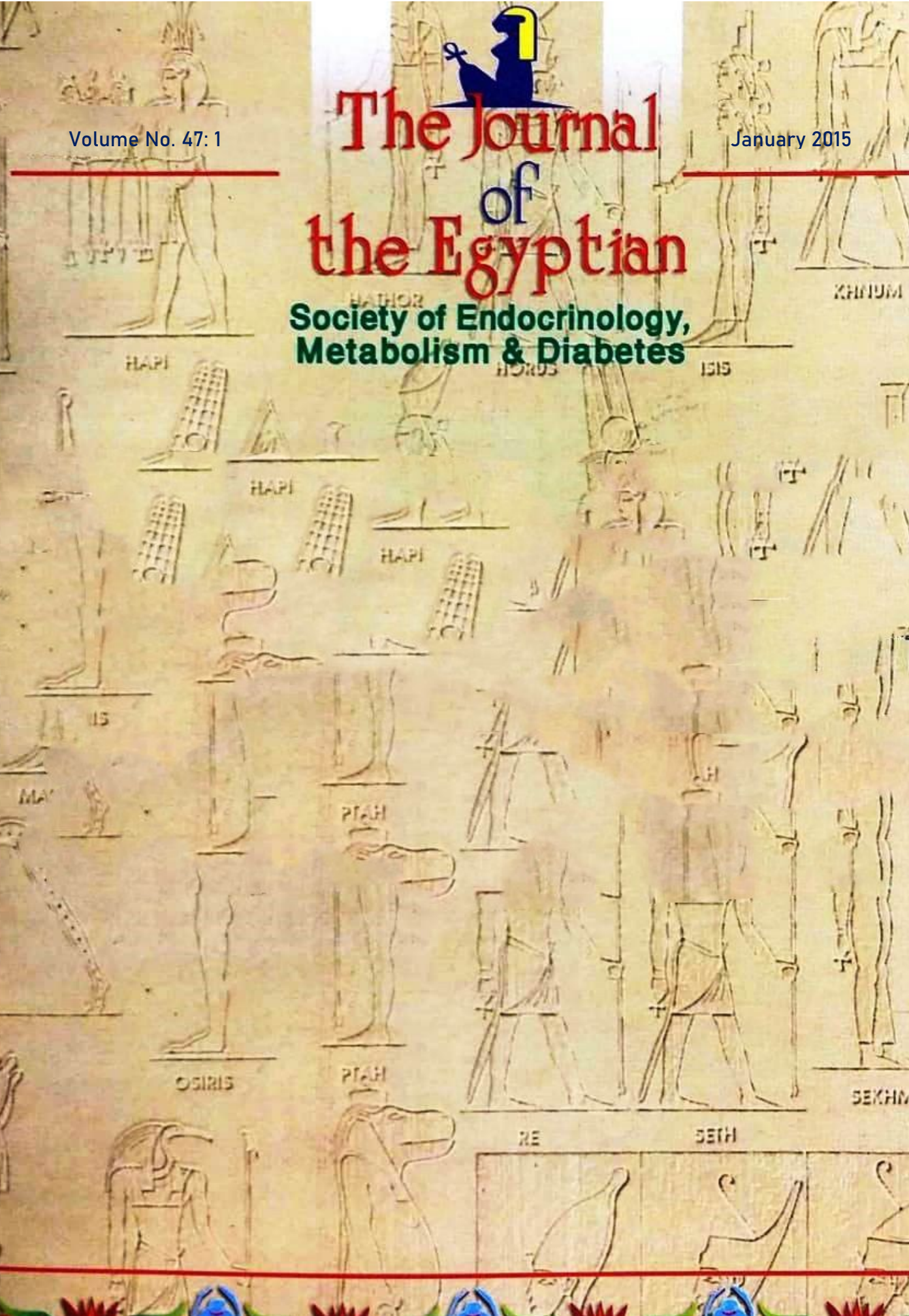
Volume No. 47: 1

January 2015



The Journal of the Egyptian

Society of Endocrinology, Metabolism & Diabetes

Original Article

Relationship of Metalloproteinase-1 and its Tissue Inhibitor to Neuropathic Diabetic Foot Ulcerations.

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Abstract:

Background: Matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs) play an essential role in the process of wound healing. However, their role in impaired healing of neuropathic diabetic foot ulcers is still unclear. Also peripheral nervous system dysfunction results in a well-documented up regulation of MMPs. **Objectives**: The aim of this work was to study serum levels of matrix metalloproteinase-1 (MMP-1) and its tissue inhibitor (TIMP-1) in diabetic patients with neuropathic foot ulcers in comparison to diabetic patients with and without peripheral nerve dysfunction. **Methods:** The study was conducted on eighty diabetic patients including; forty patients with neuropathic foot ulcers, twenty patients with diabetic peripheral neuropathy and twenty diabetic subjects without ulcer or neuropathy, recruited from diabetic foot clinic and diabetes outpatient clinics at Mansoura specialized medical hospital. All patients were consented and informed of the study purposes. Data were collected (including clinical, demographic and laboratory data) and serum samples were taken for measurement of serum MMP-1 and TIMP-1. All patients with ulcer were treated with debridement and appropriate offloading. Ulcer healing was assessed weekly and ulcer size reduction >40% after 4 weeks was considered good healing. Statistical analysis was carried out for all collected data using SPSS 18. Statistical significance was determined at a p-value < 0.05. **Results:** The results revealed that the MMP-1: TIMP-1 ratio is significantly higher in the neuropathy group than ulcer and diabetes groups **(**Median= 0.0284, 0.0245, 0.016; p= 0.041, 0.046 respectively). Ulcer duration was positively correlated with TIMP-1 (r = 0.594, p < 0.001) and negatively correlated with MMP-1: TIMP-1 ratio (r = -0.37, p= 0.019). MMP-1, MMP-1: TIMP-1, and MMP-9: TIMP-1 ratios were significantly increased in poor healers (Median = 160ng/ml, 0.0184, 0.0135 respectively) than in good healers (Median= 100ng/ml, 0.0112, 0.0097 respectively) (p= 0.009, 0.015 and 0.003 respectively). **Conclusion:** MMP-1 and TIMP-1 seem to have an important role in neuropathic diabetic foot ulcerations. Serum levels of MMP-1and MMP-1: TIMP-1 ratio could be used as predictors of wound healing in diabetic patients with neuropathic foot ulcers.

Introduction:

Diabetic foot ulcers represent a major public health problem, being the leading cause of non-traumatic amputation in developed countries. Their medical treatment remains a challenge. However, better understanding of their pathophysiology would improve their management. [(1)] For wounds to heal, the extracellular matrix (ECM) needs to be laid down and then progressively remodeled to reach maturity. The enzymes primarily involved in the degenerative arm of this turnover process are the matrix metalloproteinases MMPs. They comprise a family of some distinct but structurally related enzymes that, when acting together, can degrade almost all ECM components.[(2,3)] They help in elimination of damaged protein, destruction of the provisional extracellular matrix, remodeling of granulation tissue, angiogenesis control and also regulation of some growth factors activity.[(1)] Their activities are also tightly regulated by their inhibitors, the tissue inhibitors of metalloproteinases (TIMPs).[(4,5)] Abnormal MMPs activity has been implicated in many diseases characterized by disorganization of the extracellular matrix, such as rheumatoid arthritis and tumor development, and their importance as a therapeutic approach will be dependent upon careful analysis of their role in different disease stages. Overall, an imbalance between the MMPs and TIMPs is a consistent finding in non-healing wounds.[(6, 7)]

The aim of this work was to study serum levels of MMP-1 and its TIMP-1 in diabetic patients with neuropathic foot ulcers in comparison to diabetic patients with and

without peripheral nerve dysfunction and study their role in ulcer healing.

Subjects and Methods:

The study included eighty diabetic patients selected from Diabetic Foot Clinic and diabetes outpatient clinics in Specialized Medical Hospital, Mansoura University, from April 2013 to November 2013. The study was ethically approved and all patients were given written consent to participate in the study.

Patients were classified into 3 groups:

Group I (Ulcer group): Forty patients with neuropathic diabetic foot ulcer (DFU) (24 males and 16 females), with mean age (43.6 ± 9.3) years.

Group II (Neuropathy group): Twenty patients with peripheral nerve dysfunction without foot ulceration (9 males and 11 females), with mean age (42.1 ± 9.65) years.

Group III (Diabetic group): Twenty diabetic patients without peripheral nerve dysfunction and without ulcers (11 males and 9 females), with mean age (40.6 ± 10.65) years.

Exclusion criteria:

Smokers; patients with anemia (Hemoglobin <10mg/dl); Patients with, liver, renal and ischemic heart diseases; diabetic patients with foot ulcerations of different etiology rather than neuropathy including ischemic ulcers and patients with infected diabetic foot ulcers.

All patients were subjected to thorough history taking and clinical examination.

Diagnosis of PN was based on loss of pressure perception (at two or more sites) using a monofilament test and/or loss of vibration perception (>50 Volts) using the neurothesiometer. Doppler ultrasound was used to measure the ankle/brachial pressure index (ABPI) and toe/brachial index (TBI) to exclude patients with PAD. Patients with ABPI < 0.9 and TBI < 0.7 were excluded.

Ulcers were graded on a 1 to 3 scale according to the Texas Grading System. We used gridded paper to measure the size of the ulcers and ulcer area reduction on follow up. Patients with ulcers are followed up for 4 weeks and according to ulcer size reduction they were subdivided into two subgroups (Good healers and Poor healers; Patients with ulcer size reduction >40% were considered good healers) (8,9).

Laboratory analysis:

Six ml of venous blood was obtained in the fasting state from every patient and distributed as follow: one ml into EDTA tube for determination of CBC (using automated counter, Sysmex KX-21, USA) and HbA1c (Using Dimension Xpand plus chemistry autoanalyzer, Siemens); Five ml into plain tube and left to clot then serum was separated into two aliquots, one used for routine analysis (glucose, liver profile, lipid profile and creatinine) using commercially available kits and the other was stored at -20°C for MMP-1 and TIMP-1 analysis. Estimation of MMP-1and TIMP-1 were done using Raybio^R Human MMP-1and TIMP-1ELISA Kits, Ray-Biotech, Inc. USA for quantitative detection of MMP-1 and TIMP-1 respectively by enzyme linked immunosorbent assay.

Statistical Analysis:

The collected data were organized, tabulated and statistically analyzed using software statistical computer package (SPSS) version 16. For quantitative data, the range, mean and standard deviation were calculated. For qualitative data, comparison between two groups and more was done using Chi-square test ($\chi 2$). For comparison between means of two groups of parametric data, student t-test was used. For comparison between more than two means, the F value of analysis of variance (ANOVA) was calculated, where Scheffe test was performed to compare between each two means if F value was significant. Correlation between variables was evaluated using Pearson's correlation coefficient (r). Sensitivity, Specificity, positive predictive and negative predictive values and accuracy of measuring serum level of MMP-1 and TIMP-1 as prognostic markers were calculated. Significance was adopted at < 0.05 for interpretation of results of tests of significance.

Results:

Demographic and clinical data in the study groups (table I) showed that the duration of diabetes was significantly higher ($p< 0.001$) in the ulcer and neuropathy groups than in diabetes group (Median 15, 12.5 versus 2.5 years respectively). Body mass index (BMI) in the ulcer group (33.93 ±7.3 kg/m^2) was statistically different ($p= 0.03$) when compared with neuropathy and diabetes groups (31.76 ± 3, 30.33 + 4.37 kg/m^2 respectively). VPT

was significantly higher (P < 0.001) in the ulcer and neuropathy groups in comparison to the diabetes group (43.55 ± 4.47, 40.9 ± 7.91 vs. 1 5.4± 3.19 volts, respectively).

Table (II) showed the laboratory data in the study groups: There were insignificant differences in MMP-1 or TIMP-1 among the three different groups. However, there was a significant difference in MMP-1: TIMP-1 ratio in neuropathy group (Median= 0.0284) and diabetes group (Median= 0.0245) when compared to ulcer group.

Correlation between MMP-1, TIMP-1, MMP-1: TIMP-1 ratio and clinical variables in Ulcer group (table III) showed that Age and TBI was significantly correlated with MMP-1 levels (r = -0.327, p = 0.039; r = -0.366, p= 0.02 respectively). Also their VPT was significantly correlated with TIMP-1 levels (r = -0.457, p= 0.003). Ulcer duration was significantly correlated to MMP-1: TIMP-1 ratio (r = - 0.37, p = 0.019), and also showed a highly positive correlation with TIMP-1 (r = 0.594, p < 0.0001). By dividing patients with foot ulcer into poor and good healers (table IV): Poor healing was associated with significantly higher MMP-1 and MMP-1: TIMP-1 ratio (p= 0.009, 0.015, respectively). Table (V) showed analysis of ROC curve results denoting sensitivity, specificity, positive predictive value, negative predictive value and accuracy of MMP-1, MMP-1: TIMP-1 ratio in predicting healing. MMP-1 more than 130 ng/ml was 80% sensitive and 80% specific and MMP-1: TIMP-1 ratio more than 0.0116 was 100% sensitive and 60% specific for predicting poor healing among patients with diabetic foot ulcers.

