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Serum heat shock protein 27 as a potential marker of diabetic nephropathy and its association with traditional risk factors in Egyptian patients with type 2 diabetes

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Heat shock protein 27 (HSP27) has been involved in different kidney diseases playing protective as well as counter-protective roles. Stressful conditions that include oxidative stress as diabetes and chronic kidney disease promote cells to over express HSP27. In the present study, investigation of serum Hsp27 levels in type 2 diabetic Egyptians with traditional risk factors for diabetic nephropathy (DN), a microvascular complication in type 2 diabetes mellitus, as well as in diabetics with evident diabetic nephropathy was performed. Our study included three diabetic groups: diabetic control subjects (DC), diabetic group with one or more of the traditional risk factors for diabetic nephropathy (DR): Hypertension and /or dyslipidemia, and evident diabetic nephropathy group (DN), which was further sub-grouped into three DN stages according to the decrease in the estimated glomerular filtration rate. Serum Hsp27 levels were significantly higher in patients with traditional risk factors for DN and with evident DN than in diabetic control subjects ($p < 0.01$). Moreover, serum HSP27 showed increased area under the curve with increasing risk factors for DN. Likewise, serum Hsp27 levels showed higher sensitivity and area under the curve over creatinine and microalbuminuria. In conclusion, our results showed that serum HSP27 may be used as an early marker for diagnosis of diabetic nephropathy as serum Hsp27 levels appear to be associated with the risk for DN as well as with manifest DN.

Keywords: Diabetic nephropathy, Heat shock proteins, Hsp27, Type 2 Diabetes mellitus, Risk factors.

INTRODUCTION

Heat and oxidative stress are environmental stressors that subsequently lead cells to over-express a group of highly conserved proteins known as the heat shock proteins (Hsps). HSPs may also have other vital roles in addition to their function as molecular chaperones, facilitating the correct folding of nascent peptides and the refolding of denatured or mis-folded proteins (Georgopoulos and Welch, 1993). Furthermore, impaired expression of HSPs by insulin sensitive tissues is associated with insulin resistance and type 2 diabetes mellitus (T2DM) leaving those tissues susceptible to oxidative stress induced damage (Hooper and Hooper, 2009).

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Type 2 diabetes mellitus has quickly become a global health problem due to rapidly increasing population growth, aging, urbanization and increasing prevalence of obesity and physical inactivity. There is, therefore, an urgent need to prevent diabetes and its complications (Dabla, 2010). The prevalence of insulin resistance, a consistent feature of metabolic syndrome (Zimmet *et al.*, 2005) and obesity (Kahn and Flier, 2000), is increasing worldwide. The action of reactive oxygen species within the cell nucleus is one of the molecular mechanisms of hyperglycemia induced effects on inflammation and vascular complications (Wright *et al.*, 2006). It has been proposed that oxidative stress is one pathogenic factor underlying the onset and progression of insulin resistance

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and diabetes and consequently on vascular complications (Brownlee, 2005; Ceriello and Motz, 2004; Evans *et al.*, 2003).

Hsp27 is a member of the small HSP family that is over-expressed when cells are exposed to oxidative stress (Mehlen *et al.*, 1995). Several mechanisms were proposed to show how Hsp27 enables cells to adapt to exposure to oxidative stress including the up-regulation of glucose-6-phosphate dehydrogenase and glutathione peroxidase and by decrease intracellular iron levels (Preville *et al.*, 1999; Arrigo *et al.*, 2005).

In vitro studies have shown that Hsps are released from cells after exposure to stress (Child *et al.*, 1995; Liao *et al.*, 2000), which would explain their presence in serum in vivo and why they may stimulate an autoimmune response (Xu, 2002). Studies have reported that antibody titres to some Hsps, such as Hsp60, are related to circulating antigen concentrations (Xu *et al.*, 2000) and that there are elevated concentrations of auto-antibodies to HSPs in patients with atherosclerosis (Xu *et al.*, 1993), and furthermore, elevated concentrations of serum Hsp60 are associated with higher risk of coronary heart disease (Zhang *et al.*, 2008).

Diabetes is the major cause of end-stage renal disease (ESRD) both in the U.S. and around the world and has enormous medical, social and economic consequences. Diabetes affects the kidney in stages. At the onset of diabetes, the kidney grows large and the glomerular filtration rate (GFR) becomes disturbed (Dabla, 2010). Risk factors as: Poorly controlled glucose levels, blood pressure (BP), as well as cholesterol activate inflammatory mediators, and patients with a genetic predisposition progress to advanced stage nephropathy (Balakumar *et al.*, 2009). Moreover, these patients are at the greatest risk for cardiovascular disease (CVD), morbidity and premature mortality caused by the combination of DM and chronic kidney disease (CKD), thus imposing a large burden on both patients and health care costs (Gaede *et al.*, 2008; Keith *et al.*, 2004).

