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Is there any association between Serum anti-HSP27 antibody level and the presence of metabolic syndrome; population based case-control study

Fatemeh Sadabadi^{1*}, Alireza Heidari-Bakavoli^{2*}, Habibollah Esmaily³, Susan Darroudi¹, Maryam Tayefi⁴, Zahra Asadi⁵, Seyed Mohammad Reza Parizadeh⁶, Shima Tavalaei⁶, Najmeh malekzadeh¹, Kiana Hosseinpour Moghaddam⁷, Azam Rastgar Moghadam¹, Amir Hosein Sahebkar⁸, Narges Fereydouni¹, Elham Barati¹, Mahmoudreza Azarpazhooh², Seyyed Javad Hosseini⁸, Mohammad Tayyebi², Mahmoud Ebrahimi², Gordon A. Ferns⁹, Majid Ghayour-Mobarhan^{5,6**}, Mohsen Mouhebat^{2**}

1. "Department of modern sciences and technologies, Faculty of medicine, Mashhad university of medical sciences, Mashhad, Iran

2. Cardiovascular Research Center, Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran

3. Department of Biostatistics & Epidemiology, Faculty of Health, Management & Social Determinants of Health Research Center, Mashhad University of Medical Sciences, Mashhad, Iran

4. Clinical Research unit, Mashhad University of Medical Sciences, Mashhad, Iran

5. Department of Nutrition, Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran

6. Metabolic Syndrome Research Center, Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran

7. Department of biology, Faculty of sciences, Ferdowsi University of Mashhad, Mashhad, Iran

8. Department of Medical Biotechnology, Faculty of medicine, Mashhad University of medical sciences, Mashhad, Iran

9. Brighton & Sussex Medical School, Division of Medical Education, Falmer, Brighton, Sussex, UK

* Equally contributed as first authors; ** Equally contributed as Corresponding authors

Corresponding authors: Majid Ghayour-Mobarhan, MD, PhD; Biochemistry of Nutrition Research Center, Faculty of Medicine, Mashhad University of Medical Science, Mashhad, Iran, Postal code: 99199-91766; Email: ghayourm@mums.ac.ir.

Mohsen Mouhebat, MD; Cardiovascular Research Center, Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran. Postal code: 99199-91766; Email: Mouhebatim@mums.ac.ir.

Abstract

Background: Heat shock protein 27 (HSP27) is an intracellular chaperone constitutively expressed in many cell types including cardio myocytes and endothelial cells. Circulating levels of HSP27 and anti-HSP27 antibody are higher in patients with CVD. Anti-HSP27 antibody concentrations were also reported to be increased in atherogenesis. We aimed to evaluate serum anti-HSP27 antibody titers in individuals with, or without, MetS in the MASHAD study cohort with large sample size in 6,568 subjects. **Methods:** Participants with MetS were identified from MASHAD cohort (n=3358) using the IDF criteria, and the control group were those individuals who did not meet these criteria (n=3210). In-house enzyme-linked immune sorbent assay (ELISA) method was used for measuring Anti-HSP27 antibody levels. The two groups were matched for age, sex and smoking habit. **Results:** As expected, there were significant differences in height ($p=0.004$), waist and hip circumference, weight, BMI, systolic and diastolic blood pressure, TGs, TC, HDL-C, Hs-CRP, glucose, with the presence of diabetes mellitus, hypertension, hyperlipidemia ($p<0.001$) between the two groups. Serum HSP27 antibody titers did not show significant difference between the groups with and without metabolic syndrome ($p=0.740$). **Conclusion:** In conclusion, our results revealed serum anti-HSP27 antibody titers were not statistically different between individuals with and without MetS. However, it is possible that drug treatment may affect antibody titers and confound our findings in this population sample. .

