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Heat Shock Protein 60: A Marker of Cardiovascular Diseases in Type 2 Egyptian Diabetic Patients

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ABSTRACT

Background: Hyperglycaemia and hyperinsulinaemia, hallmarks of the postprandial state, have been associated with increased oxidative stress contributing to vascular injury. The development of cardiovascular disease is a main complication of diabetes mellitus. Heat sock protein 60 is over-expressed after cells exposure to stressful conditions that include oxidative stress like diabetes as well as cardiovascular diseases. The association between serum heat sock protein 60 levels and development of diabetic complications is unknown. **Objectives:** evaluate the potential value of serum heat sock protein 60 level in the development of cardiovascular complications in Egyptian patients with type 2 diabetes mellitus. **Patients and methods:** Heat sock protein 60 in 18 diabetic control patients and 62 diabetic patients with different risks to cardiovascular disease was determined using AssayMax Human heat sock protein 60 ELISA kit. All groups were age and sex matched. **Results:** Heat sock protein 60 concentrations were significantly higher in diabetic patients with diferent risks to cardiovascular disease compared to diabetic control group ($P<0.05$). Moreover, its level showed higher sensitivity and area under the curve compared to common traditional markers (ratio 1 & ratio 2) of cardiovascular disease. **Conclusion:** Our results showed that presence of Heat sock protein 60 in diabetic patients was associated with the development of cardiovascular disease complications. Heat sock protein 60 showed superiority in sensitivity compared to other traditional biochemical parameters so could serve as an early marker for diagnosis of diabetic cardiovascular disease complications.

Key Words: *Cardiovascular disease, Clinical, Heat-shock protein 60, Type 2 diabetes mellitus*

INTRODUCTION

More than 11% of Egyptian population suffers from type 2 diabetes which is considered an important cause of premature mortality in Egypt, it is responsible for 2.4% of all life losses and also considered as the sixth cause of disability burden in Egypt.[1]

Diabetes mellitus is characterized by hyperglycemia which results from defects in insulin secretion, insulin action, or both. Long-term complications of diabetes may be microvascular (e.g. retinopathy, nephropathy, and neuropathy) or macrovascular complications (like atherosclerotic cardiovascular, peripheral arterial and cerebrovascular disease).[2]

Diabetes mellitus increases the risk of developing cardiovascular diseases which lead to disability and death in these patients. The metabolic abnormalities that contribute to vascular dysfunction in diabetes include hyperglycemia, increased free fatty acids, and insulin resistance which decrease bioavailability of nitric oxide (NO), increased oxidative stress, disturbances of intracellular signal transduction and activation of receptors for AGEs and these lead finally to the cellular events causing atherosclerosis.[3]

Cardiac biomarkers are biological analytes which can be detected in the bloodstream at increased levels during the continuum of cardiovascular diseases or immediately after myocardial damage. The ideal cardiac biomarkers should be highly specific for cardiac

tissue, absent from non-myocardial tissue and easily accessible to achieve high diagnostic sensitivity.[4]

Biomarkers of atherosclerotic platelet activity include: cytokines (Interleukins; IL1, IL6, IL8, IL10, IL18, sCD40 ligand, myeloperoxidase, adhesion molecules sICAM-1, sVCAM-1, soluble intercellular adhesion molecule; C-reactive protein (CRP), acute phase reactants Fibrinogen, soluble vascular, white blood cells, erythrocyte sedimentation rate, adiponectin, pregnancy associated plasma protein A, lipoprotein associated phospholipase A2, placental growth factor, Cystatin C and heat shock proteins.[5]

Heat shock proteins (Hsps) -also called molecular chaperones- a heterogeneous group of molecules with a variety of functions. They are considered as stress proteins as many of which related to the response to stress and to protein folding. A division of Hsps typically involved in supporting the correct folding of nascent polypeptides and in the refolding of partially denatured proteins resulting from cellular stress.[6,7,8] Hsps can be classified into six families, the small Hsps (sHsp), Hsp 40, 60, 70, 90 and Hsp110, based on their molecular mass.[9]