Barutta *et al.* showed that in early experimental diabetes, diabetes-induced cell damage can be counterbalanced through HSP over-expression in the outer medulla resulting in cytoprotection. By contrast, lack of HSP induction in the diabetic glomeruli may subject glomerular cells to injury (Barutta *et al.*, 2008). Increased cell death or damage can occur during progression of CKD as indicated by the elevated serum levels of cell death markers (Ankersmit *et al.*, 2001; Roth *et al.*, 2011), which could also lead to a release of HSP into extracellular space (Musial and Zwolinska, 2011).

The aim of the present study was to assess the association between serum HSP27 and the traditional risk factors for DN in order to identify additional diagnostic tool that would allow the early identification of diabetic patients at risk for developing diabetic nephropathy.

MATERIAL AND METHODS

Subjects

72 patients with T2DM attending the out clinic of National Institute of Diabetes and Endocrinology, Kasr EL Einy, Cairo, Egypt, were involved in a cross-sectional survey.

The inclusion criteria were age 30–60 years old. Exclusion criteria included smokers and patients suffering from urinary tract infection, acute inter-current infection, a known history of other chronic diseases such as cancer, hyperthyroidism, Alzheimer disease, chronic analgesic abuse, and chronic gluco-corticoids treatment.

Diabetic patients were divided into three groups: diabetic control group (DC) which included 14 patients suffering from T2DM only without any traditional risk factor for DN (male/female ratio, 8/6; age, 47.00 ± 2.28 years); diabetic risk group (DR) of 28 diabetic patients who had one or more of the known risk factors for Diabetic nephropathy (male / female ratio, 13/15; age, 48.36 ± 1.66 years) and diabetic nephropathy group (DN) of 30 diabetic patients (male to female ratio, 15 /15; age, 49.93 ± 1.77 years).

DR group patients were selected by having one or more risk factor for DN but without overt microalbuminuria and this group was subdivided into: diabetic hypertensive (H) group which included 9 patients with Blood Pressure (BP) >140/90, diabetic dyslipidemia (L) group which included 11 patients and combined hypertensive and dyslipidemia (H+L) group which included 8 patients.

DN group included diabetic patients with microalbuminuria < 300 mg/dl but no evidence of dialysis or kidney transplantation. According to the estimated glomerular filtration rate (eGFR); DN group was further subdivided into: diabetic nephropathy stage 1 –DN1- normal GFR with microalbuminuria (6 patients), diabetic nephropathy stage 2 -DN2- GFR of 60-89 ml/min with microalbuminuria (9 patients) and diabetic nephropathy stage 3 –DN3- GFR of 30-59 ml/min with microalbuminuria (15 patients).

The staging of diabetic kidney disease (DKD) was evaluated by rate of decline of estimated glomerular filtration rate (eGFR) as calculated by The Cockcroft-Gault (C-G) equation for creatinine clearance, described in 1976 (Cockcroft and Gault, 1976). In this formula, CrCl approximates GFR, if the serum creatinine is stable, taking into account age-related decline in body weight and sex.

$$\text{CrCl (mL/min)} = \frac{(140 - \text{age (years)}) \times \text{weight (in kg)}}{72 \times \text{Scr level (mg/dL)}}$$

if female, this result is multiplied by 0.85

The C-G equation is easiest to use and approximates eGFR from CrCl, whereas the other equations use computer-assisted calculations to derive eGFR (Dharmarajan *et al.*, 2012).

All patients provided written informed consent to participate in this study. The study protocol was approved by the ethics review board of National Institute of Diabetes and Endocrinology.

Laboratory data

Blood samples were collected after 12 hours of fasting, centrifuged, and kept at -50°C until analysis. Fasting blood sugar (FBS) measurement was carried out using the hexokinase method using a Bayer Advia 1650 analyzer, while glycated haemoglobin, HbA1c, was analyzed by ion-exchange HPLC technique using Bio-Rad D-10 Hemoglobin testing system. Cholesterol, HDL-C, LDL-C, and triglycerides were determined using direct enzymatic methods (Greiner Diagnostic GmbH, Germany). Kidney function tests: serum creatinine and urea were measured for all participants.

Random urine spot sample was collected from each patient for measurement of microalbuminuria (turbidimetric assays) and urine creatinine using ADVIA[®] 1650 clinical chemistry system, Siemens, Germany to calculate the albumin to creatinine ratio (ACR).

Determination of serum Hsp27

Serum HSP27 levels were measured using The AssayMax Human Hsp27 ELISA kit (Assaypro, USA, Catalog No. EH5001-1 and purchased from Indomedix, Egypt) according to the manufacturer's directions. Briefly, a monoclonal antibody specific for HSP27 was pre-coated onto 96-well microtitre plate. Samples and standards were incubated along with a polyclonal HSP27 antibody in the microtiter plate. After incubation and a wash step, a horseradish peroxidase enzyme/IgG antibody conjugate was added. After another incubation and wash to remove unbound substances, an enzyme substrate was added and color was generated that was proportional to the amount of HSP27 present in the sample.

Statistical analysis

Statistical analyses were performed using Graph pad prism 5. All results are expressed as the mean \pm standard error of mean. To analyze two sets of data, parametric unpaired t-student test followed by Welch's correction was used.