Table (I): Demographic and clinical data in the study groups

		Group I (Ulcer group) 40 patients	Group II (Neuropathy group) 20 patients	Group III (Diabetes group) 20 patients	P	P1	P2	P3
Age (Years; Mean ± SD)		43.6 ± 9.3	42.1 ±9.65	40.6 ±10.65	0.3	NS		
Sex	**Male (%)**	24(60%)	9 (45%)	11 (55%)	0.5	NS		
	Female (%)	16 (40%)	11 (55%)	9 (45%)				
DM duration (Years; Median, IQR)		15 (10-18.5)	12.5 (9-19.5)	2.5 (2-3)	≤0.001	0.98	≤0.001	≤0.001
DM Therapy	**Insulin (%)**	30 (75%)	17 (85%)	8 (40%)	≤0.001	NS		
	OHG (%)	2 (5%)	3 (15%)	12 (60%)				
	Insulin+ OHG (%)	8 (20%)	Nil	Nil				
BMI (Mean ± SD)		33.93 ± 7.3	31.76 ± 3	30.33 ±4.37	0.03	0.55	0.03	0.4
Waist circumference (Centimeters; Mean ± SD)		112.1 ±8.48	108 ± 8.5	108.6±9.79	0.3	NS		
VPT (Volts; Mean ± SD)		43.55 ± 4.47	40.9 ± 7.91	15.4 ± 3.19	≤0.001	0.16	≤0.001	≤0.001
ABPI (Mean ± SD)		1.14 ± 0.1	1.13 ± 0.11	1.1 ± 0.1	0.3	NS		
TBI (Mean ± SD)		1.23 ± 0.13	1.2 ± 0.13	1.29 ± 0.12	0.06	NS		
HTN	**Yes (%)**	16 (40%)	8 (40%)	8 (40%)	1	NS		
	No (%)	24 (60%)	12 (60%)	12 (60%)				

Where, SD = Standard deviation; IQR = Interquartile range; P = Significance; NS = Non-Significant; P1 = Significance between groups I, II ; P2 = Significance between groups I,III ; P3 = Significance between groups II,III

Table (II): Laboratory data in the study groups

	Group I (Ulcer group)	Group II (Neuropathy group)	Group III (Diabetes group)	P	P1	P2	P3
HbA1c (%; Mean±SD)	8.42 ± 1.47	8.89 ± 1.40	8.35 ± 1.52	0.4	NS		
Serum creatinine (mg/dl ; Mean ± SD)	0.97 ± 0.19	0.97 ± 0.24	0.91 ± 0.17	0.5	NS		
Serum cholesterol (mg/dl ; Mean ± SD)	198.9 ± 36.28	197.7 ± 31.9	199.05 ± 24.52	0.9	NS		
Serum triglycerides (mg/dl ; Mean ± SD)	163.95 ± 39.86	160.85 ± 47.3	169.4 ± 40.21	0.8	NS		
Serum MMP-1 (ng/ml; Median, IQR)	150 (79-185)	140 (110-270)	180 (140-210)	0.15	NS		
Serum TIMP-1 (ng/ml; Median, IQR)	10000 (4050-12250)	9750 (4750-11000)	7000 (1900-10000)	0.08	NS		
MMP-1: TIMP-1 (Median, IQR)	0.016 (0.0094-0.0348)	0.0284 (0.0113-0.0414)	0.0245 (0.016-0.07)	0.049	0.041	0.046	0.7

Table (III): Correlation between MMP-1, TIMP-1, MMP-1: TIMP-1 ratio and clinical variables in Ulcer group

	MMP-1		TIMP-1		MMP-1:TIMP-1	
	r	P	r	P	r	P
Age	-0.327	0.039*	0.07	0.666	-0.202	0.212
DM Duration	0.271	0.091	-0.132	0.417	0.192	0.236
HbA1c	0.15	0.356	-0.274	0.087	0.25	0.12
BMI	-0.078	0.630	-0.117	0.473	-0.015	0.927
VPT	0.011	0.944	-0.457	0.003*	0.303	0.058
ABPI	-0.230	0.154	0.143	0.378	-0.166	0.305
TBI	-0.366	0.02*	-0.219	0.174	-0.116	0.477
Ulcer size	0.192	0.234	-0.062	0.704	0.193	0.232
Ulcer duration	0.002	0.993	0.594	0.000*	-0.370	0.019*
Creatinine	0.265	0.098	-0.004	0.979	0.131	0.420
S.Cholesterol	0.199	0.219	-0.003	0.985	0.074	0.651
S.Triglycerides	0.284	0.075	-0.142	0.382	0.281	0.08

*** =** Significant (<0.05)

Table (IV): Relationship of MMP-1, TIMP-1and MMP1:TIMP1 ratio to healing in patients with ulcers

	Healing				P
	Poor		Good		
	Median	Range	Median	Range	
MMP_1	160	70-190	100	40-140	0.009
TIMP1	5000	3800-13000	12500	3800-14000	0.2
MMP1:TIMP1	0.0184	0.012-0.0428	0.0112	0.0028-0.0315	0.015

Test used: Mann-Whitney
P significance when <0.05

Table (V): Sensitivity, specificity, positive predictive value, negative predictive value and accuracy of MMP-1and MMP-1: TIMP-1 ratio as predictors of poor healing

	Area under curve (CI 95%)	Cut-off value (ng/ml)	Sensitivity (%)	Specificity (%)	Positive predictive value	Negative predictive value	Accuracy
MMP-1	84.0(63.3-100)	>130	80	80	80	80	80
MMP1:TIMP1	82.0(63.3-100)	> 0.0116	100	60	71.4	100	80

Discussion:

Matrix metalloproteinases and their tissue inhibitors have been previously studied in diabetic foot ulcers, both in wound fluid (Muller et al., 2008; Liu et al., 2009) and in serum.[12]

In our study, there were no significant differences in MMP-1or TIMP-1levels between the studied groups. Howev er, MMP-1: TIMP-1 ratio was significantly different (p=0.041) in both group 2 and 3 in comparison to ulcer group (0.284, 0.0245 versus 0.016). Age of the patients of the ulcer group was negatively correlated with MMP-1 (r=-0.327, p=0.039). Similar correlation was obtained by others[13] and this was explained by the superimposition of a disease processes in the older subject may result in different ECM remodeling than that is seen in a younger subject. Also, Bonnema et al., (2007) studied the effect of age on MMP-9 and TIMP-1. They found that, as subject age increased, MMP-9 decreased and TIMP-1 increased.[14]

All participating subjects were overweight or obese evidenced by the abnormally increased BMI in all the study groups. BMI of patients within the ulcer group (33.93 + 7.3) was significantly higher than that in diabetes group (30.33 +4.37) (p= 0.03). Sohn et al., (2011) studied the association between BMI and foot ulceration risk. They found that higher BMI was significantly related to DFU occurrence.[15] BMI was not significantly correlated with MMP-1 or TIMP-1 in our DFU group. In another study enrolled diabetic subjects without foot ulcers, and followed them for the occurrence of foot ulcer. They found that increased body weight, along with other risk factors such as insensitivity to 5.07 monofilament, were significantly related to foot ulcer risk. [16]

Incorporating vibration perception threshold (VPT) testing into clinical practice has the potential to significantly improve the outcomes in patients with DPN, thereby reducing the

socio-economic burden of this common and challenging disease.[17] In our study, VPT was significantly higher in group 1 and 2 than in diabetic patients without DPN and without ulcers (43.55 ± 4.47, 40.9 ± 7.91, and 15.4 ± 3.19 volts, respectively) (P<0.001). Jayaprakash et al., (2011) carried out a study to evaluate the usefulness of VPT measurement in patients with DPN. They found that VPT was positively correlated with the patients' symptoms and signs.[18] In another study that aimed to assess the ability of VPT to predict the development of DFUs, VPT < 15 volts had a cumulative incidence of foot ulceration of 2.9% compared with 19.8% in patients with a VPT > 25 v. They concluded that VPT is an effective predictor of the risk of foot ulceration in diabetes.[19] Studies correlating MMP-1 or TIMP-1 to VPT is lacking in the literature. However, TIMP-1 in our patients with foot ulcers had a significant negative correlation with their VPT measurement (r= -0.457, p= 0.003).

In healing of wounds, ECM needs to be laid down then must be able to undergo degradation and remodeling to form a mature tissue with appropriate strength. Proteases, namely matrix metalloproteinases are known to degrade almost all the extracellular matrix components. They are known to be involved in fibroblast and keratinocyte migration, tissue re-organization, inflammation and remodeling of the wound tissue.[20] MMP-1 is the major collagenase implicated in wound healing: it has been shown that its specific proteolysis of type I collagen (an essential component of the dermis) is essential for keratinocyte migration. [21]

In our study, ulcer duration was positively correlated with TIMP-1 (r= 0.594, p<0.001) and negatively correlated with MMP-1: TIMP-1 ratio (r= -0.37, p= 0.019). Moreover, the process of ulcer healing was assessed by measurement of the ulcer size after 4 weeks and according to ulcer size reduction, patients were subdivided into good healers (Ulcer size reduction > 40%) and poor healers (Ulcer size reduction < 40%). We correlated both sub-groups to MMP-1and MMP-1: TIMP-1 ratio. We found that, poor healing was significantly related to higher levels of MMP-1and MMP-1: TIMP-1 ratio (p= 0.009 and 0.015 respectively). Moreover, MMP-1 level > 130ng/ml had 80% sensitivity and 80% specificity and MMP-1: TIMP-1 ratio > 0.0116 showed sensitivity 100%, specificity 60% in the prediction of poor ulcer healing

We hypothesize that higher level of MMP-1 is harmful to the healing process. It has been suggested that the contribution of increased MMPs and MMPs to TIMPs ratio to poor wound healing can be explained by the deleterious effects exerted by excess MMPs on the healing process, which is believed to be related to exaggeration of ECM degradation[22] and/or prolongation of the inflammatory phase of healing.[23] According to the available literature, studies investigating the relationship of MMPs or TIMPs to Diabetic foot ulcers (DFUs) have always focused on the gelatinases (MMP-2 and MMP-9) rather than any other MMPs isoform[6,11, 12, 24] and used the wound fluid, rather than serum, as sampling methods.[11,24] Our study is considered a unique one that explored the relationship of serum MMP-1 and TIMP-1concentrations to neuropathic DFUs and correlated these parameters to some clinical variables. Ladwig et al. (2002) described a higher level of activated MMP-9 in a group of poorly healing pressure ulcers compared to a group of good healers. In their 56 patients, the MMP-9/ TIMP-1 ratio was positively correlated with poor healing, which underlies the deleterious effect of an MMP-9 excess in chronic wounds.[25] Also, Li et al., (2013) studied the serum levels of MMP-9 in patients with DFUs and they found that the serum MMP-9 level decreases as the ulcer duration increases, and in agreement with us, they found that the MMP-9: TIMP-1 ratio is significantly related to poor healing.[12] Only

Muller et al., (2008) studied the relationship of MMP-1: TIMP-1 ratio to neuropathic DFUs but they found no significant differences in MMP-1: TIMP-1 ratio nor MMP-9: TIMP-1 ratio between good and poor healers.[10] Their study differs from ours in that they only enrolled 16 subjects and used wound fluid as a sampling method.

In conclusion: MMP-1and MMP-1: TIMP-1 ratio seem to have an important role in neuropathic diabetic foot ulcerations. They might be used as predictors of wound healing in diabetic patients with neuropathic foot ulcers and better understanding of this area will help in the development of possible therapeutic strategies.

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Original Article

Study of Plasma Omentin-1 Level in Type 2 Diabetic Patients with and Without Microvascular Complications.

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Abstract:

Background: Adipose tissue produces several hormones and cytokines termed adipokines that have widespread effects on carbohydrate and lipid metabolism. Omentin-1 is a newly identified adipokine that is highly and selectively expressed in visceral adipose tissue relative to subcutaneous adipose tissue. In some recent studies, it was shown to be decreased in obese and in insulin resistant diabetic patients. **Aim:** In this study, intending to increase the knowledge about omentin-1 and its relation with type 2 diabetes mellitus, insulin resistance and obesity, It is planned to point out the relationship between serum plasma omentin-1 level in type 2 diabetic patients with and without microvascular complications. **Subjects & Methods:** The study included 60 type 2 diabetic patients and 30 age matched control subjects. All participants subjected to the following: BMI, WC, nerve conduction velocity, fundus examination and laboratory investigations including: fasting blood glucose, glycosylated hemoglobin, Lipid profile including; Total cholesterol, serum triglycerides, low density lipoproteins cholesterol, high density lipoproteins cholesterol, Blood urea and creatinine. High sensitivity CRP urine analysis for albumin/creatinine ratio. Plasma level of omentin-1. **Results:** Diabetic group with microvascular complications had significantly lower plasma omentin-1 levels than the control group ($p<0.001$). Positive correlations were obtained between high density lipoprotein cholesterol and omentin-1 levels, and negative correlations between body mass index, fasting blood glucose, HbA1c, HS CRP and omentin-1 levels. **Conclusion:** omentin-1 levels were significantly low in diabetic patients with microvascular complications. The data may point toward a role of omentin-1 in insulin resistance and type 2 diabetes mellitus with microvascular complications.

Keywords: Omentin-1, diabetes mellitus, microvascular complications.

Introduction:

The microvascular complications of diabetes may already be present at the day of diagnosis. This may have devastating consequences, including blindness, end stage renal disease and lower limb amputation. The global increase in the prevalence of diabetic microvascular complications and consequently, significant increase in health care expenditure Recently, a new protein omentin (also named omentin-1, intelectin, intelectin-1, endothelial lectin and intestinal lactoferrin receptor) has been identified as a major visceral (omental) fat secretory adipokine. It was then found that omentin-1 is predominantly expressed in visceral but not in subcutaneous adipose tissue, with the omentin-1 mRNA being 150 times higher in the visceral adipose tissue[(1)]. Lean subjects had significantly higher plasma omentin-1 levels than obese and overweight subjects[(2)]. It is interesting to note that the omentin-1 gene is localized on a chromosomal region of 1q22–q23, where it was reported a

presence of linkage to type 2 diabetes in various populations. Visceral obesity is reportedly more pathogenic than subcutaneous obesity in promoting insulin resistance, type 2 diabetes, and microvascular complications. In addition, It has been shown that omentin-1 plasma level are decreased in type 2 diabetic patients. It was found that omentin decreases in vitro migration and angiogenesis in human endothelial cells (EC) induced by human sera, c-reactive protein (CRP) and vascular endothelial growth factor (VEGF). Thus omentin appears to be a 'protective adipokine' that it induces vasodilatation and inhibits EC migration, vascular inflammation and angiogenesis. As well as reducing endothelial dysfunction. As a secretory factor, omentin-1 may be a novel hormone that is likely to act as both an endocrine factor to modulate systemic metabolism, including insulin action in subcutaneous adipocytes, and an autocrine and paracrine factor to regulate visceral adipose biology locally[3,4].

Studies have demonstrated that omentin-1 enhances insulin action by stimulating insulin-mediated glucose uptake by subcutaneous as well as omental adipocytes in vitro[5]. There are few sudies about obesity, diabetes mellitus and omentin-1. Lean subjects had significantly higher plasma omentin-1 levels than obese and overweight subjects[6,7]. Decreased plasma omentin-1 levels were reported in type 1 and type 2 diabetes[8-10] and in patients with impaired glucose regulation[11,12] Keeping in mind that omentin-1 levels may be predictive of the metabolic consequences or co-morbidities associated with obesity and glucose metabolism.

Aim of the Work:

The study was designed to study the changes in the circulating plasma Omentin-1 level in type 2 diabetic patients with and without microvascular complications, determine the relationship of circulating omentin-1 levels with diabetic retinopathy, in subjects with type 2 diabetes and controls and investigate the possible association of Omentin-1 with some anthropometric and metabolic parameters in such diabetic patients.

Subjects and Methods:

Subjects:

The study will enroll ninety age matched subjects selected from outpatient clinic of the diabetes and metabolism unit, Alexandria Main University Hospital. Subjects were divided into 3 groups:

Group (I): will include 30 type 2 diabetic patients with history of microvascular complications (Diabetic Retinopathy, polyneuropathy and/or nephropathy).

Group (II): will include 30 newly diagnosed type 2 diabetic patients without complications.

Group (III): will include 30 healthy subjects as a control.

Methods:

All subjects included in the study were subjected to the following:

- History taking including an interview questionnaire comprising the following items: Diabetic history, screening for microvascular complications, history of renal disease (dialysis or transplantation), of laser photocoagulation, of numbness, prickling sensation or burning pain at leg or feet, screening for macrovascular complications & screening for associated risk factors: History of hypertension & Smoking status with calculation of smoking index.
- Physical examination: Anthropometric measurements including height and weight measurements then calculation of body mass index (BMI), Waist circumferences, Vital signs, Sensory function: a) Superficial sensation b) Deep sensation.
- Fundus examination: Retinal blood vessels fluorescein angiography

- Nerve conduction velocity.
- Laboratory investigations including: Fasting blood glucose, Glycosylated hemoglobin (HbA1c), Lipid profile including, Total cholesterol, Serum triglycerides, Low density lipoproteins cholesterol, High density lipoproteins cholesterol, Urine analysis for albumin/creatinine ratio, Blood urea and creatinine, High sensitivity CRP by immunoturbidemetric method & Plasma level of Omentin-1by ELISA technique

Results:

The study was performed on 90 subjects 73 of them were males and 17 were females presented to outpatient clinic, internal medicine department, Alexandria main university hospital.