Hsp 60 can be found in the chloroplast of plants and mitochondrion and cytosol of mammalian species.[10] It is also found in the cytosol independently of mitochondrial release. Both, mitochondrial and cytosolic forms of Hsp 60 can function in pro-survival or pro-apoptotic pathways, depending on the cellular situation.[11] Some studies suggest that Hsps are also associated with atherosclerosis initiation and progression.[11,12,13] In vivo data support the involvement of human Hsp60 strongly in the pathogenesis of coronary artery disease.[14,15,16,17]

The present study was designed to study the circulating level of Hsp 60 in type 2 Diabetic Egyptian patients with one or more risk factors to cardiovascular complications, and comparing the level of Hsp 60 in them with diabetic patients without any risk to cardiovascular disease in a trail to explore the role of serum Hsp 60 levels in the prediction of the progression of diabetic patients into cardiovascular complications. In addition, study the correlations between serum Hsp 60 levels and body mass index (BMI), age, gender and metabolic parameters.

MATERIALS AND METHODS

The study comprised 80 Egyptian subjects (39 males and 41 females), recruited from the clinical pathology department at National institute for Diabetes and Endocrinology (NIDE). All the subjects underwent careful physical examination, detailed history, and laboratory investigations before inclusion in this study to exclude any condition that may interfere with the studied parameters. Patients were divided into two main groups: 18 diabetic control group (D) with no evidence of microalbuminuria or any cardiovascular complications and 62 diabetic patients with one or more risk factors for CVD which is further divided into: 8 diabetic with microalbuminuria (M), 9 hypertensive diabetic group (H), 12 Diabetic with dyslipidemia (L), 9 diabetic with hypertention and dyslipidemia (H+L), 8 diabetic with microalbuminuria and dyslipidemia (M+L), 9 diabetic with microalbuminuria and hypertention (M+H) and 7 diabetic with microalbuminuria, dyslipidemia and hypertention (M+H+L). Definition and selection of type 2 diabetes were done according to American Diabetes Association criteria.[18] All groups were age- and sex-matched. The study was approved by the ethical committee of National Institute of Diabetes and Endocrinology, Cairo, Egypt. The characteristics of the patients were listed in Table 1.

The exclusion criteria were (age below 30 and above 65 years, smokers, urinary tract infection, history of chronic analgesic abuse, chronic glucocorticoids ,certain chronic diseases as colorectal cancer, hyperthyroidism (T3, T4, TSH), Alzheimer disease, medications that may affect the accuracy of the lipid profile test results, including corticosteroids, estrogens or androgens, oral contraceptives, haloperidol and niacin, alcohol, drug abuse, pregnancy and any acute / chronic inflammatory disease). Standing height and body weight were measured in light clothing without shoes.

Blood samples were drawn from all subjects after overnight fasting for the determination of the investigated parameters. Samples handling, storage and preparation was done according to manufacturers' instructions.

Laboratory analyses

Fasting plasma glucose analysed using hexokinase-glucose-6-phosphate dehygrogenase method presented by Kunst et al.,[19] serum triglycerides (TG) and serum total cholesterol (TC) were assayed by enzymatic-colorimetric, end point method[20]. Serum high-density lipoprotein cholesterol (HDL-C) was assayed according to the precipitation, phosphotungestic acid method[21] and serum low-density lipoprotein cholesterol (LDL-C) were determined according to the precipitation, Heparin/Citrate method.[22] Serum creatinine and serum urea levels were assayed using Jaffè enzymatic colorimetric, end point method.[23,24] All serum biochemical parameters were measured using Spectrophotometer 1200 UNICO Instruments.inc.USA. Microalbuminuria was measured in random urine samples taken from the patients by turbidimetric assay[25] and was measured by ADVIA® 1650 clinical chemistry system and was expressed as A/C ratio (mg/g creatinine). Atherogenic ratios (TC / HDL-C and LDL-C / HDL-C) were calculated and BMI was calculated also as weight divided by squared height (in kilograms per square meter).