Keywords: serum anti-HSP27 antibody, Metabolic syndrome, MASHAD study cohort

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Introduction

Metabolic syndrome (MetS), also called Reaven syndrome, or Syndrome X, is characterized by several risk factors of cardiovascular disease including central obesity, dyslipidemia, impaired glucose tolerance, high blood pressure (1-4), and a proinflammatory and prothrombotic state (5). MetS increases the risk of diabetes mellitus, coronary heart disease, stroke and other conditions including; fatty liver (3), asthma, ovarian cysts (4) and some cancers (6).

The prevalence of MetS varies from <10 % to 84 % dependent on the definition used and population investigated (7). Genetic background, physical activity, diet, smoking, and family history of diabetes affect the risk of MetS (8). The prevalence of MetS is also affected by age; this increases with age group in most populations: 6.7% in the 20-29 years and 43.5% in the 60-69 years of age (9).

Heat shock proteins (HSPs) are intracellular proteins highly conserved, that have chaperonin activities, refolding denatured proteins. Several characteristic features of MetS (impaired glucose tolerance, hypertension, dyslipidemia) may be associated with oxidative stress that can induce the expression of HSPs (10, 11). HSP27 is a small HSP expressed and presented in a lot of cell types including endothelial cells and cardiomyocytes. The latter possess cardio-protective properties (12). Several studies have reported that the circulating levels of HSP27 and anti-HSP27 antibody are higher in CVD patients including acute coronary syndrome (ACS) and documented coronary artery disease (CAD) (13-15). Anti-HSP27 antibody concentrations were also reported to be increased in atherogenesis (16, 17). Since the response of immune system to HSPs may contribute to the development of atherosclerosis, the production of anti-HSP27 antibodies has been proposed to be a marker of inflammation (18),

We aimed to evaluate serum anti-HSP27 antibody titers in individuals with, or without, MetS in the cohort study of **Mashhad Stroke and Heart Atherosclerotic Disorder (MASHAD)**. Individuals were recruited into this study using the stratified cluster random sampling method. The cohort comprised 9,704 participants aged 35–65, who were selected from an urban population located within **Northeastern Iran**. The MASHAD study has been one of the first cohorts of CVD in the Middle East designed to estimate the incidence of cardiovascular diseases and assess the effect of different environmental, nutritional, psychosocial and genetic risk factors, started in 2007 and continued until 2020(19).

Material and Methods

Subject

This research was approved by the Mashhad University of Medical Sciences Ethics Committee in accordance with the Declaration of Helsinki (Ethical code no. IR.MUMS.REC.1386.250), Informed consent was obtained from all individuals recruited to the study (19). All participants with MetS were identified from MASHAD cohort (n=3358) using the IDF criteria, and the control group were those individuals who did not meet these criteria (n=3210).

Study design

The International Diabetes Federation (IDF) criteria, were: if three of the following five criteria were met: (1) abdominal obesity: waist circumference >94 cm in men and >80 cm in women; (2) hypertriglyceridemia ≥ 150 mg/dl or specific treatment; (3) serum HDL-C <40 mg/dl in men and <50 mg/dl in women or specific treatment; (4) blood pressure (HBP) $\geq 130/85$ mmHg or specific treatment; (5) high fasting glucose ≥ 100 mg/dl or treatment with anti-diabetic drugs(5). We excluded individuals who had CVD before entering the study and pregnant and breastfeed-

ing women. Also full drug history was taken from each subject because of the probable potential confounding effects of drugs on Hsp27 antibody titers. Participants taking glucocorticoids, nonsteroidal anti-inflammatory (NSAIDs), anticoagulant drugs and psychiatric medications were excluded. A high proportion of patients with MetS were prescribed anti-diabetic drug, anti-hypertensive agent and statins and were not excluded from the study.

Blood sampling and Biochemical measurements

At the start of the study, a fasted blood sample (after 12 hours fast) was obtained from all participants. Anthropometric parameters, including: weight, height, waist circumference, hip circumference, body mass index (BMI), blood pressure, etc. were measured and recorded. Demographic data were collected using a questionnaire. Physical activity level (PAL) is the numerical variable expressing an individual's daily physical activity, assessed from James and Schofield human energy requirement equations.