Table (I) Comparison between the three studied groups according to fundus exam

	Micro-vascular complication (n = 30)		Newly diagnosed (n = 30)		Control (n = 30)		χ^2	^{MC}p
	No.	%	No.	%	No.	%		
Fundus Exam								
Normal	0	0.0	24	80.0	30	100.0		
Non prolifertive diabetic retinopathy	20	66.7	6	20.0	0	0.0	83.048*	<0.001*
Prolifertive diabetic retinopathy	10	33.3	0	0.0	0	0.0		
Sig.bet. Grps	p_1 <0.001*, p_2<0.001*, p_3= 0.024*							

χ^2:value for Chi square
MC: Monte Carlo test
Sig. bet. grps was done using Fisher Exact test or Monte Carlo test
p_1: p value for comparing between Micro-vascular complication and Newly diagnosed
p_2: p value for comparing between Micro-vascular complication and Control
p_3: p value for comparing between Newly diagnosed and Control
*: Statistically significant at p ≤ 0.05

Table (II) Comparison between the three studied groups according to plasma omentine.1

	Micro-vascular complication (n = 30)	Newly diagnosed (n = 30)	Control (n = 30)	F	P
Plasma Omentine.1					
Min. – Max.	0.57 – 0.61	0.61 – 0.71	0.71 – 3.43		
Mean ± SD	0.59 ± 0.01	0.66 ± 0.03	1.05 ± 0.71	11.096*	<0.001*
Median	0.59	0.65	0.79		
Sig.bet. Grps	p_1= 0.516, p_2<0.001*, p_3<0.001*				

F: F test (ANOVA), Sig. bet. grps was done using Post Hoc Test (LSD)
p_1: p value for comparing between Micro-vascular complication and Newly diagnosed
p_2: p value for comparing between Micro-vascular complication and Control
p_3: p value for comparing between Newly diagnosed and Control
*: Statistically significant at p ≤ 0.05

Table (III) Correlation between Plasma omentine.1 with different parameters in each group

		Plasma omentine.1		
		Micro vascular complication	**Newly diagnosed**	**Control**
Age	**R**	-0.158	0.213	0.040
	P	0.406	0.259	0.835
Duration	**r_s**	-0.256	0.132	-
	P	0.173	0.485	-
Weight	**R**	-0.565*	0.115	-0.067
	P	0.001	0.543	0.724
BMI	**R**	-0.781*	0.181	-0.148
	P	<0.001	0.337	0.436
WC	**R**	-0.541*	-0.546*	-0.231
	P	0.002	0.002	0.220
SBP	**R**	0.129	0.267	-0.111
	P	0.495	0.154	0.558
Fundus exam	**r_s**	-0.537*	0.212	-
	P	0.002	0.260	-
Nerve conduction velocity	**r_s**	-0.269	0.240	-
	P	0.151	0.201	-
FBS	**r_s**	0.161	-0.487*	-0.047
	P	0.394	0.006	0.806
HbA1C	**R**	0.095	-0.166	-0.367*
	P	0.617	0.381	0.046
Urea	**r_s**	0.098	0.004	-0.246
	P	0.606	0.985	0.190
Creatinine	**R**	-0.223	-0.013	-0.162
	P	0.236	0.944	0.391
Alb/Creat ratio	**r_s**	-0.393*	-0.078	0.253
	P	0.031	0.681	0.178
HS-CRP	**r_s**	-0.589*	-0.513*	0.352
	P	0.001	0.004	0.056
LDL	**R**	-0.296	-0.012	-0.153
	P	0.112	0.952	0.419
HDL	**R**	0.454*	0.621*	0.088
	p	0.012	<0.001	0.643
TG	**r_s**	-0.196	-0.228	0.260
	p	0.298	0.225	0.165
Cholesterol	**r**	-0.610*	-0.800*	-0.036
	p	<0.001	<0.001	0.850

r: Pearson coefficient

r_s: Spearman coefficient

*: Statistically significant at $p \leq 0.05$

Discussion:

How omentin-1 levels are influenced by glucose levels and vice versa glucose levels by omentin-1 levels warrant elucidation[8-10]. Decreased omentin-1 levels were reported in subjects with impaired glucose regulation, type 1 and type 2 diabetic[13] individuals. In this study, diabetic patients had statistically significant lower omentin-1 levels than control subjects. Between the three groups of diabetic and control groups the lowest omentin-1 levels were found in diabetic patients with microvascular complications. The results demonstrated that omentin-1 levels decreased in diabetic individuals and decreased further when diabetes mellitus was combined with microvascular complications. The serum omentin-1 level in diabetic retinopathy group was significantly lower than that of the newly diagnosed diabetic group (P<0.001). Statistical significant difference was found between control group and diabetic retinopathy group (P<0.001). Statistical significant difference was found between control group and newly diagnosed diabetic patients group (P = 0.024) In agreement with our results, Hideyuki Yamawaki, Naoya Tsubaki found that omentin-1 inhibits TNF-α induced vascular cell adhesion molecule (VCAM)-1 expression via preventing the activation of P38 and JNK at least in part through inhibition of superoxide production. Omentin plays an anti-inflammatory role through inhibition of TNF-α induced superoxide production in vascular smooth cells [14,15].

The serum omentin-1 level in diabetic neuropathy group was significantly lower than that of the newly diagnosed diabetic group (P<0.001). Statistical significant difference was found between control group and diabetic neuropathy group (P<0.001). Statistical significant difference was found between control group and newly diagnosed diabetic patients group (P = 0.002).

The serum omentin-1 level in diabetic nephropathy group was significantly lower than that of the newly diagnosed diabetic group (P<0.001). Statistical significant difference was found between control group and diabetic nephropathy group (P<0.001). Statistical significant difference was found between control group and newly diagnosed diabetic patients group (P = 0.001). Ho Ra1, Ji Han Yoo2, found that albuminuria is a marker of endothelial dysfunction and may influence on alterations in microvasculature of retina and kidneys[16].

The serum omentin-1 level was significantly lower in the newly diagnosed type 2 diabetic group (P<0.001). the results of G. Gürsoy study goes hand in hand with our results who proved the same result.

A significant negative relation was found between serum omentin-1 and body mass index and waist circumference in diabetic patients . Celia M. de Souza Batista,1,2 Rong-Ze Yang,1 Mi-Jeong Lee found that Central obesity and the accumulation of visceral fat are risk factors for the development of type 2 diabetes and cardiovascular disease[17-19]. Omentin is a protein expressed and secreted from visceral but not subcutaneous adipose tissue that increases insulin sensitivity in human adipocytes[20].

A significant negative relation between serum omentin-1 and fasting blood sugar and blood glucose and HbA1c in diabetic patients with microvascular complications.

A significant negative relation between serum omentin-1 and high sensitive CRP in diabetic patients with microvascular complication.

A significant positive relation between serum omentin-1 and HDL-cholesterol in diabetic patients microvascular complications. In concordance with our results G. Gürsoy study demonstrated that omentin-1 levels decreased in diabetic individuals and decreased further when diabetes mellitus was combined with insulin resistance[21,22]. They also found positive correlation between omentin-1 and age, HDL-C levels.

For the time being, it is difficult to say whether high glucose and insulin levels are the cause or the result of low omentin-1 levels and with which mechanisms they effect omentin-1 levels. Further studies are needed.

Conclusion:

The study showed that omentin-1 levels are lower in type 2 diabetics with microvascular complications. Since type 2 diabetes mellitus is closely related to visceral adipose tissue

amount and diabetes has declared to be a state of inflammation it was reasonable to further investigate the role of omentin-1 in type 2 diabetic patients. According to the study, it may be said that glucose and insulin levels as well as insulin resistance may have a repressive effect on omentin-1 levels. Decreased omentin-1 levels may contribute to the underlying pathophysiology of microvascular complications of diabetes mellitus. Future studies probably with bigger sample size will be required to address the link of omentin-1 with metabolic disturbances such as obesity, insulin resistance and microvascular complications .

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Original Article

Omentin-1 and its Relation to Arterial Stiffness and Carotid Intima- Media Thickness in Type 2 Diabetic Patients

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Abstract:

Purpose: Atherosclerosis is a major complication of type 2 diabetes mellitus. Omentin-1 is a novel adipokine implicated in body metabolism. Carotid intima media thickness (CIMT) is an established marker for atherosclerosis and a predictor of cardiovascular events. Omentin was found to be reduced in obese subjects and to correlate negatively with BMI, waist circumference and increased CIMT. There are few studies about relation between omentin level and CIMT in diabetics and none of them studied the relation of omentin to the presence of macrovascular complications. That is why we conducted the current research to assess the serum level of omentin-1 in type 2 diabetic subjects versus control and to identify its relationship with atherosclerosis and the presence of macrovascular complications in the diabetic group. **Methods:** This study enrolled 60 subjects divided into three groups. Group (1): included 20 type 2 diabetic patients with history of macrovascular complications, Group (2): included 20 newly diagnosed type 2 diabetic patients without history of macrovascular complications, Group (3): included 20 healthy subjects as control. Assessment included thorough history taking, complete clinical examination, ankle/brachial index, carotid ultrasound, laboratory investigations including metabolic profile and plasma omentin-1 by ELISA. **Results:** Omentin-1 level is significantly lower in subjects with diabetes and macrovascular complications than the newly diagnosed diabetic patients and control group. glycosylated hemoglobin, fasting blood glucose, high sensitive C-reactive protein, body mass index, waist circumference and CIMT have negative correlations with omentin-1 level, while ankle/brachial index is positively correlated. **Conclusions:** Omentin-1 level is lowermost in diabetic patients with macrovascular complications. It is an independent negative predictor of CIMT.

Keywords: Omentin-1, Atherosclerosis, Carotid IMT, ankle/brachial index, Diabetes.

Introduction:

Diabetes Mellitus is a very common metabolic disease occurring in different age groups due to insulin resistance, deficiency or both. It has a rapidly increasing prevalence in both developing and developed countries that is amounting to an epidemic. Whereas 382 million people were diagnosed as diabetics in 2013, it is expected to increase to 592 millions by the year 2035[1].Type 2 diabetes comprises more than 90% of all diabetics worldwide[2].

People with diabetes are at high risk of developing macrovascular complications especially coronary heart disease and stroke, thus becoming the leading cause of mortality and morbidity among these people with nearly 50% of diabetics dying from cardiovascular disease[3]. Atherosclerosis is considered the central pathological mechanism of macrovascular complications. It starts very early and progresses throughout life. Evidence shows that in addition to the classical risk factors like dyslipidemia and dysglycemia, chronic inflammation plays a pivotal role in the development of atherosclerosis[4].

Abdominal obesity is associated with low grade inflammation. A myriad of evidence shows that Obesity, especially of central distribution, has been linked to cardiovascular disease[5,6]. Visceral adipose tissue acts as an endocrine organ secreting bioactive substances, termed adipokines, which regulate adipose tissue function and influence glucose metabolism

and energy balance at the systemic level[7]. Alteration of the level of these adipokines in obese subjects contributes to the development of a chronic inflammatory state that impairs normal adipose tissue function and participates to many adverse outcomes like atherosclerosis[8]. Adipokines demonstrate different properties; some have pro-inflammatory activity thus promoting insulin resistance and atherosclerosis, while others have anti-inflammatory and insulin sensitizing effect and could be considered as "protectors"[9].

Omentin-1 is one of the recently discovered adipokines secreted selectively from omental[10] and epicardial adipose tissue[11]. It is a secretory hydrophilic glycoprotein consisting of 313 amino acids and 1-linked oligosacharides, its basic structural unit is a 120-kDa homotrimer in which 40-kDa polypeptides are bridged by disulfide bonds and it is considered as a new type of Ca2+ -dependent lectin with affinity for galacto-furanosyl residues which are constituents of pathogens and dominant immunogens[12]. A homolog of omentin-1 has been identified that shares 83% amino acid identity with omentin-1 and was referred to as omentin-2, however omentin-1 was shown to be the major circulating isoform in human plasma[12].

Regarding carbohydrate metabolism, Omentin-1 has no effect on basal glucose uptake, however, it enhances insulin-stimulated glucose uptake in adipocytes. Also it increases AKT phosphorylation in the absence and presence of insulin[13].

Recent studies noticed that omentin-1 is involved in many chronic inflammatory processes[14], the defense system against microorganisms[13], iron metabolism[13] and many other diseases including cardiovascular disease[15]. It is found in several recent studies that omentin-1 correlates positively with high-density lipoprotein cholesterol while it is correlates negatively with BMI, waist circumference fasting blood glucose[16,17] and increased intima media thickness IMT[18].

Carotid intima-media thickness [CIMT], a measurement of the tunica intima and tunica media of the carotid, has been frequently used since the mid-1990s as a method of assessment of atherosclerosis. It is done usually by external ultrasound but it can be measured by other methods like internal, invasive ultrasound catheters. IMT is considered an atherosclerotic disease marker in humans, also to track the regression, arrest or progression of atherosclerosis. An IMT greater than 1.1mm is indicative of atherosclerosis and increased risk of cardiovascular disease[19]. In 2010 the American Heart Association and the American College of Cardiology claimed the use of IMT on intermediate risk patients[20].

There are few studies about relation between omentin level and CIMT in diabetics but none of them studied the relation of omentin to the presence of macrovascular complications. That is why we conducted the current research to assess the serum level of omentin-1 in type 2 diabetic subjects versus control and to identify its relationship with atherosclerosis and the presence of macrovascular complications in the diabetic group.

Subjects and Methods:

This is a cross-sectional study performed to assess the level of serum omentin-1 and its relationship with atherosclerosis (presented as increased carotid intima media thickness and decreased ankle brachial index) and the presence of macrovascular complications in type 2 diabetic patients. It enrolled 60 subjects selected from outpatient clinics of Alexandria main university hospital and Alexandria Police hospital. The selected subjects were divided into 3 groups. Group (1) included 20 type 2 diabetic subjects with history of one or more macrovascular complications e.g. ischemic heart disease, transient ischemic attack, stroke and peripheral arterial disease (PAD). Group (2) included 20 subjects type 2 newly diagnosed diabetic patients without history of macrovascular complications. Group (3) comprises 20 healthy subjects of matched age and sex as a control.

The study was performed according to the guidelines of the Helsinki Declaration and

approved by the Ethics Committee of the Faculty of Medicine- Alexandria University. All participants who freely accepted to participate in the study were given a patient information sheet and signed a written informed consent.

Subjects with peripheral vascular disease due to causes other than diabetes, known history of coronary heart disease without diabetes, severe uncontrolled hypertension, severe cardiac decompensation, end stage renal disease, Familial hypercholesterolemia, Connective tissue diseases and vasculitis were excluded from this study.