Table 1. Clinical and laboratory characteristics of the studied groups

Markers	D (n=18)	M (n=8)	H (n=9)	L (n=12)	H+L (n=9)	M+L (n=8)	M+H (n=9)	M+H+L (n=7)
Gender	8F/10M	5F/3M	5F/4M	5F/7M	5F/4M	5F/3M	4F/5M	4F/3M
BMI (kg/m^2)	28.717±0.939	33.22±2.27	34.556±1.486	28.908±1.228	33.122±1.886	30.9±1.664	33.167±2.889	32.057±1.996
Age (years)	47.667± 2.016	48.375±1.614	50.222±1.913	50.222±2.715	48.667±2.703	46.125±1.563	49.333±3.424	49.424±3.637
FBG (mg/dl)	176.44±12.25	212.5± 19.82	229.4±23.65	205.25±21.63	233.1±25.03	259.13±33.83	241±35.26	226.29±37.8
Glycated Hb(%)	8.5±0.513	8.813± 0.693	9.711±0.815	9.067±0.746	9.544±0.67	10±0.694	9.467±1.055	8.9±1.04
A/C ratio	17.056±1.74	73.5±23.38 **a**	14.522±2.229 **b**	13.392±2.092 **b**	16.044±2.336 **b**	105.13±22.4 **a,c,d,e**	46.556±2.887 **g**	92.571±23.4 **a,c,d,e**
Creatinine (mg/dl)	0.9888± 0.0359	1.358± 0.132 **a**	0.9744±0.056 **b**	1.043±0.058	0.9889±0.0658	1.619±0.109 **a,c,d,e**	1.361±0.11 **a,c,e**	1.34± 0.086 **a**
Urea (mgldl)	25.606± 1.758	24.733±1.541	28.238±3.187	24.426±2.245	27.118±1.046	26.595±4.17	27.287±3.055	39.796±3.688 **a,b,d.g**
Total cholesterol (mg/dl)	172.94± 4.39	169.63± 6.25	165±8.16	225.33±5.8 **a,b,c**	225.33±6.02 **a,b,c**	221.88±4.97 **a,b,c,f**	159.78±6.264 **d,e**	243.86± 11.6 **a,b,c,f**
Triglycerides (mg/dl)	166.29±5.12	136.25±10.21	162.22±5.95	220.17±10.74 **a,b**	224.67±10.86 **a,b**	227.5±11.86 **a,b**	178.67±5.55	270.14± 45.4 **a,b,c,f**

F: Female, M: Male, **FBG**: Fasting blood glucose, **BMI**: Body mass index, **D**: Diabetic group, **M**: Diabetic with microalbuminuria group, **H**: Diabetic hypertensive group, **L**: Diabetic dyslipidemic group, **H**+**L**: Diabetic hypertensive & dyslipidemic group, **M**+**L**: Diabetic dyslipidemic with microalbuminuria group, **M**+**H**: Diabetic dyslipidemic with microalbuminuria group, **M**+**H**+**L**: Diabetic hypertensive & dyslipidemic with microalbuminuria group**.** The values are expressed as mean ± SEM. **a:** compared to D group, **b**: compared to M group, **c**: compared to H group, **d**: compared to L group, **f**: compared to M+H group and **g**: compared to M+L group. All results are considered significant at $P<0.05$, using parametric ANOVA test followed by Tukey-Kramer multiple comparisons test.

The A1c % was measured in whole blood with ion-exchange high-performance liquid chromatography fully automated system with Bio-Rad D-10 hemoglobin testing system (Bio-Rad Laboratories, Hercules, CA) intended for the percent determination of A1c% in human whole blood using HPLC technique.[26]

Determination of serum Hsp 60

Serum Hsp 60 levels were measured using commercially available kit: Hsp 60 ELISA kit (AssayPro ®, USA Catalog No. EH5505-1) and was purchased from Mediamedix, Egypt. It is a quantitative sandwich enzyme immunoassay technique in which the color generated was proportional to the amount of Hsp 60 present in the sample. ELISA procedures were done by automated ELISA system (The DiaSorin ETI-Max 3000 system, Italy) according to the manufacturer's instructions.