Analysis of variance with non-parametric Mann-Whitney-test was used for comparisons among groups. P values less than 0.05 were considered significant for all tests. Spearman's correlation analysis was used to analyze interrelationship between serum HSP27 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

Tables 1 and 2 show the clinical characteristics of all the participants groups and subgroups, respectively. Age, body mass index (BMI), FBG, A1C and serum urea levels showed no significant differences among groups. Similarly, no significant differences were noted considering age, FBG, A1C and serum urea levels among the different subgroups of Risk and DN patients. BMI was only significantly higher in hypertensive diabetic patients (H) than diabetic control (DC) and dyslipidemia (L) ones ($p < 0.05$), while there was no significant difference in BMI among DN subgroups (Table 2).

With regard to lipid profile, serum TC, TG levels and TG/HDL were significantly higher, while HDL level was significantly lower in diabetic risk (DR) than DC ($p < 0.01$). LDL and LDL/HDL were significantly higher in DR than DC ($p < 0.05$). TC and TG levels were significantly higher in dyslipidemia diabetic patients than DC ($p < 0.001$ and 0.01 , respectively) and hypertensive ones ($p < 0.001$ and 0.05 , respectively). At the same time TC and TG levels were significantly higher in combined hypertensive and dyslipidemia (H+L) than diabetic control patients ($p < 0.01$). There was no significant difference among DN subgroups with respect to TC and TG. Table 2 shows that LDL/HDL was significantly higher in dyslipidemia and H+L patients than diabetic control ($p < 0.001$ and $p < 0.01$, respectively) and Hypertensive individuals ($p < 0.01$ and $p < 0.05$), respectively. LDL/HDL was significantly higher in DN3 than DN1 ($p < 0.01$). Also, TG/HDL was significantly higher in dyslipidemia and H+L patients than diabetic control ($p < 0.001$, 0.01) and Hypertensive ones ($p < 0.01$ and $p < 0.01$, respectively) but there was no significant difference in DN subgroups considering TG/HDL.

Considering kidney function tests, Table 1 shows that microalbuminuria was significantly higher in DN patients than DC ($p < 0.01$) and DR cases ($p < 0.001$). Similarly, ACR was significantly higher in DN group than DC and DR groups ($p < 0.001$). Serum creatinine was significantly higher in DN patients than DC ($p < 0.05$) and Risk ones ($p < 0.01$). eGFR was significantly lower in patients with DN than Diabetic control ($p < 0.01$) and Risk individuals ($p < 0.001$). Subjects of DN2 and DN3 subgroups, differed significantly from DC group for microalbuminuria ($p < 0.05$ and $p < 0.01$) and ACR ($p < 0.001$ for both subgroups), while there were no significant differences considering microalbuminuria and ACR among individuals of risk group. Serum creatinine remained insignificant among risk subgroups, while it was significantly higher in DN3 individuals compared to diabetic control ($p < 0.001$) and DN1 ones ($p < 0.001$), furthermore DN2 patients differed significantly from DN1 for serum creatinine ($p < 0.05$).

Table 1. Demographic, clinical and biochemical characteristics of study subjects groups.

Parameters	Diabetic control group (DC)	Diabetic risk group (DR)	Diabetic nephropathy group (DN)
n	14	28	30
Gender (female %)	43%	54%	50%
Age (years)	47.00 ± 2.28	48.36 ± 1.66	49.93 ± 1.77
BMI (Kg/m ²)	28.42 ± 0.99	31.90 ± 0.98	32.19 ± 1.17
FBG (mg %)	189.92 ± 14.29	210.43 ± 12.37	224.20 ± 15.71
A1C %	9.02 ± 0.67	9.22 ± 0.45	9.07 ± 0.43
Lipid profile (mg %)			
TC	174.29 ± 4.82	206.04 ± 6.56**	190.60 ± 6.91
TG	161.14 ± 37.48	207.75 ± 41.33**	192.47 ± 57.20
HDL	51.07 ± 2.92	32.75 ± 3.42**	41.63 ± 3.48
LDL	91.00 ± 4.59	128.54 ± 8.33*	109.27 ± 8.51
LDL/HDL	1.88 ± 0.17	5.58 ± 0.63*	3.92 ± 0.60
TG/HDL	3.42 ± 0.38	8.46 ± 0.85**	6.16 ± 0.73
Microalbuminuria (mg/dl)	22.79 ± 2.43##	21.29 ± 2.76###	87.57 ± 14.88
ACR (mg/g cr.)	18.93 ± 1.85###	14.49 ± 1.39###	78.90 ± 10.62
Serum creatinine (mg %)	1.03 ± 0.04#	1.03 ± 0.04##	1.35 ± 0.08
Urea (mg %)	23.35 ± 1.71	27.76 ± 1.32	29.20 ± 1.88
eGFR (ml/min)	98.03 ± 1.97##	99.09 ± 1.96###	83.19 ± 8.21

Values are presented as mean ± SEM

#, ##, ###: significant from DN group at p < 0.05, 0.01 and 0.001

*, **: significant from Diabetic control at p < 0.05, 0.01. Using Kruskal-Wallis Test (Nonparametric ANOVA) followed by Dunn's Multiple Comparisons Test.

Table 2. Demographic, clinical and biochemical characteristics of study subjects sub groups.