All participants were subjected to the following:

- **Thorough history taking** by a physician including an interview questionnaire covering the following items:
- History of diabetes (type, duration, medications).
- History of macrovascular complications
- History of associated other risk factors (hypertension, dyslipidemia, smoking, family history of DM, hypertension, dyslipidemia, cardiovascular disease and obesity).
- Physical examination:
- Anthropometrics measurements e.g. weight, height, body mass index (BMI), waist circumference and Waist/hip (W/H) ratio.
- Vital signs (heart rate, systolic and diastolic blood pressure).
- Vascular assessment.

- **Ankle/brachial index (ABI):**

We estimate the systolic blood pressure in the two brachial arteries and also the systolic blood pressure in posterior tibial arteries and dorsalis pedis arteries in both lower limbs by inflation of the 30-40 cm cuff then deflating it gradually till appearance of wave on the continous wave Doppler device. According to higher ankle pressure (HAP) method; ABI is calculated as followed:

ABI= the higher of the ankle arterial SBP / the higher of the two brachial SBP[21].

- **Doppler Carotid Artery to determine the carotid intima media thickness**:[22].

Ultrasonographic scanning of the carotid arteries was performed using Philips ClearVue 350 at a transducer frequency of 7-12 MHz at Radiology Department, Alexandria Main University Hospital. Extracranial carotid arteries in the neck were scanned bilaterally in the longitudinal and in the transverse plane. This provided images of the common carotid artery, the carotid bulb, and parts of the internal and external carotid arteries. Scanning was performed by a single experienced ultrasonographer.

Carotid IMT was defined as the distance from the leading edge of the first echogenic line to the leading edge of the second echogenic line on the scans, with the first line representing the lumen–intimal interface and the second line representing the collagen-containing upper layer of the adventitia. The site with the greatest thickness in the longitudinal plane was detected along the vessel from the common carotid artery to the internal carotid artery bilaterally.

- **Laboratory investigations:**

Venous blood samples were obtained after an overnight fasting; another sample was obtained 2 hours postprandial for PPBG measurement. All fasting blood samples were divided into two aliquots: the first part was collected in vacutainer tube containing Na2EDTA for the assay of HbA1C; the second was collected in plain vacutainer tube and centrifuged (3000 rpm) for serum preparation. Serum was used for measuring total cholesterol, TG, LDL-c, HDL-c, glucose, urea, creatinine, hs-CRP and omentin. Serum samples for omentin assay were kept at -80°C till being assayed. Serum omentin-1 concentration was measured using Human Enzyme-linked immunosorbent assay (ELISA) kits (AVISCERA BIOSCIENCE INC, Santa Clara, CA, USA). Serum samples for omentin were diluted and assayed according to manufacturer instructions. All samples were measured in Alexandria Main University Hospital Laboratory.

- Fasting plasma glucose [23].
- Blood Urea and serum creatinine [24].
- Glycated heamoglobin (HbA1c) [23].

- Total serum cholesterol [25].
- High density lipoprotein cholesterol (HDL- Cholesterol) [25].
- Low density lipoprotein cholesterol (LDL- Cholesterol) [25].
- Serum triglycerides [25].
- High sensitivity CRP (hs-CRP) [26].
- Plasma level of omentin-1 by ELISA [27].

Statistical analysis of the data: [28]

Data were analyzed using IBM SPSS software package version 20.0. Qualitative data were described using number and percent. Quantitative data were described using mean and standard deviation for normally distributed data while abnormally distributed data was expressed using median, minimum and maximum. Comparison between different groups regarding categorical variables was tested using Chi-square test. For abnormally distributed data, Mann-Whitney Test was used to analyze two independent populations. If more than two populations were analyzed Kruskal Wallis test to be used. Correlations between two quantitative variables were assessed using Spearman coefficient. For normally distributed data, comparison between the three studied groups were analyzed using F-test (ANOVA) and Post Hoc test (Scheffe) for pair-wise comparisons, Significance of the obtained results was judged at the 5% level.

Results:

Demographic and clinical characteristics of the study subjects are summarized in table (I) that showed no significant difference between the three groups regarding age, sex, exercise and smoking habits. However, There was significant difference regarding DM duration ($p<0.001$), history of hypertension and dyslipidemia ($p<0.001$), weight ($p<0.001$), BMI ($p<0.001$), waist circumference ($p<0.001$), W/H ratio ($p<0.001$), systolic BP ($p=0.041$), heart rate ($p=0.015$) and ankle brachial index (ABI) ($p<0.001$). Also, there was a significant difference ($p<0.05$) between diabetics with history of macrovascular complications versus newly diagnosed diabetics without history of macrovascular complications regarding diabetes duration, history of hypertension, BMI, W/H ratio and ABI. Moreover, there was a significant difference ($p<0.05$) between newly diagnosed diabetics without history of macrovascular complications versus controls regarding ABI and also regarding BMI, history of dyslipidemia and history of hypertension.

Different laboratory findings in addition to the Carotid intima media thickness (CIMT) measurements are summarized in table (II). There was significant difference between the studied groups regarding omentin-1($p<0.001$), CIMT ($p<0.001$), hs-CRP ($p<0.001$), FPG ($p<0.001$), PPPG ($p<0.001$), HbA1c ($p<0.001$), total cholesterol ($p<0.001$), LDL-c ($p<0.001$), HDL-c ($p=0.002$), TG ($p<0.001$) and creatinine ($p=0.031$).

Omentin-1 was significantly lower in diabetics with history of macrovascular complications than in newly diagnosed diabetics without history of macrovascular complications ($p<0.05$) and significantly lower in the later group compared to control ($p< 0.05$). Conversely, CIMT and CRP were significantly higher in diabetics with history of macrovascular disease versus those without ($p<0.05$), and still higher in the later group than control ($p< 0.05$).

As shown in table (III),(IV) in all diabetics, omentin-1 correlated negatively with CIMT, BMI, HbA1c, FPG, PPPG, total cholesterol, LDL-c, TG, hs-CRP and W/H ratio, yet correlated positively with ABI. Regarding CIMT, it correlated negatively, in all diabetics, with omentin-1 level and ABI, while correlated positively with BMI, HbA1c, FPG, PPPG, total cholesterol, LDL-c, TG, hs-CRP and W/H ratio.

In Multivariate linear regression analysis using Omentin-1 as dependent variable, CIMT and HbA1C were the independent predictors; Table (V), while on using CIMT as the dependent variable, omentin-1 was the only independent predictor; Table (VI).

As shown in figure (1): omentin-1 level correlates negatively with carotid intima-media thickness (CIMT) in all diabetic patients.

Table (I): Comparison between the study groups according to demographic and clinical data

	DM cases			
	With history of macrovascular complications (n=20)	**Newly diagnosed without history macrovascular complications (n=20)**	**Control (n=20)**	**p**
Sex (male/female)				
Male	13 (65%)	14 (70%)	15 (75%)	0.788
Female	7 (35%)	6 (30%)	5 (25%)	
Age (years)	56.15 ± 6.98	55.25 ± 5.58	53.70 ± 4.26	0.397
DM duration (years)	11.5 †∂ (0.5 - 31)	2† (0.25 - 5)	0 (0 – 0)	<0.001*
History Hypertension	20 †∂ (100%)	15† (75%)	0 (0%)	<0.001*
History dyslipidemia	19 † (95%)	19† (95%)	0 (0%)	<0.001*
Exercise	5† (25%)	10 (50%)	12 (60%)	0.072
Smoking	8 (40%)	10 (50%)	10 (50%)	0.765
Weight (kg)	96.25† ± 12.87	88.70 ± 9.39	81.65 ± 6.23	<0.001*
Height (m)	1.78 ± 0.08	1.80 ± 0.07	10.83 ± 0.06	0.176
BMI (kg/m2)	30.23†∂ ± 2.82	27.46† ± 2.13	24.44 ± 1.50	<0.001*
Waist Circumference (cm)	97.55† ± 8.48	91.55 ± 6.19	91.55 ± 3.56	<0.001*
Hip Circumference (cm)	97.95 ± 5.80	96.45 ± 5.84	96.50 ± 3.25	0.577
W/H ratio	0.99†∂ ± 0.05	0.95 ± 0.02	0.94 ± 0.01	<0.001*
Systolic BP (mmHg)	138.50† ± 10.40	134.50 ± 12.34	129.75 ± 8.96	0.041*
Diastolic BP (mmHg)	90.0 ± 12.14	86.50 ± 11.37	81.75 ± 7.48	0.053
HR (beat/minute)	75.0† ± 8.60	77.20 ± 8.52	82.20 ± 5.87	0.015*
ABI	0.48†∂ ± 0.13	0.81† ± 0.11	1.10 ± 0.15	<0.001*

Values was expressed as (mean ± SD) for normally distributed parameters, median (Min. – Max.) for abnormally distributed parameters and percentage for qualitative values

†: Significant difference (p<0.05); diabetics (with or without history of macrovascular complications) versus control

∂: Significant difference (p<0.05); diabetics with history of macrovascular complications versus newly diagnosed diabetics without history of macrovascular complications

*: Statistically significant at p ≤ 0.05

BMI: body mass index, W/H ratio: waist/hip ratio, ABI: ankle brachial index.

Table (II): Comparison between the studied groups according to laboratory investigations and CIMT

	DM			
	With history macrovascular complications (n=20)	**Newly diagnosed without history macrovascular complications (n=20)**	**Control (n=20)**	**p**
Omentin-1 (ng/L)	1105†∂(1078–1218)	1355†(122-1428)	1561 (1428–6866)	<0.001*
CIMT (mm)	1.56†∂ ± 0.18	1.03† ± 0.21	0.67 ± 0.12	<0.001*
Hs-CRP (mg/L)	7.31†∂ ± 0.93	4.17† ± 0.67	1.59 ± 0.70	<0.001*
FPG (mg/dl)	222.1†∂ ± 43.21	145.1† ± 10.95	82.05 ± 8.21	<0.001*
PPPG (mg/dl)	330.5†∂ ± 56.44	180.40† ± 11.89	115.20 ± 12.59	<0.001*
HbA1c (%)	10.10†∂ ± 1.13	7.62† ± 0.72	5.09 ± 0.40	<0.001*
Urea (mg/dl)	42.80 ± 5.04	41.60 ± 4.77	40.35 ± 3.57	0.237
Creatinine (mg/dl)	1.0 ± 0.23	0.99 ± 0.19	0.85 ± 0.17	0.031*
Total Cholesterol (mg/dl)	331.8†∂ ± 46.45	232.05† ± 41.20	188.05 ± 7.44	<0.001*
LDL-c (mg/dl)	243.35†∂ ± 42.94	158.8† ± 36.02	109.45 ± 8.07	<0.001*
HDL-c (mg/dl)	37.9 † ± 8.60	38.4† ± 8.19	45.55 ± 3.73	0.002*
TG (mg/dl)	253.2†∂ ± 23.57	185.05† ± 16.09	142.0 ± 6.75	<0.001*

Values was expressed as (mean ± SD) for normally distributed parameters or median (Min. – Max.) for abnormally distributed parameters

†: Significant difference (p < 0.05); diabetics (with or without history of macrovascular complications) versus control

∂: Significant difference (p < 0.05); diabetics with history of macrovascular complications versus newly diagnosed diabetics without history of macrovascular complications

*: Statistically significant at p ≤ 0.05

CIMT: carotid intima media thickness, Hs-CRP: high sensitivity C reactive protein, FPG: fasting plasma glucose, PPPG: postprandial plasma glucose, LDL-c: low-density lipoprotein cholesterol, HDL-c: high density lipoprotein cholesterol. TG: triglycerides.

Table (III): Correlation between Omentin-1 and different clinical and laboratory parameters in each group

	DM							
	With history macrovascular complications (n=20)		Newly diagnosed without history macrovascular complications (n=20)		Control (n=20)		All diabetics	
	r_s	P	r_s	P	r_s	p	r_s	p
CIMT (mm)	-0.587*	0.007	-0.738*	<0.001	0.077	0.746	-0.902*	<0.001
ABI	0.576*	0.008	0.568*	0.009	-0.219	0.354	0.881*	<0.001
Waist circumference (cm)	0.248	0.291	-0.424	0.062	0.046	0.847	-0.311	0.051
W/H ratio	-0.113	0.634	-0.349	0.132	-0.033	0.891	-0.450*	0.004
BMI (kg/m^2)	0.195	0.410	-0.313	0.178	0.291	0.214	-0.462*	0.003
Systolic BP (mmHg)	0.390	0.089	-0.196	0.408	-0.148	0.534	-0.052	0.743
Diastolic BP(mmHg)	0.207	0.382	-0.299	0.200	-0.014	0.952	-0.151	0.352
Heart rate(beat/min)	0.234	0.321	-0.094	0.694	0.293	0.210	0.158	0.331
HbA1C (%)	-0.590*	0.006	-0.444*	0.050	-0.041	0.865	-0.864*	<0.001
FPG (mg/dl)	-0.560*	0.010	-0.588*	0.006	0.258	0.272	-0.877*	<0.001
PPPG (mg/dl)	-0.546*	0.013	-0.415	0.069	0.224	0.342	-0.870*	<0.001
Urea (mg/dl)	-0.304	0.193	0.035	0.885	-0.280	0.231	-0.191	0.238
Creatinine (mg/dl)	0.149	0.530	-0.102	0.669	0.071	0.766	-0.016	0.924
Total cholesterol (mg/dl)	-0.074	0.757	-0.558*	0.006	0.135	0.571	-0.748*	<0.001
LDL-c (mg/dl)	-0.164	0.489	-0.609*	0.004	0.194	0.412	-0.765*	<0.001
HDL-c (mg/dl)	-0.123	0.606	0.132	0.580	0.465*	0.039	0.074	0.650
TG (mg/dl)	-0.467*	0.038	-0.235	0.318	-0.103	0.665	-0.838*	<0.001
Hs-CRP (mg/L)	-0.789*	<0.001	-0.322	0.166	0.201	0.396	-0.889*	<0.001

rs: Spearman coefficient

*: Statistically significant at p ≤ 0.05

CIMT: carotid intima media thickness, ABI: ankle brachial index, BMI: body mass index, W/H ratio: waist/hip ratio, FPG: fasting plasma glucose, PPPG: postprandial plasma glucose, LDL-c: low-density lipoprotein cholesterol, HDL-c: high density lipoprotein cholesterol. TG: triglycerides, Hs-CRP: high sensitivity C reactive protein.