Statistics

Statistics were done using GraphPad Instat (Graph software Inc., V 3.05, Ralf Stahlman, Purdue Univ.), to test significance of differences between groups. Appropriate graphs were plotted using GraphPad Prism 6 (Graphpad software Inc., V 6.00, USA). Correlation co-efficient was done using least square method. The accuracy indices were calculated according to Reed et al.[27] P value less than 0.05 was considered statistically significant. Spearman's correlation analysis was used to analyze interrelationship between serum Hsp 60 levels and other clinical parameters. SPSS software (Statistical Package for the Social Sciences, version 10, SPSS Inc, Chicago, Ill, USA) was used to make ROC curve.

RESULTS

Clinical data of all subjects are shown in Table1. Concerning age, sex and BMI (kg/m^2) using Tukey-Kramer multiple tests, no significant variation was verified between all the studied groups. Fasting blood glucose level (FBGL) and glycated hemoglobin (A1c%) showed no significant difference between the different studied groups.

Serum TC and serum TG were significantly higher in L, H+L, M+L and M+H+L groups compared to D group ($P<0.05$). Also serum TC and serum TG were significantly higher in L, H+L, M+L and M+H+L groups was significantly higher compared to M group ($P<0.01$).

Serum TC level was significantly higher in L, H+L, M+L and M+H+L groups compared to H group ($P<0.001$) while TG level was elevated significantly in

Table 2. Diagnostic tools for diabetic cardiovascular complications

Markers	D (n=18)	M (n=8)	H (n=9)	L (n=12)	H+L (n=9)	M+L (n=8)	M+H (n=9)	M+H+L (n=7)
HSP 60 (ng/ml)	0.6557± 0.0791	3.8728±0.499 **a**	3.18888±0.47 **a**	2.939±0.5655 **a**	2.2066±0.318 **a**	2.1685±0.595 **a**	3.697±0.585 **a**	1.2257±0.265
TC/HDL (Ratio 1)	3.444±0.215	2.973±0.157	3.244±0.291	11.229±0.543 **a,b,c**	10.478±1.179 **a,b,c**	11.844±0.481 **a,b,c,f**	3.222±0.221 **d,e**	11.241±0.698 **a,b,c,f**
LDL/HDL (Ratio 2)	1.857±0.138	1.49±0.122	1.62±0.251	7.638±0.346 **a,b,c**	7.322±0.992 **a,b,c**	8.363±0.61 **a,b,c,f**	1.471±0.168 **d,e**	7.743±0.598 **a,b,c,f**

F: Female, **M**: Male, **FBG**: Fasting blood glucose, **BMI**: Body mass index**, D**: Diabetic group, **M**: Diabetic with microalbuminuria group, **H**: Diabetic hypertensive group, **L**: Diabetic dyslipidemic group, **H**+**L**: Diabetic hypertensive & dyslipidemic group, **M**+**L**: Diabetic dyslipidemic with microalbuminuria group, **M**+**H**: Diabetic dyslipidemic with microalbuminuria group,**M**+**H**+**L**: Diabetic hypertensive & dyslipidemic with microalbuminuria group, The values are expressed as mean ± SEM. **a:** compared to D group, **b**: compared to M group, **c**:compared to H group, **d**: compared to L group, **f**: compared to M+H group and **g**: compared to M+L group. All results are considered significant at $P<0.05$ using parametric ANOVA test followed by Tukey-Kramer multiple comparisons test.

M+H+L group only compared to H group ($P< 0.001$). Moreover, it was higher in M+H group compared to L group and H+L ($P<0.001$). Finally, TC was significantly higher in M+L and M+H+L groups while TG was significantly higher only in M+H+L group compared to M+H group ($P <0.01$).