Biochemical parameters measured included: fasting blood glucose (FBG), lipid profile including serum total cholesterol (TC), triglycerides (TGs), high density lipoprotein cholesterol (HDL-C) and serum hsCRP, and were measured using commercial kits and an Alycon auto analyzer (ABBOTT, Chicago, IL, USA). LDL-C (Low-density lipoprotein cholesterol) concentrations were estimated using the Friedewald formula (20).

Measuring of anti-HSP27 antibody

In-house enzyme-linked immune sorbent assay (ELISA) method was used for measuring Anti-HSP27 antibody levels. In brief, 50-ng recombinant human HSP27 antigen was added to 50 μ L carbonate buffer pH= 9.6 and then micro-titer plates (Nunc Maxisorp, Nunc) were coated. The plates were incubated at 4 °C under humid con-

ditions for 18 hours. After this time, the wells were washed three times with buffer phosphate saline with 0.05 % Tween-20 (PBST). For blocking stage and decreasing non-specific binding, wells were filled and incubated with 2% goat serum in PBS. The wells were incubated for 30 min in 37 °C and 30 min at room temperature. After washing (three times with PBS), 100 µL of diluted serum 1:100 with 2% goat serum in PBS was added to each well in duplicate and incubated 30 min at room temperature. Wells were washed six times (four times in PBST and two times in PBS). Then peroxide conjugated-goat anti-human IgG (Sigma-Aldrich, Poole, UK) diluted 1:500 with 2% goat serum in PBS. 100 µL of diluted anti-human IgG was placed in each well. In this step the incubation time was 30 min at room temperature. Finally, each well was washed six times (four times in PBST and two times in PBS).

The tetramethyl benzidine (TMB) substrate was prepared [100 µL of 6mg/mL TMB in dimethyl sulfoxide (DMSO)] and 100 µL of TMB and 3 µL H₂O₂ was added to 10 mL of acetate buffer, 50 mM and pH 4.5. Finally, 100 µL of substrate solution was added per well and the plate was incubated for 15 min in the dark at room temperature. To terminate the reaction, 50 µL of 3 M HCl was added. The optical density of each well was evaluated at 450 nm using Lab systems iEMS Reader MF microtiter plate reader. The reference wavelength was 620 nm.

Statistical analysis

SPSS version 16 was used for all statistical analyses. Variables with normally and none normal distribution were expressed as means ± SD, median and interquartile range respectively. The groups were matched according to algorithms for pair-matching based on the propensity score by age, sex and smoking ($P=0.231$, 0.798 and 0.718 respectively) (21). Comparison of groups was accomplished by ANOVA, or Kruskal-Wal-

lis tests, and a Bonferroni correction applied for multiple comparisons. In order to assess the independent effects of taking medication and PAL on MetS and its components on measures of serum anti-HSP27 antibody levels, multiple regression analyses were performed. The level of statistical significance was $p<0.05$.

Result

Baseline anthropometric, demographic and biochemical characteristics between participants with MetS and controls are shown in Table 1. As expected, there were significant differences in height ($p=0.004$), weight, waist and hip circumference, BMI, systolic and diastolic blood pressure, TGs, TC, HDL-C, Hs-CRP, glucose, and the presence of diabetes mellitus, hypertension, hyperlipidemia and PAL between two groups ($p<0.001$). There was not significant difference in serum HSP27 antibody titers and LDL-C between the two groups ($p=0.740$ and $p=0.694$ respectively).

There were significant differences between those with and without MetS in prescription for medication such as anti-diabetic drug, anti-hypertensive agent, statins ($p<0.001$).

Anti-HSP27 levels were compared in groups that were positive for different numbers of MetS criteria as showed in Table 2, there were no significant differences in serum anti-HSP27 titers with increasing components of MetS.