Table (IV): Correlation between CIMT and different clinical and laboratory parameters in each group

	DM							
	With history macrovascular complications (n=20)		Newly diagnosed without history macrovascular complications (n=20)		Control (n=20)		All diabetics	
	r_s	P	r_s	p	r_s	p	r_s	P
Omentin-1 (ng/L)	-0.587*	0.007*	-0.738*	<0.001*	0.077	0.746	-0.902*	<0.001*
ABI	-0.479*	0.033*	-0.547*	0.013*	-0.225	0.341	-0.853*	<0.001*
Waist circumference (cm)	-0.230	0.329	0.240	0.308	0.151	0.525	0.281	0.079
W/H ratio	-0.112	0.639	0.200	0.399	-0.098	0.682	0.375*	0.017*
BMI (kg/m^2)	-0.230	0.329	0.032	0.894	0.269	0.251	0.364*	0.021*
Systolic BP (mmHg)	-0.003	0.989	0.322	0.167	0.200	0.398	0.206	0.203
Diastolic BP(mmHg)	-0.085	0.721	0.348	0.133	-0.052	0.826	0.206	0.201
Heart rate (beat/min)	-0.208	0.380	-0.052	0.827	0.256	0.276	-0.184	0.256
Creatinine (mg/dl)	-0.039	0.869	0.171	0.472	0.191	0.419	0.047	0.776
Total cholesterol (mg/dl)	0.293	0.210	0.469*	0.037*	0.196	0.409	0.748*	<0.001*
LDL-c (mg/dl)	0.346	0.136	0.523*	0.018*	0.349	0.132	0.761*	<0.001*
HDL-c (mg/dl)	0.311	0.182	-0.211	0.372	0.0	1.000	-0.010	0.953
TG (mg/dl)	0.055	0.819	0.474*	0.035*	-0.139	0.560	0.791*	<0.001*
Hs CRP (mg/L)	0.485*	0.030*	0.523*	0.018*	-0.219	0.353	0.854*	<0.001*

rs: Spearman coefficient

*: Statistically significant at p ≤ 0.05

CIMT: carotid intima media thickness, ABI: ankle brachial index, BMI: body mass index, W/H ratio: waist/hip ratio, FPG: fasting plasma glucose, PPPG: postprandial plasma glucose, LDL-c: low-density lipoprotein cholesterol, HDL-c: high density lipoprotein cholesterol. TG: triglycerides, Hs-CRP: high sensitivity C reactive protein.

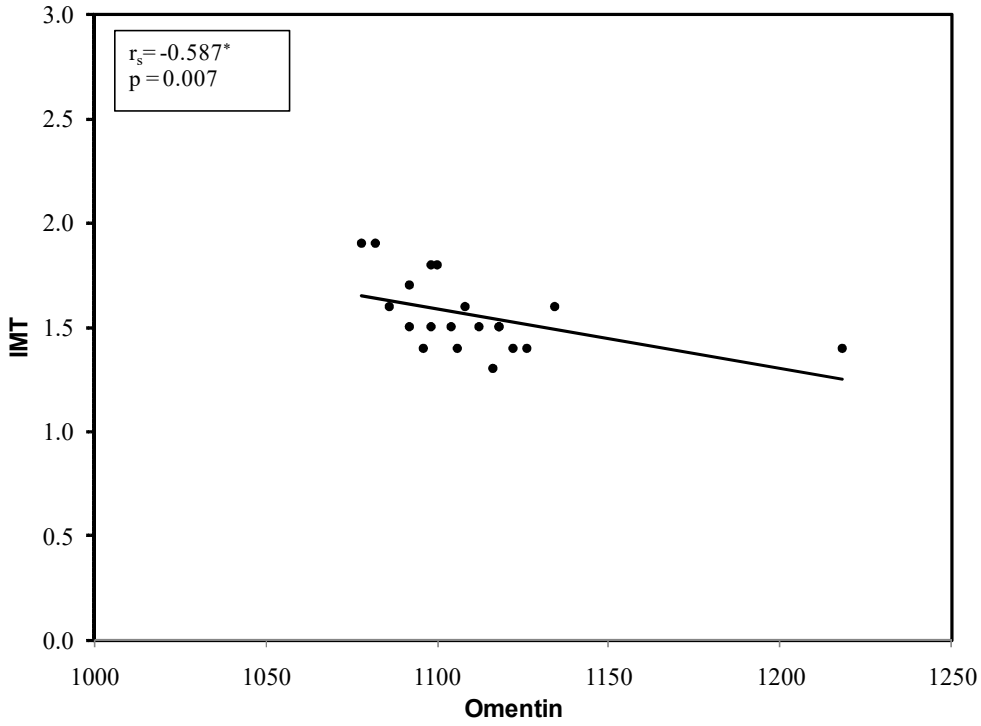

Figure (1): Correlation between Omentin with IMT

r_s: Spearman coefficient

*: Statistically significant at p ≤ 0.05

Table (V): Multivariate linear regression analysis of variables associated with Omentin-1 levels

	B	SE	t	p
CIMT	-140.01	45.37	3.086*	0.005*
ABI	-23.467	87.41	0.268	0.790
BMI	-1.237	3.75	0.330	0.744
W/H ratio	-288.92	224.72	1.286	0.209
HbA1C	-27.92	13.5	2.067*	0.048*
FPG	0.820	0.52	1.577	0.126
PPPG	-0.459	0.34	1.361	0.184
Total cholesterol	-0.497	0.58	0.862	0.396
LDL-c	0.216	0.70	0.311	0.758
TG	-0.293	0.42	0.697	0.491
Hs CRP	-2.115	15.11	0.140	0.890
R = 0.957, R^2 = 0.916, F = 27.744*,p <0.001*				

B: Unstandardized Coefficients, SE: Standard error, t: t-test of significance, R: coefficient or regression, R2: coefficient or regression, F: F test (ANOVA), *: Statistically significant at p ≤ 0.05, CIMT: carotid intima media thickness, ABI: ankle brachial index, BMI: body mass index, W/H ratio: waist/hip ratio, FPG: fasting plasma glucose, PPPG: postprandial plasma glucose, LDL-c: low-density lipoprotein cholesterol, HDL-c: high density lipoprotein cholesterol. TG: triglycerides, Hs-CRP: high sensitivity C reactive protein.

Table (VI): Multivariate linear regression analysis of variables associated with CIMT

	B	SE	t	p
Omentin-1	-0.002	0.001	3.232*	0.003*
Ankle brachial index	-0.506	0.295	1.716	0.097
BMI	-0.019	0.013	1.467	0.153
W/H ratio	-0.051	0.818	0.062	0.951
HbA1C	-0.057	0.049	1.163	0.254
FPG	0.001	0.002	0.389	0.700
PPPG	0.000	0.001	0.265	0.793
LDL-c	0.000	0.001	0.267	0.791
TG	0.000	0.001	0.168	0.868
Hs-CRP	0.034	0.053	0.636	0.530
R = 0.920, R^2 = 0.847, F = 16.021*,p <0.001*				

B: Unstandardized Coefficients, SE: Standard error, t: t-test of significance, R: coefficient or regression, R2: coefficient or regression, F: F test (ANOVA), *: Statistically significant at p ≤ 0.05, CIMT: carotid intima media thickness, ABI: ankle brachial index, BMI: body mass index, W/H ratio: waist/hip ratio, FPG: fasting plasma glucose, PPPG: postprandial plasma glucose, LDL-c: low-density lipoprotein cholesterol, HDL-c: high density lipoprotein cholesterol. TG: triglycerides, Hs-CRP: high sensitivity C reactive protein.

Discussion:

The relationship between DM and atherosclerotic diseases is well established[29]. That is why diabetes is considered a cardiovascular disease[30]. Adipokines are implicated in several body functions including cardiovascular functions[31] and insulin resistance[32]; this could be triggered through obesity induced inflammation and signaling pathways[33]. An abundance of adipokines has been associated with development of atherosclerosis and cardiovascular diseases, while others could be protective[34-37]. Omentin-1, also named intelectin-1, is a secretory protein newly identified in 2005. It is selectively expressed in visceral adipose tissue[36]. It was found that decreased omentin-1 levels are associated with increasing obesity and insulin resistance in human body. The combination of obesity and diabetes mellitus, the so-called ‘‘diabesity’’, is increasing the risk of cardiovascular complications[15].

Our study showed that serum omentin-1 level in newly diagnosed diabetics without history of macrovascular complications is significantly lower than the control group and it is much lower in diabetics with history of macrovascular complications. This is in agreement with Pan et al.[38] who found that serum omentin-1 levels were decreased in subjects with impaired glucose regulation. Moreover, this is consistent with Zhang et al.[39] who demonstrated that serum omentin-1 levels significantly decreased in type 2 diabetes patients compared to normal controls. Our results are also supported by a study done by Yoo et al.[40] that rev ealed that serum omentin-1 levels were significantly decreased in type 2 diabetes patients than control.

We also noticed that omentin-1 level was inversely correlated with HbA1c. This is in agreement with Gursoy et al.[41] and Kilic et al. [42] It was demonstrated that recombinant omentin enhanced the uptake of glucose in isolated adipocytes and dramatically increased the insulin induction of Akt/PKB phosphorylation[13]. Pan et al. speculated that decreased serum omentin-1 levels observed in Type 2 diabetes might cause a reduction of insulin-stimulated glucose uptake in visceral and subcutaneous adipocytes or other insulin-sensitive tissue[38]. Moreover, Tan et al.[43] showed that plasma glucose and insulin levels could regulate omentin-1 synthesis directly or indirectly. Again, these facts can elucidate the negative correlation, observed in our study, between

HbA1c and Omentin-1, which was maintained in the multivariate analysis.

In the current study, we demonstrated that omentin-1 is negatively correlated with CIMT in both diabetics and controls. In conformity with our results, Liu et al.[44] showed that omentin-1 was inversely correlated with CIMT in subjects with metabolic syndrome. Moreover, Shibata et al.[18] proved that Circulating omentin-1 levels correlated negatively with max-CIMT and mean-CIMT in 100 apparently healthy Japanese men. In addition, Yoo et al. showed that low circulating omentin-1 was proved as an independent determinant of carotid plaque existence among type 2 diabetic patients[40] as it was reduced in type 2 diabetes patients with carotid plaque compared to those without.

In harmony with the above mentioned studies, Shang et al.[45] demonstrated that serum omentin-1 levels were inversely associated with the presence and angiographic severity of CAD in Metabolic Syndrome patients and that serum omentin-1 might be a potential biomarker to predict the development and progression of CAD in such a cohort of patients. This was supported by Liu et al.[44] who showed that levels of omentin-1 were lower in Metabolic Syndrome than in controls and reduced further in [metabolic syndrome and atherosclerosis] compared with [metabolic syndrome without atherosclerosis].

In contrast to our results, only one recent study has shown that elevated omentin-1 is a strong predictor of cardiovascular events independently from the presence and extent of baseline CAD[46]. On the other hand, Kadoglou et al.[47] showed that low omentin-1 level was positively associated with either low carotid plaque echogenicity index score [GSM score] or carotid-related symptoms; but not after multivariate analysis. They recommended that further investigations were required to study the association of low serum omentin-1 with carotid plaque echolucency.

From our data, ankle/brachial index was positively correlated with circulating omentin-1 level in diabetic patients, however, this was not maintained in the multivariate analysis. This came in consistency with both Yoo et al.[40] who confirmed the positive correlation between omentin and ankle /brachial index after studying the impact of circulating omentin-1 level on arterial stiffening in patients with type 2 diabetes mellitus. Also Bubenek et al.[48] found that ankle/brachial index was significantly correlated positively with omentin-1 during analyzing the results of their study which was performed to assess the gene expression of omentin in peripheral occlusive arterial disease patients .

In this study, we showed that hs-CRP was negatively correlated with serum omentin-1 level in the study groups. This finding came in consistency with results from Tan et al.[15] and Yilmaz et al.[49] studies. Moreover, Moreno-Navarrete et al.[50] demonstrated a negative association between omentin and circulating IL-6 and CRP; both of them are related endothelial dysfunction and atherosclerosis.

A vasodilator effect of omentin-1 was detected on isolated blood vessels. It is suggested that omentin-1 directly induces endothelium-dependent vasodilatation, mediated via endothelium- produced nitric oxide [NO], as it promotes activation of the Akt signaling pathway, modulating the function of NO synthase in the endothelium[51]. It is also reported that omentin suppresses TNF-α stimulated cyclooxygenase-2 expression in cultured endothelial cells through its ability to reduce c-Jun N-terminal kinase activation, thus modulates vascular inflammatory state[52]. Moreover, it was shown that circulating omentin contributed independently to the variance of endothelium-dependent vasodilatation after controlling for age and CRP in subjects with impaired glucose tolerance[51].

In addition, Omentin-1 causes inhibition of endothelial ICAM-1 and VCAM-1 expression via interruption of NF-Kß signaling pathway and suppression of adhesion of monocytes to TNF-α activated endothelial cells[53]. Recently, Xie et al.[54] investigated the effects of omentin-1 on arterial calcification and bone metabolism in vivo and concluded that omentin-1 ameliorates arterial calcification and bone loss in vivo through the regulation of the RANK signaling pathway. All these actions at the molecular level help us explain our results that presume a protective role for omentin-1 against atherosclerosis.

Our results showed also that plasma omentin-1 levels were inversely correlated with BMI and waist circumference. In other words, decreased omentin-1 levels were associated with increasing general and central obesity. These findings are in congruence with the results published by de Souza Batista et al. [16] and Moreno-Navarrete et al.[17] Also, Cai et al.[55] found that the omentin-1 mRNA was negatively correlated with body weight, WHR and BMI.

Another finding in our study that omentin-1 was inversely correlated with total cholesterol, LDL-c and TG. Similarly, in a study done by Saremi et al.[56], there were inverse correlations between omentin-1 and each of total cholesterol and triglyceride. Consonant with this, Shibata et al.[18] showed that Plasma levels of omentin-1 correlated negatively with total cholesterol levels.

Conclusions and Limitations:

Omentin-1 level is significantly lower in diabetic patients with macrovascular complications than newly diagnosed diabetics without complications and the controls. Omentin-1 is an independent negative predictor of CIMT, and thus could be considered as an indicator for atherosclerosis. HbA1c and CIMT are the independent factors that negatively influence serum omentin-1 level. This study results are is limited by its cross-sectional design and the relatively small number of subjects.

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Original Article

Study of the Association of Serum Cystatin C and Plasma Fibronectin with Diabetic Nephropathy in Patients with Type 2 Diabetes Mellitus.

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Abstract:

Background: Diabetic nephropathy is the most frequent cause of end-stage renal disease (ESRD), thus many markers was studied for early diagnosis of diabetic nephropathy. This study was done to evaluate clinical usefulness of serum cystatin C levels and plasma fibronectin for early detection of renal impairment in patients with type 2 diabetes and to evaluate the association between serum cystatin C and plasma fibronectin with albuminuria. **Methods:** A prospective, randomized, double-blinded study was performed on 50 type2 diabetic patients divided into two groups, groub1 included 25 type2 diabetic patients with nephropathy and group2 include 25 type2 diabetic patients without nephropathy as a control group. Serum cystatin C, plasma fibronectin, Serum creatinine, serum lipids, glycosylated hemoglobin and urine analysis included albumin creatinine ratio were measured. GFR was estimated by Cockcroft and Gault formula. **Results:** The study showed a statistically significant difference between the two studied groups according to serum cystatin C for early detection of diabetic nephropathy (P<0.001). On the other hand according to plasma fibronectin there was no statistically significant difference between the two studied groups (P=0.960). Regarding albuminuria there was a significant correlation between cystatin C and albumin creatinin ratio in diabetic nephropathy group (P<0.001) but there was no significant correlation with plasma fibronectin (P=0.334). **Conclusion:** Serum cystatin C was significantly higher in albuminuric type2 diabetic patients compared to normoalbuminuric patients and can be also used as an early marker of of diabetic nephropathy. More studies needed to be applied on plasma fibronectin as our study results that it could be a weak marker for early diabetic nephropathy.

Keywords: Diabetic nephropathy, Cystatin C, Fibronectin, Albuminuria.