Serum HDL-C was significantly lower and serum LDL-C was significantly higher L, H+L, M+L and M+H+L groups compared to D group ($P<0.001$), and in L, H+L, M+L and M+H+L groups compared to M group ($P<0.001$). The same significant pattern occurred in L, H+L, M+L and M+H+L groups compared to H group ($P<0.001$), in M+H group compared to L group ($P<0.001$), in M+H compared to H+L group ($P<0.001$) and finally in M+L and M+H+L groups compared to M+H group ($P<0.001$).

Cardiovascular risk ratios (1&2) both were significantly higher in L, H+L, M+L and M+H+L groups compared to D, M and H groups ($P<0.001$), and significantly higher in M+L and M+H+L groups compared to M+H group ($P<0.001$) but significantly lower in M+H compared to L and H+L groups ($P<0.001$) (Table 2).

Regarding Kidney Function our results showed that M, M+L, M+H and M+H+L groups were significantly higher compared to D group ($P<0.05$, $P<0.001$, $P<0.05$ and $P<0.05$ respectively). M+L and M+H groups were significantly higher compared to H and H+L groups ($P<0.001$and $P<0.05$ respectively), while, M+L group alone was significantly higher compared to L group ($P<0.001$), but H group was significantly lower compared to M group ($P<0.05$). The serum urea level in M+H+L group was significantly higher compared to D, M, L and M+L groups ($P<0.05$). The mean serum value of ACR in M, M+L and M+H+L groups were significantly higher compared to D group ($P<0.01$, $P<0.001$ and $P<0.001$ respectively). M+L and M+H+L groups were significantly higher compared to H, L and H+L groups ($P<0.001$). In contrast H, L and H+L groups were significantly lower compared to M group ($P<0.05$, $P<0.01$and $P<0.05$ respectively) and H group was significantly lower compared to M+L ($P<0.05$).

Hsp 60 showed significant increases in M, H, L, H+L, M+L and M+H groups compared to D group ($P<0.01$, $P<0.05$, $P<0.01$, $P<0.01$, $P<0.05$, $P<0.05$ and $P<0.05$ respectively). Serum level of Hsp 60 showed no significant difference between males and females of the same group or between different studied groups (Table 2 and Figure 1).

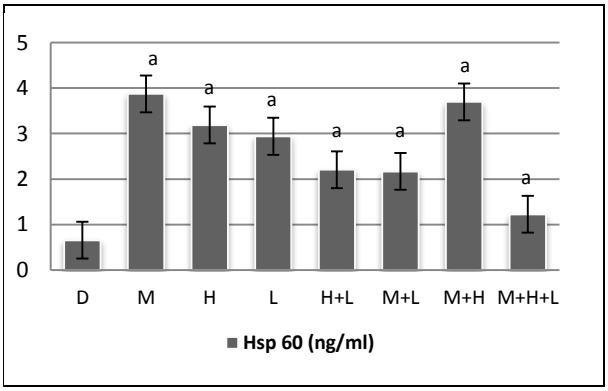

Figure 1. Hsp 60 (ng/ml) of D: Diabetic group, M: Diabetic with microalbuminuria group, H: Diabetic hypertensive group, L: Diabetic dyslipidemic group, H+L: Diabetic hypertensive & dyslipidemic group, M+L: Diabetic dyslipidemic with microalbuminuria group, M+H: Diabetic dyslipidemic with microalbuminuria group, M+H+L: Diabetic hypertensive & dyslipidemic with microalbuminuria group. The values are expressed as mean ± SEM. **a:** compared to D group. Results are considered significant at $P<0.05$ using parametric ANOVA test followed by Tukey-Kramer multiple comparisons test)

Simple linear regression analysis using Hsp 60 as dependent variable showed that, Hsp 60 showed a

statistically significant direct correlation with BMI (Figure 2) and ACR (Figure 3).