Parameters	DC group		DR group			DN group		
		Hypertensive	Dyslipidemia	H+L	DN1	DN2	DN3	
n	14	9	11	8	6	9	15	
Age (years)	47.00 ± 2.28	50.22 ± 1.91	44.64 ± 2.87	51.38 ± 3.43	43.00 ± 2.53	49.11 ± 3.11	52.20 ± 2.55	
BMI (Kg/m ²)	28.42 ± 0.99	35.23 ± 4.46 ⁺	28.91 ± 1.33	31.99 ± 1.71	37.07 ± 4.67	32.36 ± 1.26	30.15 ± 1.00	
FBG (mg%)	189.92 ± 14.29	210.43 ± 12.37	224.20 ± 15.71	225.89 ± 23.94	196.17 ± 32.72	270.22 ± 37.10	207.80 ± 16.28	
A1C %	9.02 ± 0.67	9.51 ± 0.85	8.92 ± 0.80	9.31 ± 0.71	9.51 ± 0.85	8.92 ± 0.80	9.31 ± 0.71	
Lipid profile (mg %)								
TC	174.29 ± 4.82	166.11 ± 8.43 ⁺⁺⁺	231.25 ± 24.23 ^{***}	226.73 ± 6.02 ^{**}	160.67 ± 14.66	190.00 ± 8.34	202.93 ± 10.32	
TG	161.14 ± 37.48	167.89 ± 8.62 ⁺	224.00 ± 10.99 [*]	230.25 ± 10.56 ^{**}	192.33 ± 43.28	190.11 ± 10.58	193.93 ± 12.06	
HDL	51.07 ± 2.92	53.22 ± 3.83 ^{+++oo}	20.55 ± 1.06 ^{***}	26.50 ± 5.91 ^{**}	54.83 ± 4.64	44.44 ± 7.71	34.67 ± 4.24	
LDL	91.00 ± 4.59	79.44 ± 10.84 ⁺⁺⁺	154.27 ± 6.68 ^{***}	148.38 ± 11.12 ^{**}	67.50 ± 7.33 ^{††}	107.67 ± 14.39	126.93 ± 12.21	
LDL/HDL	1.88 ± 0.17 ^{+++oo}	1.62 ± 0.26 ^{+++oo}	7.65 ± 0.38	7.19 ± 1.12	1.29 ± 0.19 [†]	3.88 ± 1.14	5.00 ± 0.88	
TG/HDL	3.42 ± 0.38 ^{+++oo}	3.27 ± 0.27 ^{+++oo}	11.25 ± 0.90	10.46 ± 1.29	2.96 ± 0.19	6.06 ± 1.39	7.62 ± 1.10	
Micro-albuminuria (mg/dl)	22.79 ± 2.43	21.56 ± 6.14	22.73 ± 4.46	19.00 ± 3.76	45.00 ± 14.60	92.00 ± 19.49 [*]	101.93 ± 26.26 ^{**}	

Table 2 Cont.

ACR (mg/g cr.)	18.93± 1.85	14.63± 2.27	13.15± 2.28	16.18 ±2.65	41.50 ± 4.22	93.56 ± 26.58 ^{***}	85.07 ^{***} ±13.06
Serum creatinine (mg%)	1.03 ± 0.04 ^{†††}	1.02± 0.06	1.07 ± 0.06	0.99 ± 0.08	0.74 ± 0.03 ^{†††}	1.31 ±0.09 [^]	1.61 ± 0.07
Urea (mg %)	23.35± 1.71	29.95± 2.70	26.12 ± 2.44	27.53± 1.09	27.16 ± 4.43	30.79 ± 2.58	29.07 ± 3.06
eGFR (ml/min)	98.03 ± 1.97 ^{†††}	102.11± 2.96	97.16 ± 2.99	98.33 ± 4.53	163.02 ± 13.22 ^{†††}	81.13± 3.08 ^{^A}	52.50 ± 1.30

Values are presented as mean ± SEM

*, **, ***: significant from control at p<0.05, 0.01 and 0.001 respectively

+, ++, +++: significant from Dyslipidemia gp at p<0.05, 0.01 and 0.001, respectively

o, oo: significant from H+L gp. at p< 0.05 and 0.01, respectively

^, ^ ^: significant from DN1 at p< 0.05 and 0.01, respectively

†, ††, †††: significant from DN3 at p< 0.05, 0.01 and 0.001, respectively. Using Kruskal-Wallis Test (Nonparametric ANOVA) followed by Dunn's Multiple Comparisons Test.