Introduction:

Diabetic nephropathy (DN) is one of the microvascular complications of diabetes, it is characterized by a progressive increase in proteinuria and decline in GFR with a high risk of cardiovascular morbidity and mortality.[1-3] Diabetic nephropathy occurs in 20–40% of patients with diabetes and is a leading cause of ESRD worldwide. Patients with persistent albuminuria (30–299 mg/24 h) who progress to more significant levels (≥300 mg/24 h) are likely to progress to ESRD[4-7]. The terms microalbuminuria (30 – 299 mg/24 h) and macroalbuminuria (≥300 mg/24h) will no longer be used , but rather referred to as persistent albuminuria at levels (30 – 299 mg / 24 h) and levels ≥ 300 mg/24 h , with normal albumin excretion defined to be less than 30 mg / 24 h [8,9].Screening for increased urinary albumin excretion can be performed by measurement of the albumin to creatinine ratio in a random spot collection[10].Early diabetes is heralded by glomerular hyperfiltration and an increase in glomerular filtration rate (GFR). This is believed to be related to increased cell growth and expansion in the kidneys, possibly me diated by hyperglycemia itself. Increased albumin

excretion typically occurs after 5 years in type 1 diabetes. Overt nephropathy, with urinary protein excretion higher than 300 mg/day, often develops after 10 to 15 years. End stage renal disease (ESRD) develops in 50% of type 1 diabetic with overt nephropathy within 10 years[11]. Type 2 diabetes has a more variable course. Patients often present at diagnosis with increased albumin excretion because of delays in diagnosis and other factors affecting protein excretion. Without intervention, approximately 30% progress to overt nephropathy and, after 20 years of nephropathy, approximately 20% develop ESRD. Because of the high prevalence of type 2 compared with type 1 diabetes, however, most diabetics on dialysis are type 2 diabetics [12].

Cystatin C, a cysteine protease inhibitor, is freely filtered by the renal glomeruli, metabolized by the proximal tubule and identified as a promising marker of chronic kidney disease. Cystatin C is produced at a constant rate by nucleated cells and released into blood stream with a half-life of 2 hr. Its concentration is almost totally dependent on GFR.[13] Some studies have demonstrated that serum cystatin C is an early renal marker in diabetic patients, but not all studies have done so[14-16].

Fibronectins are large glycoproteins found in plasma, in extracellular matrix, and on cell surfaces. They promote cell-cell and cell-matrix interactions and thus play a role in tissue construction and reconstruction, the intravascular accumulation of cellular fibronectin reflects injury to blood vessels. Vessel wall damage with characteristic endothelial extracellular matrix changes has a role in development of microvascular complication and nephropathy in diabetic patient[17, 18].

Aim of the Study:

The aim of the study is to compare the level of serum Cystatin C and plasma fibronectin in patients with and without diabetic nephropathy and to Study the relationships between Cystatin C, fibronectin and other markers of diabetic nephropathy.

Patients and Methods:

After approval of Local Ethics Committee and with written informed consent, the present study was carried out in Alexandria University Hospital on 50 type2 diabetic patients divided into two subgroups: Subgroup I: 25 diabetic patients with diabetic nephropathy (Stage I to stage V). Subgroup II: 25 diabetic patients without diabetic nephropathy (i.e. normal GFR and urinary albumin creatinine ratio ‹ 30 mg / 24) as a control group. Evaluation of patients was carried out through proper history taking, clinical examination and routine laboratory investigations. Regarding cystatin C analysis, after 30 minutes incubation and washing, polyclonal anti-human cystatin C antibody, conjugated with horseradish peroxidase (HRP) is added to the wells and incubated for 30 minutes with captured cystatin C. Following another washing step, the remaining HRP conjugate is allowed to react with the substrate solution (TMB). The reaction is stopped by addition of acidic solution and absorbance of the resulting yellow product is measured. The absorbance is proportional to the concentration of cystatin C. A standard curve is constructed by plotting absorbance values against concentrations of cystatin C standards, and concentrations of unknown samples are determined using this standard curve. Regarding fibronectin analysis, fasting overnight venous blood sample (about 5 ml) was drawn from each control and diabetic nephropathy individuals. The blood was collected into precooled plastic or siliconized tubes containing 3.2% buffered citrate in a ratio of 1:10 (blood: anticoagulant). Centrifuge within 30 minutes after the puncture at 2000g for 10min. at 4°C. Pipette off the plasma. Store as aliquots at temperature below -30°C.

Statistical Analysis:

Data were fed to the computer and analyzed using IBM SPSS software package version 20.0.[19]

Qualitative data were described using number and percent. Quantitative data were described using range (minimum and maximum), mean, standard deviation and median. Comparison between different groups regarding categorical variables was tested using Chi-square test. The distributions of quantitative variables were tested for normality using Kolmogorov-Smirnov test, Shapiro-Wilk test and D'Agstino test, also Histogram and QQ plot were used for vision test. If it reveals normal data distribution, parametric tests was applied. If the data were abnormally distributed, non-parametric tests were used. For normally distributed data, comparison between two independent populations were done using independent t-test. Correlations between two quantitative variables were assessed using Pearson coefficient. For abnormally distributed data, comparison between two independent populations were done using Mann Whitney. Correlations between two quantitative variables were assessed using Spearman coefficient. Significance of the obtained results was judged at the 5% level.

Results:

Fifty type2 diabetic patients were enrolled in the study. All patients completed the study. Group I included 25 type2 diabetic patient with nephropathy and Group II 25 type2 diabetic patient without nephropathy. There was no significant difference in anthropometric measures (weight, height and BMI) between the two groups). There was significant difference in renal functions (creatinin, urea and albumin creatinin ratio) between the two groups. There was significant difference in GFR between the two groups. There was significant difference in cystatin between the two groups. There was no significant difference in fibronectin between the two groups. There was significant difference between Cystatin C and Albumin creatinine ratio in diabetic nephropathy group ($p<0.001$). With no significant difference between fibronectin and albumin creatinine ratio in diabetic nephropathy group ($p=0.334$).

Table (I): Comparison between the two studied groups according to anthropometrics

	Diabetic Nephropathy (n = 25)	**Control (Diabetic only) (n = 25)**	**T**	**P**
Weight (kg)				
Min. – Max.	69.0 – 97.0	70.0 – 90.0		
Mean ± SD.	80.64 ± 8.24	78.52 ± 5.74	1.056	0.296
Median	80.0	79.0		
Height (cm)				
Min. – Max.	165.0 – 184.0	160.0 – 132.0		
Mean ± SD.	174.28 ± 5.37	95.20 ± 17.61	1.045	0.301
Median	172.0	95.0		
BMI (kg/m2)				
Min. – Max.	24.16 - 29.41	21.13 - 30.10		
Mean ± SD.	26.49 ± 1.58	26.42 ± 1.91	0.140	0.890
Median	25.95	26.23		

t: Student t-tes.

Table (II): Comparison between the two studied groups according to renal function

	Diabetic Nephropathy (n = 25)	Control (Diabetic only) (n = 25)	Test of sig.	P
Creatinin (mg/dl)				
Min. – Max.	1.20 – 12.0	0.10 – 1.40		
Mean ± SD.	4.35 ± 2.82	0.88 ± 0.29	Z=6.001*	<0.001*
Median	3.0	0.90		
Urea (mg/dl)				
Min. – Max.	36.0 – 214.0	20.0 – 50.0		
Mean ± SD.	110.16 ± 43.70	38.60 ± 8.23	t=8.046*	<0.001*
Median	105.0	40.0		
ALB Creatinin(mg/dl)				
Min. – Max.	60.0 – 2597.0	7.60 – 133.0		
Mean ± SD.	417.46 ± 510.97	19.85 ± 23.88	Z=5.968*	<0.001*
Median	265.0	16.50		

t: Student t-test

Z: Z for Mann Whitney test

*: Statistically significant at p ≤ 0.05

Table (III): Comparison between the two studied groups according to eGFR

	Diabetic Nephropathy (n = 25)	Control (Diabetic only) (n = 25)	Z	P
GFR (ml/ min)				
Min. – Max.	5.83 – 62.20	52.0 – 129.20		
Mean ± SD.	27.25 ± 17.23	93.43 ± 20.19	6.005*	<0.001*
Median	27.20	89.50		

Z: Z for Mann Whitney test

*: Statistically significant at p ≤ 0.05

Table (IV): Comparison between the two studied groups according to cystatin C

	Diabetic Nephropathy (n = 25)	Control (Diabetic only) (n = 25)	T	P
Cystatin C (0.62-1.11ng/L)				
Min. – Max.	0.95 – 2.58	0.66 – 1.27		
Mean ± SD.	1.55 ± 0.41	0.95 ± 0.18	6.812*	<0.001*
Median	1.47	0.96		

t: Student t-test

*: Statistically significant at p ≤ 0.05

Table (V): Comparison between the two studied groups according to fibronectin

	Diabetic Nephropathy (n = 25)	Control (Diabetic only) (n = 25)	T	P
Fibronectin (25 - 40µg/ml)				
Min. – Max.	71.0 – 129.0	72.0 – 132.0		
Mean ± SD.	94.96 ± 15.66	95.20 ± 17.61	0.051	0.960
Median	94.0	95.0		

t: Student t-test

*: Statistically significant at p ≤ 0.05

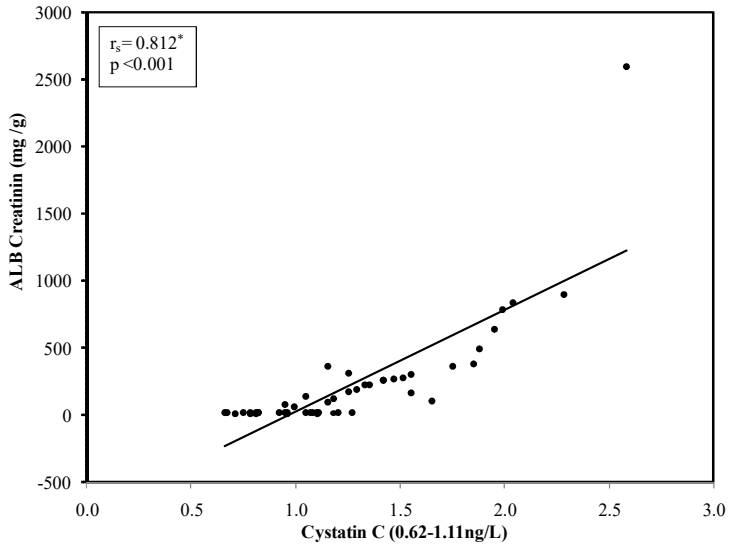

Figure (1): Correlation between cystatin C and ALB creatinine

Discussion:

Diabetic nephropathy (DN) is one of the most common microvascular complications of DM, greatly affecting the life quality and survival of the patients. As global prevalence of diabetes is steadily increasing, the number of patients with DN is expanding day by day. DN is now the leading cause of ESRD, a disease that I s described as a medical catastrophic of worldwide dimensions.[20] Morphological changes are known to start earlier DN than laboratory abnormality. Also some patients with albuminuria have normal renal structure, while some normoalbuminuric diabetic have well established nephropathic lesions. Also albumin excretion rate is a predictor of renal disease in hypertension and cardiovascular diseases, so it is not a sensitive marker for DN.[21] Measuring GFR is the best functional parameter in renal disease using creatinine clearance. This requires 24h urine collection and blood sample and measure creatinine level in blood and urine and volume of urine. There are several factors that may interfere with the accuracy of the test like incomplete collection of urine. Other methods of assessment of GFR are Cockcroft-Gault formula or MDRD but also there were not accurate methods.[22] Thus, we are still in need of identifying earlier marker early markers of DN needed to be identified. It has been reported that a decline in the renal function of patients with diabetes was not always

accompanied by an increased AER. To overcome these limitations, many clinicians additionally used creatinine in evaluating such patients. However, serum creatinine also depends on creatinine production, extrarenal elimination and tubular handling. Therefore, other biomarkers for estimation of renal function have been searched for, and one of them can be cystatin C. In our study, we measured the serum level of cystatin C and plasma level of fibronectin in different stages of DN and comparing them with ACR and eGFR to detect the usefulness of using those markers to diagnose DN and detect the progression of the disease in T2DM patients.

Data presented in our study dealt with 25type 2 diabetic patients with nephropathy (stage I to stage V). as cases and 25 diabetic patients without nephropathy (i.e. normal GFR and urinary albumin creatinine ratio (30 mg / 24) as a control group.

In our study, there was no significant correlation between serum cystatin C and the age in the control group and the patients group (P=0. 0.791).This may reflected the important of cystatin C to use as renal function test in elderly or even in very young patients. In contrast to serum creatinine is highly affected by the age. This data matched with Filler G et al[23] who found that measuring of cystatin C not affected by the age.

In contrast, Christensson AG et al[24] reported that there was significant positive correlation noticed between serum cystatin C and age.

In our study, there was no significant correlation between serum cystatin C and the sex in the control group and the patients groups (P=0.499).

This data matched with Erlandsen JE et al[25] and Uhlmann EJ et al[26] whom reported that there was no significant difference as regarding measuring cystatin C in both gender.

In contrast, some authors like Croda-Todd et al[27] and Finney H et al[28] found that there were statistically significant differences as regarding measuring cystatin C in adults' males and females and was higher in male gender than female.

From the previous data, cystatin C is another marker of renal function that has been proposed as potentially superior to serum creatinine level for estimating renal function, because it is thought to be produced at a constant rate by most nucleated cells. Moreover, cystatin C production has been reported to be not affected by age, gender, or muscle mass. In contrast serum creatinine level is commonly used to estimate renal function. Serum creatinine is not only determined by its renal excretion, but also by its production in muscular tissue, which is dependent on age, weight, and gender. Therefore, when using serum creatinine level to estimate renal function, one needs to adjust for these factors.[29]

In our study, there was a statistically significance difference between patients group and control group regarding blood urea (P<0.001). Blood urea is not an accurate marker for measuring renal function. It is filtered by glomerulus and reabsorbed by the renal tubule. Concentration of urea in serum could vary with diet, also can increased in dehydration associated with poor glycemic control that cause polyuria.

Regarding serum creatinine in diabetic nephropathy group,it ranges from 1.20 – 12.0 with a mean 4.35 ± 2.82 and in control group without nephropathy it ranges from 0.10 – 1.40 with a mean 0.88 ± 0.29.So in our study there was a statistically significant difference between patients groups and control group as P value <0.001 .

In contrast to our results, Vervoot G et al[30] showed significant decreases in serum creatinine concentrations among patient group compared to control group. These

results may be explained based on glomerular hyperfiltration that may develop at initial stages of the DN.

In our study, either serum creatinine or blood urea have no significance correlation with serum cystatin C.

The same result was reported by Plebani M, Dall'Amico R, Mussap M, et al whom reported that Cystatin C and creatinine are probably two independent markers of GFR.

In contrast to our result, Tian et al[31] reported that there was significant positive correlation between serum cystatin C and serum creatinine suggesting that serum cystatin C increased similar to serum creatinine for measuring renal function in diabetic patients.

In our study, there was a statistically significance difference between patients group and control group regarding albumin creatinine ratio (P<0.001). As classical evaluation of DN includes appearance of albuminuria, decreased creatinine clearance and increased serum creatinine.