Different models of multivariate regression for anti-HSP27 and MetS were undertaken, and the effect of using drug and PAL on anti-HSP27 levels assessed (Table 3). In model 1 (unadjusted), model 2 (adjusted for taking anti-diabetic drug, anti-hypertensive agent and statins) and model 3 (adjusted for taking anti-diabetic drug, anti-hypertensive agent, statins and PAL), no significant relation between anti-HSP27 level and MetS was observed.

Table1. Comparison of the baseline anthropometric, demographic and biochemical characteristics between participant with or without MetS

		MetS ⁺ (n=3358)	MetS ⁻ (n=3210)	p value
Age (y)		49.43±7.94	49.19±8.19	0.231
Sex N (%)	Male	1035(31.3)	1015(31.6)	0.810
	Female	2269(68.7)	2195(68.4)	
Height (m)		1.59±0.09	1.58±0.08	0.004
Weight (kg)		75.85±12.66	68.92±12.64	<0.001
Waist circumference (cm)		100.40±10.19	93.42±12.50	<0.001
Hip circumference (cm)		106.63±8.91	102.72±9.57	<0.001
BMI (kg/m2)		29.78±4.33	27.32±4.78	<0.001
Systolic blood pressure (mm Hg)		129.50±20.69	118.46±16.06	<0.001
Diastolic blood pressure (mm Hg)		83.59±12.21	76.91±10.83	<0.001
Triglycerides (mg/dl)		177(136-233) ^a	100(75-128) ^a	<0.001
Total Cholesterol (mg/dl)		199.56±40.83	189.90±38.40	<0.001
HDL-C(mg/dl)		39.11±7.67	46.07±10.52	<0.001
LDL-C(mg/dl)		118.00±38.09	118.35 ±34.43	0.694
Glucose (mg/dl)		99.28±45.23	90.43±37.46	<0.001
Hs-CRP (mg/dl)		2.03 (1.20-4.16) ^a	0.215 (0.95-3.33) ^a	<0.001
HSP27 antibody titers (OD)		0.203 (0.104-0.336) ^a	0.202(0.105-0.335) ^a	0.740
Smoking habit, N (%)	Never	2301 (69.7)	2229(69.4)	0.841
	Past	318 (9.6)	300(9.3)	
	Current	684 (20.7)	681(21.2)	
Diabetes mellitus N (%)		644(19.7)	393(12.4)	<0.001
Hypertension N (%)		1646(50.2)	763(24)	<0.001
Hyperlipidemia N (%)		3257(98.6)	2430(75.7)	<0.001
PAL	Sedentary	789(27)	496(16.9)	<0.001
	Low active	860(29.5)	741(25.2)	
	Moderately active	1032(35.3)	1231(41.8)	
	High active	239(8.2)	475(16.1)	

BMI bodymass index, LDL-C low-density lipoprotein cholesterol, HDL-C high-density lipoprotein cholesterol, Hs-CRP high-sensitivity C-reactive, ^aMedian (interquartile range) because of skewed distribution.

Serum HSP27 antibody titers were also compared according to the specific phenotypes of MetS shown in figure 1. Anti-HSP27 antibody titers were significantly higher in participants

with waist circumstances criteria than subjects without any criteria of MetS ($p<0.05$) and patients with high waist circumstances, high density lipoprotein, triglycerides and blood pressure

Table 2. Serum HSP27 antibody concentration and number of MetS components

	Components number of metabolic syndrome						
	0	1	2	3	4	5	P-value
n	271	966	1707	2181	706	82	
Anti-HSP27 (OD)	0.184 (0.102-0.315)	0.193 (0.103-0.325)	0.207 (0.107-0.340)	0.207 (0.105-0.340)	0.190 (0.098-0.324)	0.216 (0.108-0.319)	0.570