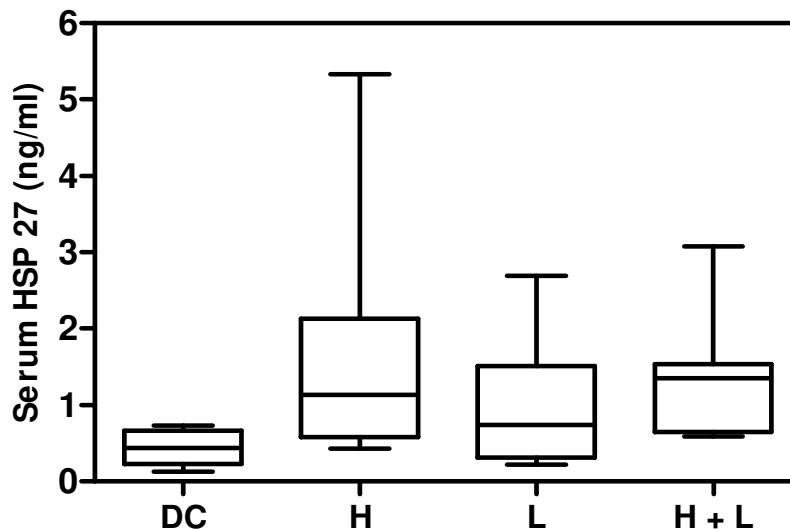


Figure 2. Box plot demonstrating the higher serum level of HSP27 (ng/ml) in Hypertensive (H) (p<0.05) and combined hypertensive & dyslipidemia (H+L) groups (p<0.05) vs. diabetic control (DC) Using Kruskal-Wallis Test (Nonparametric ANOVA) followed by Dunn's Multiple Comparisons Test.

There was no significant difference for mean eGFR between the subgroups of risk group. In contrast, eGFR was significantly different between DN3 and subjects of diabetic control (p<0.001) and DN1 (p<0.001), respectively as well as, between DN2 and DN1 subjects (p<0.01) (Table 2).

Increased serum Levels of Hsp27 in DR and DN Patients

Serum HSP27 levels in diabetic patients with risk for diabetic nephropathy were higher than in diabetic controls [1.30 ± 0.20] vs. [0.45 ± 0.06] ng/ml; p<0.01).

Likewise, HSP27 levels were higher in patients with DN than diabetic control ones [1.11 ± 0.14] vs. [0.45 ± 0.06] ng/ml; p<0.01; Figure 1), however, there was no significant difference in serum HSP27 levels between risk and DN groups.

Serum HSP27 levels were significantly higher in diabetic patients with hypertension and H+L than in diabetic controls [1.64 ± 0.51] and [1.34 ± 0.28] vs. [0.45 ± 0.06] ng/ml; p<0.05; Figure 2). Likewise, HSP27 serum levels were higher in patients with DN1 [0.90 ± 0.20] ng/ml than diabetic control subjects. However, the significant increase in HSP27 levels were in DN2 and DN3 compared to Diabetic control ones [1.31 ± 0.33] and [1.08 ± 0.19] vs. [0.45 ± 0.06] ng/ml; p<0.05; Figure 3).

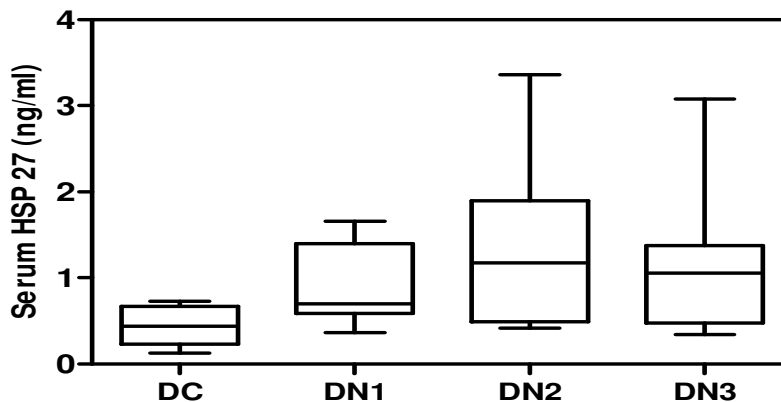


Figure 3. Box plot demonstrating the higher serum level of HSP27 (ng/ml) in diabetic nephropathy stage 2 (DN2) ($p < 0.05$) and stage 3 (DN3) groups ($p < 0.05$) vs. diabetic control (DC) Using Kruskal-Wallis Test (Nonparametric ANOVA) followed by Dunn's Multiple Comparisons Test.

Table 3. Correlation between HSP27 serum concentration and baseline characteristics for all participants

Variable	Spearman correlation coefficient	P-value
Age	-0.102	0.395
BMI	0.022	0.852
FBG	0.122	0.309
A1C	0.102	0.396
TC	0.059	0.622
TG	0.155	0.194
HDL-C	-0.041	0.735
LDL-C	0.002	0.989
LDL/HDL	0.026	0.830
TG/HDL	0.134	0.261
Microalbuminuria	-0.019	0.877
ACR	0.182	0.127
Serum creatinine	0.048	0.691
Urea	0.059	0.621
GFR	-0.143	0.231
Diabetes duration	-0.189	0.112

Effect of Gender on serum HSP27 levels

Regarding sex differences, it was found that serum HSP27 level showed no significant differences in females vs. males of the same group, in DC (0.49 ± 0.1 vs 0.42 ± 0.07), DR (1.28 ± 0.36 vs 1.32 ± 0.16) and DN (1.30 ± 0.24 vs 0.93 ± 0.13) at ($p < 0.05$) using parametric unpaired t-student test followed by Welch's correction.