Albuminuria expresses underlying endothelial dysfunction as presence of chronic hyperglycemia cause disruption of the endothelial permeability through production and activation of mediators such as ROS, VEGF and proinflammatory cytokines. This disturbance of endothelial cell podocyte communication contributes to and amplifies the endothelial lesions, leading to albuminuria.

With more progression, there is increase in urinary albumin excretion due to underlying inflammatory process with excess production of growth factor, deposition of ECM with subsequent interstitial fibrosis, and glomerulosclerosis leading to renal function deterioration. In contrast to control group with subsequent less or insignificant inflammatory process in the kidney, the level of ACR did not correlate significant with renal function.

In our study, there was strong positive correlation between serum cystatin C and ACR in patients group (P<0.001) and there was no correlation in the normoalbuminuric control group (P=0.517).

In agreement with these results, Mojiminiyi et al[32] found that cystatin C was significantly higher in patients with DN than in normoalbuminuric diabetic patients, which can be explained in that there is already impaired renal function in DN patients.

In addition, Yang et al[33] reported that serum cystatin C concentration increased significantly in patients from normoalbuminuric to patients with increase albumin excretion rate.

In our study, we used Cockcroft-Gault formula for measuring eGFR. There was significant difference between the patients groups and control group (P<0.001).

The current Kidney Disease Outcomes Quality Initiative (K/DOQI) guidelines advocate creatinine based equations for estimating GFR to identify patients with potential kidney disease and to classify them into different stages on the basis of these results. These stages also include individuals with normal or near-normal GFR. Such stratification requires an accurate and precise measurement of GFR that is inexpensive, reliable, and widely available.[34] In our study, there was a significant negative correlation between eGFR with serum cystatin C (P<0.001). As cystatin C is produced at a constant rate by nucleated cells and released into the blood stream with a half-life of ~2 h and is freely filtered and almost completely taken up and degraded, but not secreted, by proximal tubular cells, so with the occurrence of pathological changes in diabetic kidney the filtration capacity is decreased with subsequent retention of cystatin C and increased serum level.

The result of previous studies of the role of cystatin C in detecting early renal failure in

diabetic patients was contradictory. Some authors showed that cystatin C was more effective than creatinine in detecting initial reduction of GFR in T2DM as well as in T1DM as Mussap M et al[34], Xia LH et al[35], and Harmoinen et al[36] showed that serum cystatin C was more sensitive than serum creatinine for estimation of GFR in T2DM patients and Tan et al[37] showed the same in T1DM patients.

In contrast to these result, Oddoze et al[38] found that serum creatinine was better than serum cystatin C for the estimation of GFR in albuminuric and proteinuric diabetic patients. Oddoze et al selected a heterogeneous group of type 1 and type 2 diabetic patients.

In general, unlike healthy subjects, diabetic patients are continuously exposed to the various metabolic and hemodynamic risks associated with this disease. Recent studies have mainly focused on tubular damage, which is known to correlate with acute kidney injury in patients with DN. (39) Some cross-sectional studies have reported that several tubular markers increase more in diabetic patients than in healthy controls, and this correlated with the severity of albuminuria.[40]

In our study, there was statistically significant relationship of serum cystatin C in patients groups in comparison to control group (P<0.001).

In my opinion the strong correlation between serum cystatin C in the patients group was due to decrease eGFR in this group in comparing to normoalbuminuric control group. From our study, we can consider that serum cystatin C can be used as albuminuria for detection of DN.

In contrast to our study, Jeon YK[41] found that in normoalbuminuric patients, the cystatin C levels of serum was significantly increased in patients with GFR ≤ 60 mL/min/1.73 m2 than those with GFR > 60 mL/min/1.73 m2. It was thought that this increment was probably due to the tubular phase before glomerular manifestation. He suggested that the cystatin C levels of serum was related to subclinical tubular impairment and can be an earlier measurable marker of renal involvement before onset of albuminuria.

Another marker which can be increase in type 2 diabetic patients is fibronectin, and in this study we show the relation between plasma fibronectin and other markers of diabetic nephropathy. Plasma levels of circulating cellular fibronectin could reflect matrix changes and hence vessel wall damage in patients with diabetes, high levels of plasma fibronectin may be the effect of a widespread endothelial injury of small vessels, which early involves the renal glomerular microvasculature and may affect urinary albumin excretion and development of nephropathy.

In our study, There was statistically significant correlation between plasma fibronectin and (age and HbA1c), (P=0.016, <0.001 respectively).

There was no statistically significant correlation between plasma fibronectin and ACR, eGFR and s.Cr (P=0.334, 0.736, 0.152 respectively).

In agreement with our study, Takahashi et al.[42] found no relation between plasma fibronectin in diabetic patients and albuminuria.

On the other hand, DeGiorgia et al.[43] found a significant correlation between the PF with non-insulin dependent diabetes mellitus. Although Skrha et al. did not evaluate the intact plasma fibronectin; they found a positive correlation between the plasma free N-terminal fibronectin 30-kDa domain and albuminuria.

More studies needed to be applied on plasma fibronectin as our study results that it could be a weak marker for early diabetic nephropathy.

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Original Article

The Role of Urinary Connective Tissue Growth Factor in Early Detection of Diabetic Nephropathy in Elderly Type 2 Diabetics.

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Abstract:

Levels of connective tissue growth factor (CTGF; CCN-2) in plasma are increased in various fibrotic disorders, including diabetic nephropathy. Recently, several articles have reported a strong increase of urinary CTGF excretion (U.CTGF) in patients with diabetic nephropathy. **Objective:** This study evaluated U.CTGF in elderly patients with type 2 diabetics and its correlation with kidney functions tests, and degree of albuminuria. **Subjects:** This study included 45 elderly type 2 diabetic patients (group I) and compared with 15 elderly healthy controls (group II). **Method:** All subjects in this study were subjected to thorough history taking and clinical examination. Laboratory investigations done included: CBC, blood urea, serum creatinine, eGFR using MDRD method, ACR, complete urine analysis, fasting FBG, 2hppg, HbA1c, U.CTGF was measured by sandwich ELISA. **Results:** U.CTGF in patients with microalbuminuria (mean 5.05±1.14 pg/mg creatinine [range 3.70 – 7.50]) was significantly higher than those with microalbuminuria (mean 1.71±0.22 pg/mg creatinine [range 1.50– 2.20]), norm albuminuria (mean 0.25±0.05 pg/mg creatinine [range 0.20 – 0.30]), and control subjects (mean 0.17 ± 0.06 pg/mg creatinine [range 0.10 – 0.30]). **Conclusions:** U.CTGF level increased in elderly type 2 diabetes. The observed association of U.CTGF with albumin creatinine ratio (ACR) and glomerular filtration rate (GFR) might reflect a role of CTGF as a progression promoter in diabetic nephropathy.

Keywords: Connective Tissue Growth Factor, Diabetic Nephropathy, Elderly, T2DM.

Introduction:

Connective tissue growth factor (CTGF), a cysteine-rich, heparin-binding 38 KD growth factor protein[(1)] is expressed in mesenchymal cells such as fibroblasts and smooth muscle cells.[(2)] In these latter cell types CTGF is up-regulated by TGF-β, which acts through a distinct element in the promoter region of the CTGF gene.[(3)]

Connective tissue growth factor (CTGF) has been associated with numerous fibrotic disorders, but it is of particular interest to diabetic nephropathy. Soon after its identification, the expression of CTGF mRNA was shown to be strongly up-regulated in human mesangial cells cultured under high glucose and in renal biopsies of patients with diabetic nephropathy.[(4)]

Abbreviations:

CBC: complete blood count, e **GFR:** estimated glomerular filtration rate, **ACR:** urinary albumin creatinine ratio, **FBG:** fasting blood glucose, 2hppg: 2hours postprandial blood glucose

Connective tissue growth factor (CTGF) over expression in podocytes was critically involved in diabetes-related GBM thickening. Once induced, CTGF is able to initiate changes in ECM composition: it increased expression of fibronectin and enhanced fibronectin assembly into an insoluble matrix, increased type IV, type III and type I collagen production and up-regulated integrins on the cell surface, facilitating the deposition and assembly of ECM proteins. (5)

Understanding the different determinants of elevated U.CTGF in diabetes is essential for its proper interpretation as a biomarker. In the healthy kidney, filtered CTGF is almost completely reabsorbed in the proximal tubules by megalin-mediated endocytosis and that impairment of tubular reabsorption results in increased urinary excretion of CTGF. Reduced proximal reabsorption of U.CTGF, increased release of CTGF from damaged distal tubules also play a role with the majority of U.CTGF originating from an intrarenal source.(6)

CTGF levels were markedly increased in both plasma and urine of patients with diabetic nephropathy, but not in diabetic patients without nephropathy.(7) Therefore, we set out to analyze U.CTGF in elderly patients with type 2 diabetes and how U.CTGF levels relate to clinical parameters associated with severity of diabetic nephropathy.

Research Design and Methods:

For the present study, CTGF levels were determined in early morning voided spot urine samples from 45 elderly type 2 diabetic patients and 15 healthy control subjects. Patients were selected from the Renal and /or Diabetes Outpatient Clinics of Alexandria Main University Hospital for a study of diabetic nephropathy. Diabetic patients were categorized as having normoalbuminuria when ACR was persistently < 30 mg/g creatinine; microalbuminuria when ACR was between 30 and 300 mg/g creatinine of at least two of three consecutive visits to the outpatient clinic; macroalbuminuria if they had persistent albuminuria >300 mg/g creatinine and no other kidney or renal tract disease. Demographic and clinical data were recorded, including age, sex, and duration of diabetes, weight, height, blood pressure and medication. ACR was determined in early morning voided spot urine samples by turbidimetry.(8) In venous blood samples, serum creatinine, blood urea was determined, (9) and HbA1c was measured by affinity chromatography.(10) Estimated glomerular filtration rate (eGFR) was calculated by the Modification of Diet in Renal Disease study equation (MDRD).(11) The study was performed after approval of the ethical committee of Alexandria University. All patients and control subjects gave their informed consent.

Enzyme-linked immunosorbent assay for U.CTGF

Urine content of CTGF was determined by a sandwich ELISA using 2 distinct monoclonal antibodies against the CTGF protein. The catching and detecting monoclonal antibodies bind distinct epitopes on the N-terminal half of the protein. This assay detects both CTGF N-terminal half fragments and the full length CTGF protein with detection limit of approximately

31.2-2,000 pg/ml. The microplate reader was read at 450 nm immediately. [12]

Statistical analysis:

Data were fed to the computer and analyzed using IBM SPSS software package version 20.0.Qualitative data were described using number and percent. Quantitative data were described using range (minimum and maximum) mean, standard deviation and median. Comparison between different groups regarding categorical variables was tested using Chi-square test. For normally distributed data, comparison between the different groups were done using F-test (ANOVA) and Post Hoc test (Tukey's test) for pair wise comparison while for abnormally distributed data, comparison were done using Kruskal Wallis test and pair wise comparison was assessed using Mann-Whitney test.

Results:

U.CTGF excretion is increased in patients with diabetic nephropathy

General characteristics and clinical parameters of healthy subjects and diabetic patients are summarized in Table I. The U.CTGF/Creatinine ratio in macroalbuminuric group (mean 5.05 ± 1.14 pg/mg creatinine) was significantly higher than in microalbuminuric group (mean 1.71 ± 0.22 pg/mg creatinine) and normoalbuminuric group (mean 0.25±0.05 pg/mg creatinine) and control subjects (mean 0.17±0.06 pg/mg creatinine). A significant difference in U.CTGF/Creatinine ratio was observed between all studied groups and control ($p \leq 0.001$). There was statistically significant difference between normoalbuminuric group, microalbuminuric group and macroalbuminuric. There was statistically significant difference between microalbuminuric group and macroalbuminuric ($p < 0.001$).

U.CTGF/Creatinine correlates with albuminuria (ACR) and with declined renal function as regard eGFR (tables II,III)

U.CTGF correlated with ACR in all patients ($R=0.979$, $P < 0.001$) and also in the subgroups of microalbuminuria ($R= 0.981$, $P <0.001$) and macroalbuminuria ($R= 0.999$, $P <0.001$).

We estimated GFR using Modification of Diet in Renal Disease study method described by Levey et al.[11] U.CTGF inversely correlated with estimated GFR in all patients ($R= 0.796p < 0.001$). When subgroups of patients were examined separately, this correlation was strongest in macroalbuminuric group ($R=-0.966$, $p < 0.001$) and also present in microalbuminuric group ($R= - 0.741$, $p = 0.002$).

Correlations between U.CTGF/Creatinine and different studied clinical Parameters (tables II,III)

In this study there was significant correlation between U.CTGF/Creatinine ratio, duration, HbA1c, Creatinine, systolic, and diastolic blood pressure in all patients ($p < 0.001$).

By analysis of these data in each group of this study we found that: the Correlation between U.CTGF/Creatinine ratio with duration, HbA1c, S. Creatinine, systolic and diastolic blood pressure was statistically significant only in microalbuminuric group (duration $p = 0.001$), (HbA1c $p = 0.008$), (S. Creatinine $p = 0.015$), (systolic $p = 0.001$), (diastolic $p < 0.001$).