Table 3. Different model of multivariate regression for anti-HSP27 and MetS

	Model	OR (CI 95%)	P value
Anti-HSP27 (OD)	Model 1	1.032(0.738-1.445)	0.853
	Model 2	0.954(0.738-1.448)	0.848
	Model 3	0.954(0.676-1.347)	0.788

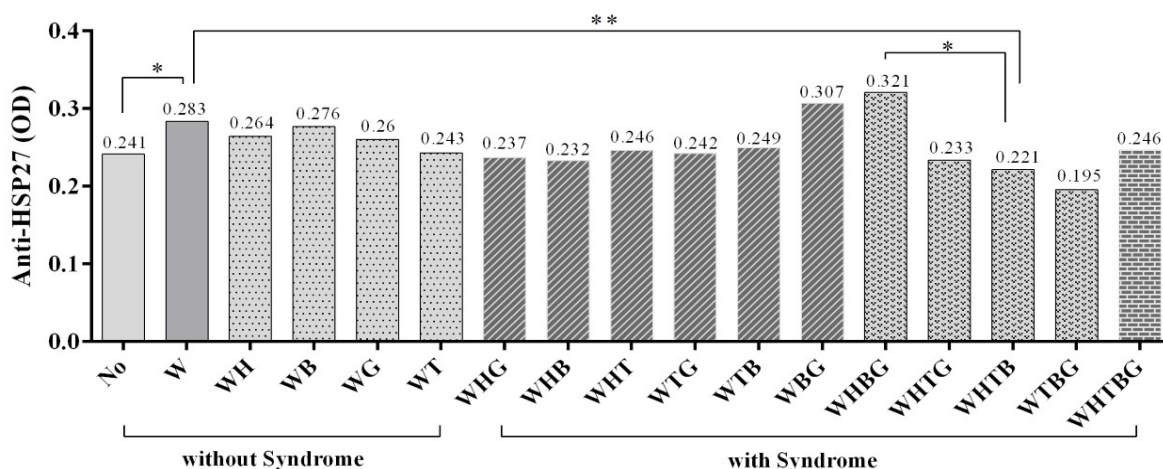
Model 1: unadjusted; Model 2: adjusted for taking anti-diabetic drug, anti-hypertensive agent and statins; Model 3: adjusted for taking anti-diabetic drug, anti-hypertensive agent, statins and PAL. PAL: Physical Activity Level.

($p < 0.01$). Also there was a significant difference in serum HSP27 antibody titers between the subjects with high waist circumferences, high density lipoprotein, triglycerides and blood pressure compared to participants with high waist circumferences, high density lipoprotein, blood pressure and glucose ($p = 0.05$).

Discussion

We have measured serum anti-HSP27 antibody titers in individuals with or without MetS recruited in the MASHAD study. Serum HSP27 antibody levels did not show significant difference between MetS positive (MetS+) and negative (MetS-) groups. The result of this study did not show additive effect in increasing MetS

component on serum anti-HSP27 antibody titers. Previous studies have shown higher HSP antibody titers, release and consequently immune response to HSPs being affected by different MetS-related risk features such as diabetes, hypertension, hyperlipidemia and smoking which is most likely due to the induction of oxidative stress (22-24). Sahebkar et al. showed an increase in anti-HSP27 antibody levels in patients with coronary heart disease (CHD) and MetS compared to individuals with CHD alone (Sahebkar et al. 2011), which is in agreement with the study of Ghayour-Mobarhan et al. (25). Kargari et al. found that obese individuals showed significantly higher anti-HSP27 antibody levels than the non-obese group (26). However, they did not find any association between anti-HSP27

**Fig. 1. Anti-HSP27 antibody according to phenotype of MetS**

W: waist circumferences, H: high density lipoprotein, T: triglycerides, B: blood pressure, G: glucose

* $p < 0.05$ ** $p < 0.01$; ^aMedian (interquartile range) because of skewed distribution.

antibody titers and either diabetes or MetS. They suggested that the previous results and inconsistencies may be because of the differences in the health status of the MetS- groups between the studies (2).