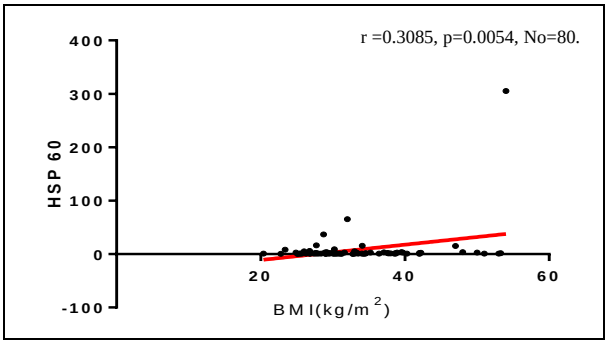

Figure 2. Linear regression between Hsp 60 (ng/dl) and BMI (kg/m^2).

The diagnostic accuracy, sensitivity and specificity of Hsp 60, Ratio 1 and Ratio 2 were 93.6%, 64.5% and 65.5% respectively, and 53.3%, 76.4% and 72.2% respectively. Receiver operating characteristics (ROC) analysis for investigated parameters where quantitatively, area under the curve "AUC" is an overall measurement of accuracy, Hsp 60 Showed the biggest AUC ± SEM (0.716 ± 0.08) compared to other traditional markers (ratio 1& ratio 2) (Figure 4).

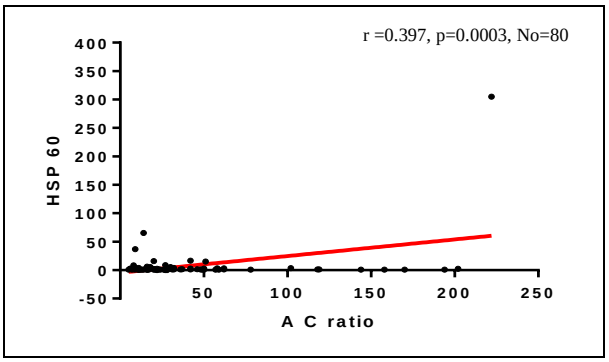

Figure 3. Linear regression between Hsp 60 (ng/dl) and ACR (mg/g).

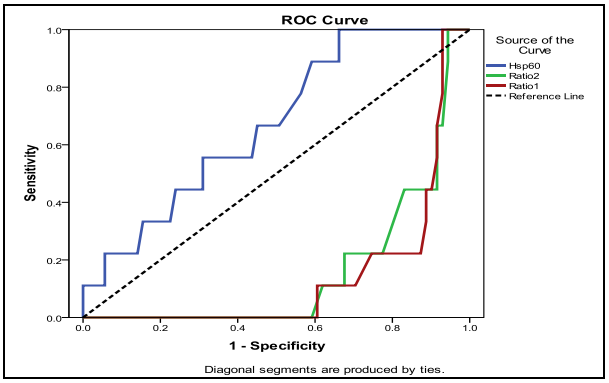

Figure 4. ROC curve of diabetic cardiovascular markers.

DISCUSSION

One of the complications arouse from the diabetes is cardiovascular macrovascular complication. Individuals with type 2 diabetes are at higher risk of CVD than those without type 2 diabetes. Individuals with the metabolic syndrome have a 61% higher risk of cardiovascular disease.[28] In diabetic patients the abnormalities of cardiovascular systems are common, and the consequences of cardiac disease in diabetes are overwhelming.[29] In addition, diabetic patients often have evidence of a cardiomyopathy that may occur even without apparent microvascular or macrovascular diseases.[30] The mechanisms underlying the development of diabetic cardiomyopathy are not fully understood although there are number of biochemical and physiological changes had been described in it.

Hsp 60, a mitochondrial protein which is critical for proper folding of key metabolic proteins and it is also found in the cytosol.[31] Serum level of Hsp 60 was reported to be significantly higher in diabetes[16], and associated with the cell membrane in stress conditions, localized in the surface of cardiac myocytes in heart failure and correlated with myocyte apoptosis.[30,31]

This work was designated to investigate the circulating level of Hsp 60 in Egyptian patients with T2DM with and without different risk factors to CVD, correlate that with some anthropometric and metabolic parameters and illuminate the potential value of Hsp 60 in the development of cardiovascular complications of diabetes.