Effect of Diabetes duration on serum HSP27 levels

Regarding duration of the disease, the mean value of HSP27 level for diabetics newly diagnosed (1.23 ± 0.20

ng/ml, $n=18$) has no statistical significant difference when compared to individuals with duration ≤ 5 years (1.01 ± 0.16 ng/ml, $n=27$) or with duration >5 years (0.94 ± 0.20 ng/ml, $n=27$) at $p < 0.05$ using using Kruskal-Wallis Test (Nonparametric ANOVA).

Correlations of Plasma Hsp27 Levels with Other Variables

Table 3 shows the results of correlation analysis undertaken between serum Hsp27 and other biochemical variables. The correlations between serum levels of Hsp27 with the other biochemical variables in all studied

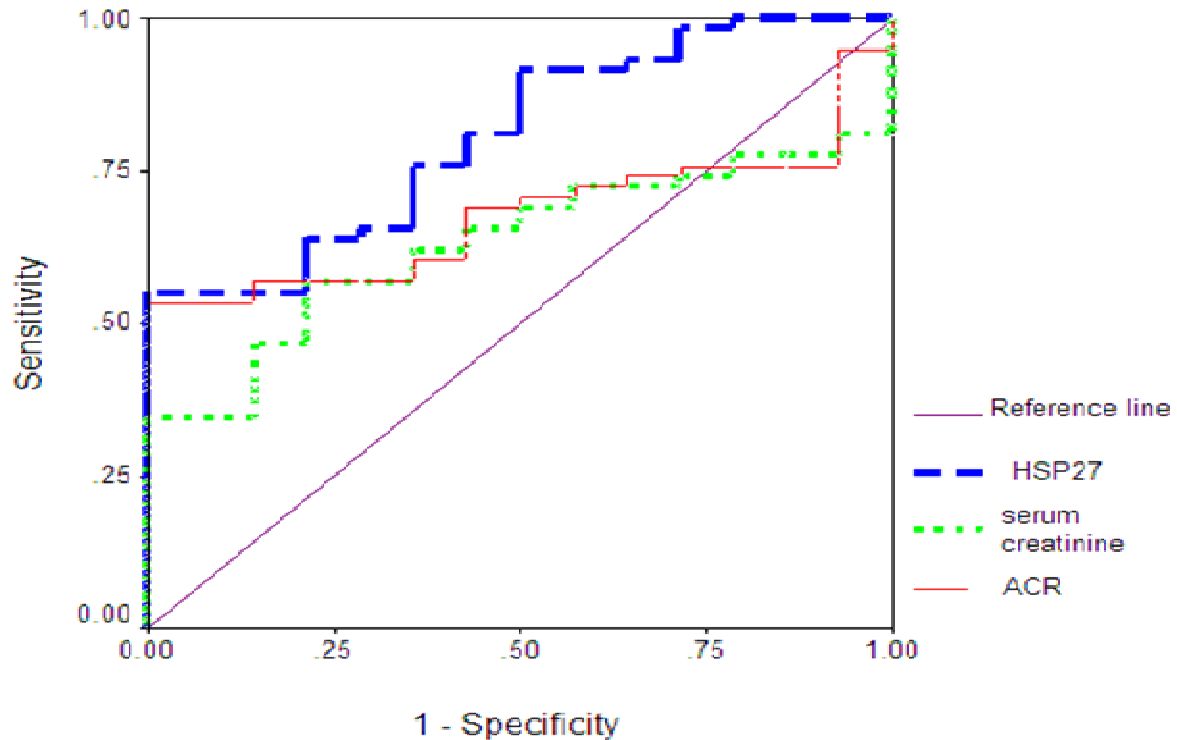


Figure 4. ROC curve for serum stress marker HSP27 compared to traditional markers for nephropathy. HSP27 shows higher AUC than all the other markers.

population were not significant.

ROC curve analysis of HSP27 serum levels

As depicted in Figure 4 ROC curve analysis revealed an area under the curve (AUC) of 0.801 ($p < 0.001$). Furthermore, HSP27 showed an increase in AUC with increasing number of risk factors which could be adducted to predict diabetic patients at higher risk of progression to DN, as ROC curve analysis displays an AUC of 0.869, ($P = 0.03$) for diabetic patients with hypertension only and AUC of 0.911 ($P = 0.002$) for diabetic patients with combined hypertension and dyslipidemia, seen in Figure 5.

Moreover, as seen in Figure 4, serum HSP27 showed higher AUC than that of ACR (0.668) and serum creatinine (0.624) as well as higher sensitivity to the stressful conditions implicated in pathogenesis of DN than ACR and serum creatinine, indicating a possibility of HSP27 serum levels to serve as an early diagnostic marker for DN before it becomes manifest.

DISCUSSION

Diabetic nephropathy, the primary cause of renal failure worldwide, affects approximately one-third of all people

with diabetes. Microalbuminuria is considered the first sign and the best predictor of progression to renal failure and cardiovascular events. However, albuminuria has several limitations. Therefore, earlier, more sensitive and specific biomarkers with greater predictability are needed (Cohen-Bucay and Viswanathan, 2012).