We measured anti-HSP antibody titers of MetS+ and MetS- individuals based on whether or not they had cardiovascular disease or diabetes. These results were non-significant either. We expect the reason for this misalignment of results may be due to the differences in the drugs taken by the two groups. Most people with MetS take cholesterol-lowering drugs (e.g. statins), blood glucose control drugs (e.g. insulin and metformin), blood pressure medications, etc. These drugs can affect the expression of HSPs.

Metformin, a metabolic stressor that potentially activates AMPK pathway, is a first-line medication to treat type 2 diabetes and it is consumed by hundred millions of people worldwide (27). Studies showed that metformin impairs the DNA-binding activity of HSF1 by the induction of ser121 phosphorylation through AMPK activation. Inhibition of HSF1 activity leads to down-regulation of HSPs (28). Statins can also cause a significant reduction in serum anti-HSPs levels. In our previous studies, we found that 40 mg/day statin therapy for 4 weeks was associated with decrease in anti-HSP antibody 60 and 70 titres in dyslipidemic patients (29, 30), while our next studies show that statin therapy did not significantly affect serum HSP60 and 70 (31). This phenomenon may be attributed to the immunomodulatory properties of statins. Since individuals with MetS often take metformin and statins because of hyperglycemia and dyslipidemia, decrease of anti-HSP27 antibody might be due to the use of these drugs in the group.

Another explanation for this result might be due to the removal of HSP27 by the immune system when it is released into the circulation. Burut et al. measured HSP27 IgG and IgM antibody

levels between the CVD and non-CVD individuals with or without glucose tolerance. They showed there was not any significant difference in HSP27 IgG antibody between the groups but HSP27 IgM antibody levels were significantly lower in the normal glucose tolerance with CVD patients, compared with normal glucose tolerance without CVD patients. They suggested that the release of HSP27 IgM antibody into circulation may lead to formation of immune complexes. This may be one reason for the clearance of released HSP27 in patients with acute coronary syndromes (32). We did not evaluate Hsp27 IgM antibody titers in this study but found similar serum HSP27 IgG antibody levels in MetS+ and MetS- group. Serum anti Hsp27 antibody titers in two groups may be affected by the formation of antigen-antibody complexes and this might have happened to our HSP27 IgG antibody of MetS+ samples.

In conclusion, our results revealed that there was no significant difference in serum HSP27 antibody titers between MetS positive and negative groups.

This may be due to the effect of their use of administered drugs with MetS individuals. This result needs further knowledge about the effect of whole drugs consumed by MetS patients on HSP27 and anti-HSP27 antibody.

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Compliance with Ethical Standards

This research was approved by the Mashhad University of Medical Sciences Ethics Committee in accordance with the Declaration of Helsinki (Ethical code no. IR.MUMS.REC.1386.250). Informed consent was obtained from all individuals recruited to the study.

Authors' contribution

FS (Conceptualization; Data curation; Formal analysis; Software; Visualization; Writing – original draft)

AHB (Conceptualization; Project administration; Visualization)

HE (Conceptualization; Formal analysis; Project administration; Software)

SD (Formal analysis; Investigation)

MT (Formal analysis; Software)

ZA (Data curation)

SMRP (Conceptualization; Writing – review & editing)

ST (Methodology)

KHM (Investigation)

ARM (Conceptualization)

AHS (Conceptualization; Methodology)

NF (Conceptualization)

EB (Methodology)

MA (Investigation)

SJH (Visualization)

MT (Conceptualization)

ME (Conceptualization)

GAF (Writing – review & editing)

MGM (Conceptualization; Project administration; Supervision)

MM (Conceptualization; Project administration; Supervision)

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Conflict of Interest

The authors have no conflict of interest to disclose.

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