). The normal distribution of the variables was checked by Shapiro-Wilk's W-test. Univariate linear regression models were used to predict the association between the mean of anti-HSP70 and other clinical and biochemical variables. Furthermore, in order to reduce potential confounders, those variables with a significance level  $< 0.2$  were entered a multiple linear regression model. The association between anti-HSP70 and other variables was examined in four different regression models. The bootstrapping method was used in univariate and multivariate regression. The p value  $< 0.05$  was considered statistically significant and analysis was performed using the IBM SPSS Statistics for windows, version 19.0 (IBM Corp., Armonk, N.Y., USA).

## 4. Results

### 4.1. Patient Characteristics

The mean age was 46.64 years in our 90-patient study population, 51 (56.71%) were female, 40 (44.4%) were diabetic, and 42 (46.7%) were hypertensive patients. According to the hemodialysis vintage, we divided our patients into three subgroups. In this regard, 23 (25.6%), 29 (32.2%) and 39 (42.2%) patients were undergoing HD for 6 - 23 months, 24 - 56 months and 60 - 120 months, respectively. The clinical and laboratory characteristics of the study participants are presented in [Table 1](#).

### 4.2. Association of Anti-Hsp70 Levels with Inflammatory Markers, Uric Acid, and Other Variables

We examined the association between serum anti-HSP70 levels and clinical characteristics and some laboratory parameters of HD patients. Analysis of clinical characteristics and other laboratory parameters revealed a significant association between serum anti-HSP70 level and

**Table 1.** The Demographic, Clinical, and Laboratory Characteristics of Hemodialysis Patients

Variable <sup>a</sup>	Mean $\pm$ SD or No. (%)
Age, y	46.64 $\pm$ 10.69
BMI	23.10 $\pm$ 4.58
Gender	
Male	51 (56.7)
Female	39 (43.3)
Kt/V	1.46 $\pm$ 0.28
Duration of hemodialysis, mo	
6 - 23	23 (25.6)
24 - 59	29 (32.2)
60 - 120	39 (42.2)
Diabetes	34 (37.8)
Hypertension	32 (35.6)
Cachexia (BMI $< 18.5$ )	16 (17.8)

Abbreviations: BMI, body mass index; BUN, blood urea nitrogen; Cr, Creatinine; Kt/V, K - dialyzer clearance of urea, t - dialysis time, V - volume of distribution of urea, approximately equal to the patient's total body water

<sup>a</sup>Mean  $\pm$  SD for quantitative and frequency (%) for qualitative variables.

diabetes mellitus (DM), hypertension (HTN), hemodialysis vintage, uric acid, hs-CRP, IL-6 and endotoxin ([Table 2](#)). There was no significant association between anti-HSP70 concentrations and other markers of kidney injury [BUN, CR, Ph, and Ca]. There were significant differences in anti-HSP70 between the three HD vintage groups ( $P < 0.001$ ). Increasing periods of HD patient groups were associated with a stepwise increase in anti-HSP70 level. The patients who underwent HD for at least 5 years had a significantly higher serum level of anti-HSP70 than those on HD for 6 - 23 months [390.86 (121.30) vs. 223.7 (108.32),  $P < 0.001$ ] and 24 - 59 months [390.86 (121.30) vs. 254 (118.10),  $P < 0.001$ ] ([Figure 1](#)).

To evaluate the association of anti-HSP70 level with serum uric acid level, a multiple regression analysis was performed. We considered the possible effect of the four models. According to all models, after adjustment for major confounding factors in model 1: DM, HTN, HD vintage model 2: model 1 + hs-CRP; model 3: model 2 + IL-6; model 4: model 3 + endotoxin, significant associations were observed between circulating anti-HSP70 and uric acid, hs-CRP, IL-6, endotoxin, and HD vintage ([Table 3](#)).

## 5. Discussion

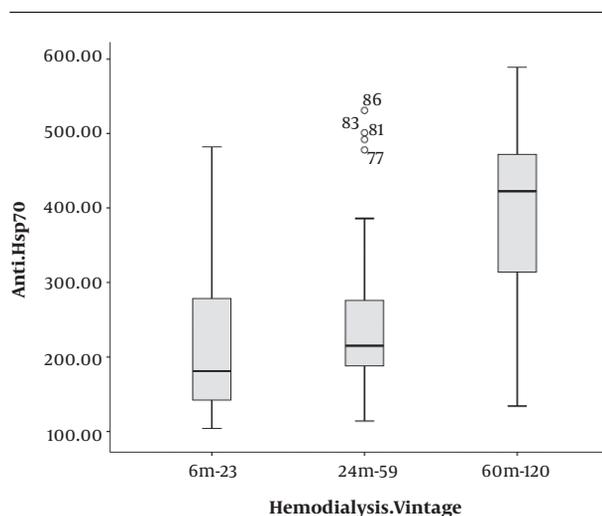
The novel finding of this cross-sectional study is that we indicated an independent relationship between serum anti-HSP70 levels and serum uric acid level in HD patients.