In this study we proposed that Hsp27 concentrations may be associated with the traditional risk for DN as a microvascular complication which involves various stresses in type 2 diabetic patients. Therefore, the association between serum Hsp27 levels and the risk for DN as well as its relation with different stages of DN was investigated.

In sight of the alarming increase in the number of people with DM, a growing number of patients with DKD, ESRD as well as CVD is forecasted (Altemtam *et al.*, 2011). Knowledge of the HSP engagement in CKD is still increasing, although not all aspects of their role in this process are wholly understood. Data showed that the intracellular forms of HSP, mainly Hsp70, delay the progression of CKD through the anti-apoptotic activity and cytoprotection, while the extracellular HSP in CKD show more variability in their action, because they may actively protect against stress conditions as Hsp27 and Hsp70 or act detrimentally like Hsp60 (Musial and Zwolinska, 2011).

In the present study, serum Hsp27 concentrations were found to be significantly higher in individuals with

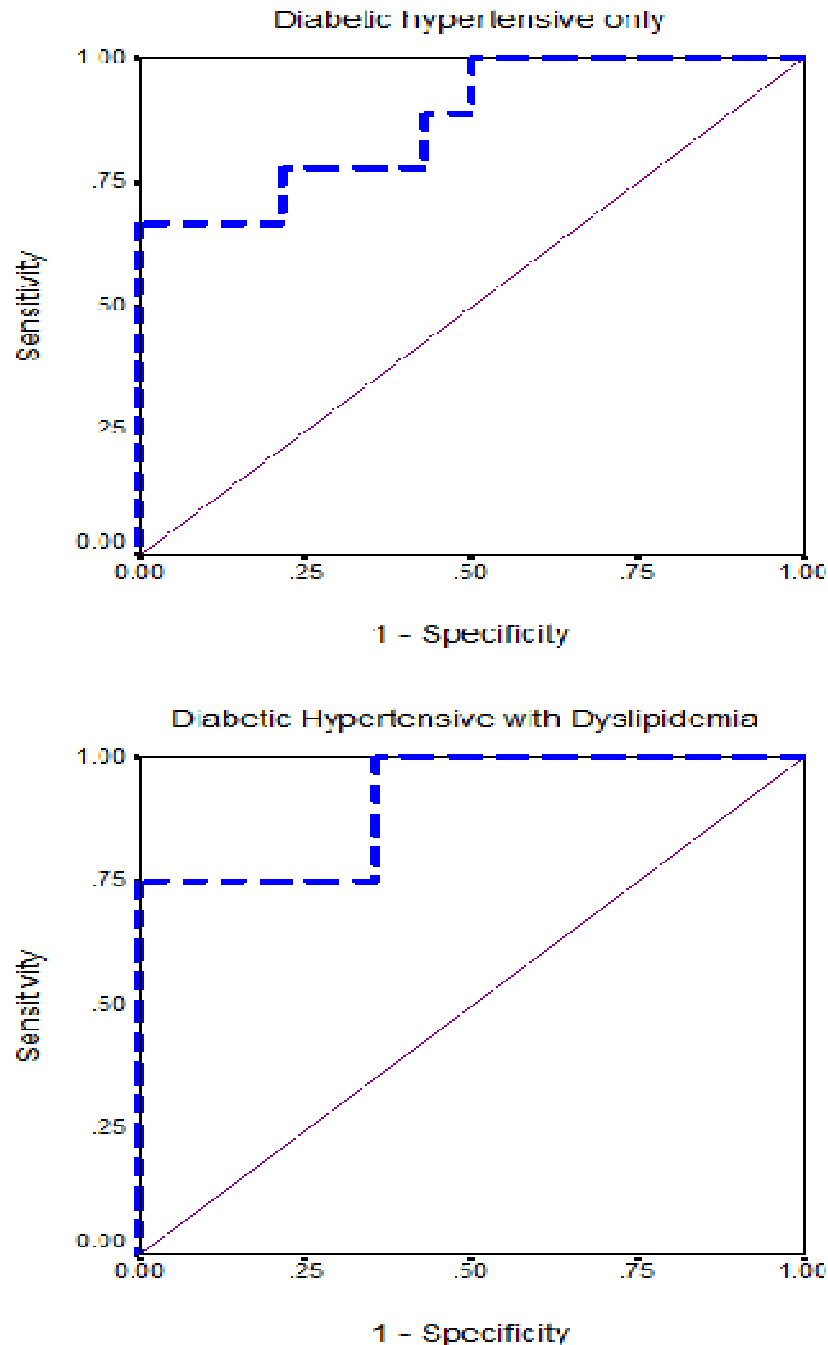


Figure 5. ROC curve for serum stress marker HSP27 in diabetic patients with only hypertension and with combined hypertension and dyslipidemia as traditional risk factors for diabetic nephropathy. HSP27 shows higher AUC in diabetic patients with combined hypertension and dyslipidemia than diabetic patients with hypertension only.

traditional risk for DN compared to diabetic control subjects. Furthermore, investigations of serum HSP27 levels in the subgroups of DR patients showed that individuals with hypertension and with combined hypertension and dyslipidemia showed significant increase in serum HSP27 compared to diabetic control

ones. An effect which was not observed in individuals with dyslipidemia only suggesting that hypertension may be related to an increased cellular expression of Hsp27 leading to the higher serum concentration.