Regarding lipid profile in this study, TC level showed significant increase in all diabetic groups with risk factors to CVD compared to diabetic group, and the same figure appear with the level of TG. These results came in agreement with many reports who demonstrated that the most commonly known lipid abnormality in diabetics is hypertriglyceridaemia, and not an increase in total cholesterol.[32,33] However, in the Lipid Research Clinics Programme it was reported that elevation in the total cholesterol level (an independent cardiovascular risk factor) has been reported in certain populations of patients.[34] The results of this study are also consistent with the findings reported by previous workers[35,36], but disagree with others[32] who reported hyper-triglyceridaemia as the main lipid abnormality in their diabetic patients.

The serum level of LDL-C was significantly increased in diabetic patients with dyslipedima, hypertension & dyslipidemia, microalbuminuria &dyslipidemia and combined microalbuminuria, hypertension &dyslipidemia at the same time serum level of HDL-C was significantly decreased in the same patient groups compared to diabetic group and as a consequence both cardiovascular risk ratios (1&2) in our study were significantly higher in dyslipedima, hypertension & dyslipidemia, microalbuminuria

&dyslipidemia and combined microalbuminuria, hypertention & dyslipidemia groups compared to diabetic group. These results came in accordance with scientific researches which demonstrated that diabetic dyslipidemia, the main lipid disorder in diabetic patients, characterized by increased serum triglycerides, numbers of small, dense LDL particles and decreased levels of HDL –C.[37,38]

Studying the serum level of Hsp 60 in the present study, showed that serum Hsp 60 level was significantly higher in microalbuminuria, hypertension, dyslipidemia, hypertension & dyslipidemia, micro-albuminuria & dyslipidemia and microalbuminuria & hypertension groups compared to diabetic group and this came in agreement with the results of Shamaei-Tousi et al who stated that there was a significantly higher amounts of a circulating levels of Hsp 60 in patients with CVD compared to those without CVD.[9] Increase serum level of Hsp 60 in hypertensive group came in accordance to the Framingham Study which showed that nearly one quarter of heart failure cases suffer from hypertension[39], and that hypertension may contribute to the development of heart failure in about 50-60% of patients. In hypertensive women, the risk of heart failure is increased by 3-fold compared to 2-fold in men. Holman et al., demonstrated that controlling blood pressure in patients with both T2DM and hypertension is important in preventing cardiovascular complications in these patients.[40]

In the present study the significant increase of serum Hsp 60 in diabetic patients showing microalbuminuria came in agreement with results of Karalliedde and Viberti who reported that microalbuminuria is a marker for generalized vascular dysfunction and increased risk for cardiovascular morbidity and mortality. Microalbuminuria has an independent relationship to renal and cardiovascular outcomes although it interacts with the traditional cardiovascular risk factors. It doubles the risk for a cardiovascular event in type 2 diabetic patients. And although increased rates of urinary albumin excretion predict target organ damage, particularly renal disease, it is also related to left ventricular dysfunction, stroke, and myocardial infarction.[41]

The results of Hsp 60 in dyslipidemic group are in agreement with Nesto-Richard who reported that there is in diabetic patients the relation between serum levels of HDL-C and triglycerides is reversed and the low serum HDL-C levels may be representing an independent risk factor for cardiovascular disease.[42]

Correlation study between Hsp 60 and other parameters studied in this work showed that there was no correlation found between Hsp 60 and any of them, a result which may suggest that Hsp 60 may act as independent risk factor for the early diagnosis of the development of CVD complications.

The above findings are consistent with ours and support what was observed in our study regarding the elevated levels of Hsp 60 in patients' serum suffering from hypertension, dyslipidemia or microalbuminuria as a stressful risk factors for CVD.

CONCLUSION

The increased serum level of Hsp 60 level in diabetic patients with one or more risk factors to CVD suggested the clinical significance of Hsp 60 as an early marker in the diagnosis of developing CVD complications in Egyptian patients with type 2 diabetes. We recommend further standardization in large-scale population in order to establish clear and definite sharp normal values of Hsp 60 that can be practically applicable in Egypt.

Conflict of Interest

The authors declare that they don't have any conflict of interest.

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