**Table 2.** Linear Regression Analysis: Anti-HSP70 as Dependent Variable, and the Other Data as Independent Variables (Total N = 90)<sup>a</sup>

Variables	B	P Value
Age, y	-1.29	0.36
Gender, female/male	20.42	0.49
Presence of diabetes	64.03	0.029
Presence of hypertension	60.11	0.047
Hemodialysis vintage, mo	87.88	0.001
BUN	1.37	0.14
CR	1.85	0.75
Phosphate	-3.5	0.64
Uric acid	26.28	0.001
Calcium	-2.25	0.90
Albumin	-8.19	0.78
hs-CRP	20.32	0.001
IL-6	5.31	0.001
Endotoxin	0.38	0.001

Abbreviations: BUN, blood urea nitrogen; CR, creatinine; hs-CRP, high sensitivity C-reactive protein; IL-6, interleukin-6

<sup>a</sup>Results are based on bootstrapping method; beta was obtained by univariate linear regression model.



**Figure 1.** Serum anti-HSP70 is significantly higher in HD patients who had been on HS for at least 5 years compared to those patients with lower HD vintage. The vertical axis represents serum anti-HSP70 levels in ng/mL, whereas the horizontal axis refers to patient groups based on hemodialysis vintage. The box and bars represent the mean and the 95% confidence interval, respectively. Serum anti-HSP70 significantly increased in HD patients with more than 5 years of HD treatment compared to the patients with 24 - 59 months and 6 - 23 months HD (390.86 (121.30) vs. 223.7 (108.32) vs. 254 (118.10), respectively;  $P < 0.001$ ).

Moreover, the detailed characterization of the patient population allowed us to identify a significant association between serum anti-HSP70 levels and serum systemic inflam-

**Table 3.** Association Between Serum Anti-HSP70 Level and Uric Acid Level Using Unadjusted and Adjusted Linear Regression Models<sup>a</sup>

Model	B	P Value <sup>b</sup>
<b>Unadjusted model</b>		
Uric acid	26.28	0.001
<b>Model 1</b>		
Uric acid	19.45	0.001
Presence of diabetes	42.74	0.067
Presence of hypertension	43.39	0.062
Hemodialysis vintage	70.31	0.001
<b>Model 2</b>		
Uric acid	21.17	0.001
Presence of diabetes	39.11	0.063
Presence of hypertension	12.69	0.537
Hemodialysis vintage	55.91	0.002
Hs-CRP	16.27	0.001
<b>Model 3</b>		
Uric acid	18.11	0.001
Presence of diabetes	21.63	0.221
Presence of hypertension	2.95	0.872
Hemodialysis vintage	42.24	0.002
Hs-CRP	12.98	0.001
IL-6	3.33	0.001
<b>Model 4</b>		
Uric acid	16.92	0.001
Presence of diabetes	6.48	0.699
Presence of hypertension	1.93	0.909
Hemodialysis vintage	43.76	0.002
Hs-CRP	11.77	0.002
IL-6	2.87	0.002
Endotoxin	0.14	0.005

Abbreviations: B, unstandardized regression coefficients; CI, confidence interval; IL-6, Interleukin-6; hs-CRP, high sensitivity C-reactive protein.

<sup>a</sup>Results are based on bootstrapping method; beta was obtained by multiple linear regression model. Adjusted variables for model 1: diabetes mellitus, hypertension, hemodialysis vintage model 2: model 1 + hs-CRP, model 3: model 2 + IL-6 model 4: model 3 + endotoxin.

<sup>b</sup>P value refers to the association between serum anti-HSP70 levels and other variables.

mation. We also noticed that the increasing periods of HD patients were associated with a stepwise increase in anti-HSP70 level.