Regarding hypertension, Rossing *et al.* reported that baseline Systolic BP was one of the main predictors of

GFR decline in patients with DKD (Rossing *et al.*, 2004). Moreover, hyperglycemia and glomerular capillary hypertension were reported to be crucial determinants in the pathogenesis of DN by producing cellular stresses on renal target cells which make them potential inducers of a stress response to counterbalance the deleterious effects of these insults (Smoyer *et al.*, 1996). Barutta *et al.* reported that diabetes and diabetes related insults differentially modulate HSP27, HSP60, and HSP70 expression/phosphorylation in the glomeruli and in the medulla which may affect the ability of renal cells to build up an effective cytoprotective response (Barutta *et al.*, 2008).

Furthermore, a recent study by Altemtam *et al.* indicated that hypertension is a strong predictor for the progression of DKD (Altemtam *et al.*, 2011). The above findings are consistent with ours and support what was observed in our study regarding the elevated levels of HSP27 in serum of patients suffering from hypertension as a stressful risk factor for DN.

Additionally, findings of interventional and observational studies suggest that early improvement in BP control in patients with both T2DM and hypertension is important in preventing cardiovascular and renal complications in diabetic patients (Holman *et al.*, 2008).

Increased serum HSP27 in patients with evident diabetic nephropathy compared to diabetic control subjects was also reported in our study. Studies revealed that oxidative stress is increased throughout the decline of kidney function (Shah *et al.*, 2007; Fujii *et al.*, 2011) which is manifested by accumulation of dysfunctional oxidized proteins (Himmelfarb *et al.*, 2000), subsequently promoting inflammation (Iadecola and Alexander, 2001) and apoptosis (Mattson, 2006), thus leads to cardiovascular complications in uremic patients (Himmelfarb *et al.*, 2000; Fujii *et al.*, 2011). Moreover, this pathological condition may be intensified through the loss of extracellular reducing substances by the kidney, which leads to induction the heat shock response (Meyer and Hostetter, 2007). That response is indicated by an increased expression of HSPs at high stress levels in order to maintain cell integrity and minimize cell injury.

HSP27 inhibits apoptosis by blocking caspase activation in addition to different antioxidant capabilities through raising levels of intracellular glutathione in addition to lowering the intracellular iron levels so as to decrease the reactive oxygen species (Arrigo *et al.*, 2005). Similarly, Vidyasagar *et al.* reported that HSP27 up-regulation as a biomarker of some disease states is probably the cell's try to survive by using HSP27 to prevent cell death or to decrease oxidative stress (Vidyasagar *et al.*, 2012).

However, cell damage or necrosis can lead to the release of intracellular HSP and in consequence to elevated HSP serum concentrations (Srivastava, 2002). Due to increased systemic oxidative stress it is not surprising to find elevated serum HSP concentrations in

patients with DN, released from disintegrated cells that alter HSP expression before death of the cells (Lebherz-Eichinger *et al.*, 2011), which agrees with the findings of the our study.

A clinical study concerning HSP27 and diabetic microvascular and /or macrovascular complications was performed. It was a cross-sectional sample of type 1 diabetic patients from the EURODIAB Prospective Complications Study. It was the first study measuring serum HSP27 in a large group of subjects and showed significantly greater age-adjusted HSP27 levels in cases with distal symmetrical polyneuropathy as well as with micro-macroalbuminuria, providing a proof that serum HSP27 levels are independently associated with distal symmetrical polyneuropathy in type 1 diabetic patients (Gruden *et al.*, 2008).

The present study is consistent with the above report concerning increase in serum HSP27 level in microalbuminuria patients; however in our study serum HSP27 levels were not age dependant. Nonetheless, the present research does not show a correlation between serum HSP27 concentration and other clinical and biochemical parameters, it should be taken into consideration that the natural history of CKD in T2DM is heterogeneous and is mainly associated with atherosclerosis (Plutzky *et al.*, 2002). The progression of CKD in T2DM extensively varies between individuals. Moreover, the risk factors that determine the prognosis of the disease have not been fully understood (Altemtam *et al.*, 2011). Confirmation of these findings by other prospective studies is warranted.

In our study the rise in serum HSP27 in diabetic hypertensive patients together with the elevated levels observed in established DN patients may suggest that the kidney cells suffering from hypertension, as a stress factor, increase expression of HSP27 as an adaptive response which helps podocytes to survive this stressor in addition to the other stressors implicated in the pathogenesis and progression of DN itself.

In conclusion, the present study showed increased serum HSP27 levels in DN patients suggesting that HSP27 may be associated with DN. Furthermore, serum HSP27 in diabetic patients with hypertension, a main risk factor implicated in the pathogenesis of DN, was elevated independently from any other common diagnostic marker for renal function. HSP27 is associated with the traditional risk for DN and thus could serve as an early marker for diagnosis of DN before it becomes evident. Further studies using larger numbers of subjects are required to confirm these findings.

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