The heat shock response is shown by an increased expression of heat shock proteins at a high-stress situation, such as raised urea concentration, to preserve the cell integrity and lessen cell injury. However, in HD patients, cell damage or necrosis due to uremic toxins, inflammation,

and high oxidative stress can lead to a release of intracellular HSP, which contributed to elevating the extracellular HSP concentration, initiating a response by the innate immune system (10, 16). It should be noted that serum HSP70 has a role in damage-associated molecular pattern and induce inflammation in various cells and tissues. Those effects are triggered via Toll-like receptors (TLR) 2 and 4, driving to the activation of nuclear factor  $\kappa$ B (NF- $\kappa$ B) signaling pathway, as well as mitogen-activated protein kinase pathways, which result in cytokine production (13). Indeed, it has been reported that increased circulating HSP70 levels were associated with inflammatory responses in various pathological statuses, including acute infections, after liver resection and coronary artery bypass grafting, following myocardial infarction, HEEP syndrome, type 2 diabetes, preeclampsia, and during aging (17-21). Clinical and epidemiological studies have demonstrated higher levels of serum HSP70 in HD patients (8, 10, 16). According to the results of this study, the relationship between serum HSP70, hs-CRP, and IL-6 levels was found in HD patients suggesting that circulating HSP70 might play a key role in the progress of the excessive systemic inflammatory response, which seems to be responsible for the complications of HD patients. In fact, the inflammation and anti-HSP70 could form a vicious cycle in which the body's inflammatory environment provoke HSP release to the circulation, and HSPs and their antibodies can increase the inflammation. Moreover, it has been postulated that uremia and oxidative stress contribute to HSP release to the circulation by producing cytokines, which provide an inflammatory environment in the body (10). However, to compare our study, there is no human study, which has investigated the association of serum anti-HSP70 with cytokine or other inflammatory biomarkers.

On the other hand, we observed that hemodialysis vintage after adjusting the confounders significantly contributed to enhancing the serum anti-HSP70. This can be normally explained because HD procedure and CKD progression had an additive effect on oxidative stress and inflammation; its prolonged use could increase the severity of releasing anti-HSP70.

In addition, an independent association between levels of HSP70 and uric acid was observed in HD patients. The association between serum anti-HSP70 level and serum uric acid level was significant when compared using a multiple linear regression adjusted for the following variables: hypertension, diabetes mellitus, hemodialysis vintage, hs-CRP, and IL-6 levels. We had noticed similar positive relationships in a previous study between serum uric acid level and the levels of anti-HSP70 ( $r = 0.4099$ ;  $P = 0.0017$ ) in early-onset preeclampsia patients (6). Moreover, Alvarez-Cabrera *et al.*, recently, demonstrated a positive correlation between serum HSP-60 and -70 and serum

uric acid level and other inflammatory response secretion (IL-1 $\beta$  and TNF- $\alpha$ ) in patients with pre-eclampsia (14). Hyperuricemia is prevalent in CKD patients, including HD patients (22). Few studies have evaluated the association between uric acid and inflammation in CKD patients (11, 23). The mechanisms by which uric acid causes inflammation seems to involve complex pathways that can induce oxidative stress and endothelial dysfunction. Studies suggest that increased serum uric acid increases the cyclooxygenase-2 activity, which results in the activation of Nod-like receptors inducing interleukin-1 $\beta$  (IL-1 $\beta$ ) which acts as a pro-inflammatory cytokine to trigger an inflammatory response (11). The above studies taken together provide strong evidence that links uric acid with a higher level of anti-hSP70, which take place by stimulating the release of inflammatory cytokines. Therefore, our findings support the theory that high serum uric acid level may intensify the systemic inflammation, which can increase serum anti-HSP70 level.

To the best of our knowledge, this is the first study that investigated the independent association of serum anti-HSP70 with serum uric acid and inflammatory biomarkers levels in HD patients. The present study used alternative statistical approaches to test the hypothesis, adjusting for important confounders in four multiple regression models. However, there are a number of limitations in the present study that the interpretation of results should be cautious. The most important limitation of our study is our inability to judge causality from the observed associations because of inherent cross-sectional and observational study design. Therefore, a well-designed cohort or randomized clinical trial study will determine the predictors leading to higher serum anti-HSP70. Second, a relatively small sample size and single-center study might reduce the statistical power of the present study. Future multi-center, prospective studies with a larger sample size are warranted to confirm these findings.

In conclusion, these findings suggest that systemic inflammation, serum uric acid level, hemodialysis vintage may have an independent role in the elevation of serum anti-HSP70 in hemodialysis patients. However, further studies are required to determine whether systemic inflammation, serum uric acid level, and hemodialysis vintage play a causative role in the elevation of circulating HSP70 levels or serum anti-HSP70.

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## Footnotes

**Authors' Contribution:** Neda Haghighat was involved in study concept, design, analysis, interpretation of data, drafting of the manuscript. Majid Mohammadshahi supervised the conduct of the study and Shokouh Shayanpour assist with data collection.

**Conflict of Interests:** The authors declare that they have no conflict of interests.

**Ethical Approval:** This protocol, approved by the Medical Ethics Committee of Ahvaz University of Medical Sciences, is in accordance with the Declaration of Helsinki (approval number: IR.AJUMF.REC.1395.812).

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**Patient Consent:** Each subject will sign an informed consent form.

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