Table (I): Comparison between the different studied groups according to different parameters

	Normal (Control) (n = 15)	Albuminuria			p
		Normo (n = 15)	Micro (n = 15)	Macro (n = 15)	
Sex					
Male	7 (46.7%)	7 (46.7%)	6 (40.0%)	8 (53.3%)	0.911
Female	8 (53.3%)	8 (53.3%)	9 (60.0%)	7 (46.7%)	
Age (in years)	62.93 ± 2.15	63.80 ± 3.63	62.67 ± 2.55	62.0 ± 1.81	0.316
Duration (in years)	-	1.0 (0.42 – 3.0)	5.0 (3.0 – 7.0)	12(10.0 – 15.0)	<0.001*
Sig.bet. Grps	-	p_1<0.001*,p_2<0.001*,p_3<0.001*			
FBG (mg/dl)	96.60 ± 8.69	190.47 ± 39.40	189.13 ± 33.12	250.33 ± 44.87	
$p_{Con.}$	-	<0.001*	<0.001*	<0.001*	<0.001*
Sig.bet. Grps	-	p_1=1.000, p_2<0.001*, p_3<0.001*			
Hb A1C %	5.0 – 5.80	8.25 ± 0.45	8.60 ± 0.76	9.78 ± 1.05	
$p_{Con.}$	-	<0.001*	<0.001*	<0.001*	<0.001*
Sig.bet. Grps	-	p_1= 0.517, p_2<0.001*, p_3<0.001*			
S. Creat. (mg/dl)	0.70 (0.60 – 0.90)	0.70 (0.60 – 0.90)	0.80 (0.80 – 1.0)	1.10 (0.70 – 3.10)	
$p_{Con.}$	-	0.744	0.003*	<0.001*	<0.001*
Sig.bet. Grps	-	p_1= 0.001*, p_2<0.001*, p_3= 0.003*			
eGFR (ml/min/m^2)	98.11 ± 8.10	100.55 ± 14.64	82.72 ± 13.25	61.48 ± 25.0	
$p_{Con.}$	-	0.977	0.061	<0.001*	<0.001*
Sig.bet. Grps	-	p_1= 0.022*, p_2<0.001*, p_3= 0.004*			
ACR (mg/g creat)	2.60(1.80 – 6.50)	25.6(14.5 – 29.6)	68.4(45.9– 136.0)	2239(509.8-15425)	
$p_{Con.}$	-	<0.001*	<0.001*	<0.001*	<0.001*
Sig.bet. Grps	-	p_1<0.001* , p_2<0.001* , p_3<0.001*			
U. CTGF (pg/ml)	0.32 (0.25 – 0.38)	0.30 (0.20 – 0.65)	2.40 (1.50 – 6.20)	3.80 (1.90 – 8.50)	
$p_{Con.}$	-	0.414	<0.001*	<0.001*	<0.001*
Sig.bet. Grps	--	p_1<0.001*, p_2<0.001*, p_3= 0.048*			
U. CTGF/Creat. (picog/mg creat)	0.20 (0.10 – 0.30)	0.30 (0.20 – 0.30)	1.60 (1.50 – 2.20)	4.80 (3.70 – 7.50)	
$p_{Con.}$	-	0.001*	<0.001*	<0.001*	<0.001*
Sig.bet. Grps	-	p_1<0.001*, p_3<0.001*, p_3<0.001*			
Systolic (mmHg)	116.0 ± 9.49	128.67 ± 5.81	141.0 ± 8.49	146.67 ± 12.49	
$p_{Con.}$	-	0.003*	<0.001*	<0.001*	<0.001*
Sig.bet. Grps	-	p_1= 0.004*, p_2<0.001*, p_3= 0.357			
Distolic (mmHg)	77.67 ± 6.23	85.0 ± 4.23	92.0 ± 5.61	91.33 ± 7.43	
$p_{Con.}$	-	0.008*	<0.001*	<0.001*	<0.001*
Sig.bet. Grps	-	p_1= 0.012*, p_2= 0.027*, p_3=0.990			

Qualitative data was expressed using number and percent and was compared using Chi square or Monte Carlo test. Normally quantitative data was expressed in (Mean. ± SD) and was compared using F test (ANOVA) while for abnormally quantitative data expressed in Median (Min. – Max.) and was compared using Kruskal Wallis test.

$p_{con.}$: value for comparing between Normal (Control) and each other groups

p_1: value for comparing between Normo albumnuria with Micro albumnuria

p_2: value for comparing between Normo albumnuria with Macro albumnuria

p_3: value for comparing between Micro albumnuria with Macro albumnuria

Table (II): Correlation between U. CTGF/Creatinine with different studied parameters in total patients.

	U. CTGF/Creatinine	
	r_s	p
Age	-0.209	0.167
Sex (male = 1, female = 2)	-0.071	0.644
Duration of diabetes	0.928*	<0.001
FBG	0.236	0.118
HbA1c	0.691*	<0.001
S. Urea	-0.019	0.901
S. Creatinine	0.781*	<0.001
eGFR	-0.796*	<0.001
ACR	0.979*	<0.001
Systolic blood pressure	0.780*	<0.001
Diastolic blood pressure	0.575*	<0.001
BMI	0.178	0.241

Table (III): Correlation between U. CTGF/Creatinine with different studied parameters in each group.

		U. CTGF/Creatinine			
		Normal (Control) (n = 15)	Albuminurea		
			Normo (n = 15)	Micro (n = 15)	Macro (n = 15)
Duration of diabetes	r_s	-	0.456	0.778*	-0.083
	p	-	0.088	0.001	0.769
HbA1c	r_s	0.031	-0.102	0.654*	-0.100
	p	0.914	0.719	0.008	0.724
S. Creatinine	r_s	-0.241	0.360	0.614*	0.267
	p	0.387	0.188	0.015	0.336
eGFR	r_s	-0.357	-0.356	-0.741*	-0.966*
	p	0.191	0.192	0.002	<0.001
ACR	r_s	0.236	0.371	0.981*	0.999*
	p	0.397	0.173	<0.001	<0.001
Systolic blood pressure	r_s	0.257	0.168	0.746*	0.163
	p	0.356	0.550	0.001	0.561
Diastolic blood pressure	r_s	0.316	0.266	0.861*	0.231
	p	0.251	0.337	<0.001	0.407

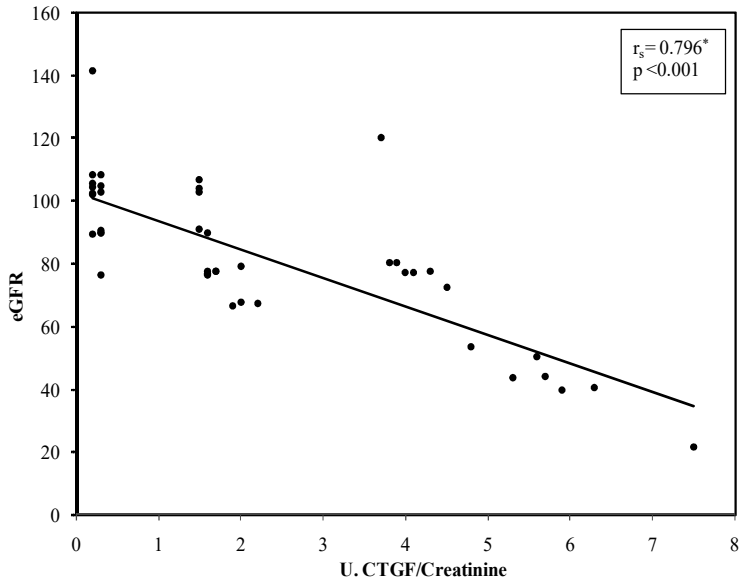

Figure (1): Correlation between U. CTGF/Creatinine with eGFR in total patients.

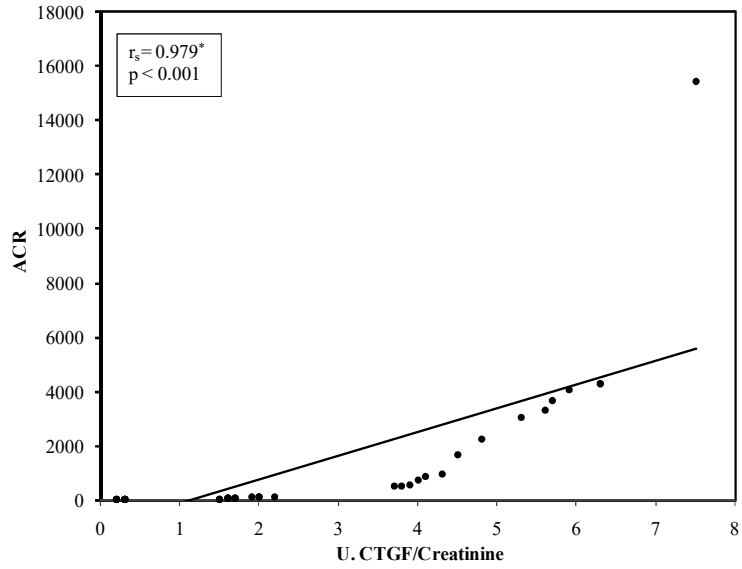

Figure (2): Correlation between U. CTGF/Creatinine with ACR in total patients.

Discussion:

In the present study, we analyzed U.CTGF excretion in 45 elderly type 2 diabetic patients and 15 healthy control subjects. Our results, confirmed that U.CTGF was significantly increased in diabetic nephropathy. To this, We added that in patients with diabetic nephropathy, U.CTGF was correlated with ACR (R=0.979, P < 0.001) and GFR (R= 0.796, p < 0.001), both are important clinical markers for severity of renal disease.

Elevated CTGF levels in the urine of patients with diabetic nephropathy was shown in multiple studies supporting our results.

In Yasuhiko Ito et al study,[13] which included 333 patients, classified into 175 type 2 diabetic patients, 130 patients with non-diabetic renal disease and 28 healthy controls at the Faculty of Medicine Nagoya University, Nagoya Kyoritsu Hospital and

Chubu Rosai hospital. They noted that U.CTGF excretion was significantly higher in patients with DM renal insufficiency and chronic renal failure of DM nephropathy than in healthy controls. Urinary excretion levels of CTGF (U.CTGF/Creatinine) were significantly higher in diabetic nephropathy patients with chronic kidney disease (CKD) stages 4 and stage 5 compared to those with CKD stages 1, stage 2 and stage 3.

Nguyen TQ et al study [14] the excretion of U.CTGF in 24 hours predicted an increase in 24 hours UAE in 318 type 1 diabetic patients and 29 normoglycemic controls. Patients were selected from the outpatient clinic at Steno Diabetes Center (Copenhagen, Denmark). They reported that U.CTGF in patients with diabetic nephropathy was significantly higher than in microalbuminuric patients and normoalbuminuric patients and control subjects.

This observation was supported also by Riser BL et al study,[15] which quantified urinary CCN2 (CTGF) excretion in patients making routine visits to either the nephrology or the endocrinology clinics of Henry Ford Hospital. Patients were divided into two groups (renal disease group included 7 patients and the group without renal disease included 6 patients) and healthy controls. CCN2 levels were determined to be consistently low among the healthy volunteers. The values from the volunteer group did not differ from the diabetic patient group without renal disease as a whole. However, there was a significant difference between the control group and the diabetic patients with renal disease.

Tam F.W.K et al study[16] included 43 adult diabetic patients (9 patients with type 1 DM, 34 patients with type 2 DM), whose attended to the Diabetic Clinic and Diabetic Nephropathy Clinic at Hammersmith Hospital, London, United Kingdom. They reported that low or undetectable levels of urinary CCN2 were observed in patients with normoalbuminuria, urinary CCN2 were significantly higher level in microalbuminuria than those with normoalbuminuria. Urinary CCN2 were significantly higher level in those with macroalbuminuria ($p<0.05$) than those with normoalbuminuria, but had a tendency to be lower than those with microalbuminuria.

Conclusion:

The main finding of this study was that, in patients with diabetic nephropathy U.CTGF was elevated and correlated with the severity of renal disease in terms of both ACR and decreased GFR. This suggests that CTGF, probably in conjunction with other factors, might act as a progression promoter in diabetic nephropathy.

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Original Article

Cytotoxin-Associated Gene (CAG) a of Helicobacter Pylori Seropositivity in Type 2 Diabetic Patients; Relation to Insulin Resistance, Beta Cell Function and Glycemic Control.

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Abstract:

Introduction: diabetes mellitus is a worldwide problem and helicobacter pylori is a chronic infection occur in diabetic patients. **Objective:** to determine the relation between H.pylori Cag-A seropositivity and HOMA-IR, HOMA-B, and glycemic control in type 2 diabetic patients. **Methods:** 91 male diabetic patients (more than 40 years) were included in the study they were subjected to full history taking , clinical examination stressing on blood pressure, BMI, WHR, and laboratory investigations including: FBG, fasting insulin, HbA1c, HOMA-IR, HOMA-B, inflammatory markers (WBCs, ESR, hsCRP), lipid profile (TG,HDL-c,LDL-c, Cholesterol) and the seroprevalence of Cag-A IgG antibodies to H.pylori was performed for all patients. **Results:** 44 Cag-A seropositive were detected. They showed a significantly higher duration of diabetes (P=0.026*),BMI (P<0.001*), inflammatory markers (WBCs P<0.001*, hsCRP P=0.025*, ESR P<0.001*), HbA1c (P<0.001*), fasting insulin (P=0.039*), HOMA-B (P=0.013*), HOMA-IR (P=0.027*), serumTG (P=0.035*) and a significantly lower HDL-c (P<0.001*) than seronegative group. **Conclusion:** inflammatory mechanism of Cag-A infection may play a role in development of IR, ISD, obesity, and poor glycemic control in type 2 diabetic patients.

Keywords: CAG, H.Pylori, T_2DM

Introduction:

Diabetes mellitus is a group of metabolic diseases characterized by hyperglycemia resulting from defect in insulin secretion, insulin action, or both. The chronic hyperglycemia of diabetes is associated with long term damage, dysfunction, and failure of different organs especially the eyes, kidneys, heart, nerves and blood vesseles.(1)

Type 2 diabetes mellitus (T2DM) continuous to be a major health problem worldwide. It is well known that T2DM is a metabolic disorder characterized by hyperglycemia, which arises from insufficient pancreatic insulin secretion, insulin resistance, and inadequate suppression of glucagon production.(1) This suppression in inadequate uptake, storage, and disposal of ingested glucose is accompanied by elevated hepatic production of glucose and profound hyperglycemia.(1)

H.pylori is a curved spiral shaped, gram-negative, bacteria about 0.5×3 µm in size and have up to 7 sheathed flagella that extend from one end and give the organism the mobility to withstand rhythmic gastric contractions and penetrate the gastric mucosa.(2) H. pylori produces a number of virulence factors, including vacuolating cytotoxin (vacA) and cytotoxin associated gene A (cagA) which causes cell injury and inflammation.(3)

The topic of extra-gastric manifestations of Helicobacter pylori infection continues to capture the attention of many researchers all over the wor ld. That it is the most common chronic infection in human and has been associated with a variety of extra-gastro-intestinal manifestations.

Infection with H.pylori causes the release of pro-inflammatory cytokines and vasoactive substances, such as (tumor necrosis factor α TNF- α, interferon-gamma, interleukin IL-1, 6, 8, 10, 12), eicosanoids as (leukotrienes, prostaglandins), and acute phase proteins (fibrinogen, C-reactive protein). It also increases inter cellular and vascular cell adhesion molecules (ICAM-1 and VCAM-1) , enhances platelet activation and platelet-leukocyte aggregation,[4] and was found to alter the apoptotic process.[5] The increased oxidative stress causes inflammation, accumulation of reactive oxygen species ROS, and oxidative DNA damage due to neutrophil infiltration.[6] This inflammatory mechanism was proved to be involved in the pathogenesis of IR.[7]

Furthermore the infection leads to reduction of vitamin B12 and folate acid concentrations, and the consequent increased of homocysteine (Hhcy) and lipid peroxide,[8] lower ghrelin [9] and increase leptin[10] levels, which are associated with impaired energy homeostasis, lipid metabolism, elevated fasting insulin levels and insulin sensitivity.[10]

Moreover, fetuin A, another acute-phase glycoprotein involved in mineralization and insulin signaling regulation.[11] Its dysregulation results in an excessive inhibition of insulin signaling in the liver and skeletal muscle.[12] H.pylori infection decreases the level fetuin A leading to defect in insulin signaling and development of insulin resistance.

Furthermore, fetuin-A levels appear significantly increased after successful H.pylori eradication treatment. The specific virulence factor cytotoxin associated gene A (CagA) has more strong relationship with the pathogenesis of type 2 diabetes that it implicated in insulin resistance and insulin secretion along with other traits that comprise the Metabolic Syndrome.[7]

Methods:

The study included 91 type 2 diabetic male patients above 40 years divided in to **Group I**: H.pylori (Cag-A) seropositive diabetic patients and **Group II**: H.pylori (Cag-A) seronegative of diabetic patients. They were selected from the outpatient clinic of the Diabetes and Metabolism Unit, Alexandria Main University Hospital. The study was approved by hospital ethics committee and written consents were obtained from all subjects after explaining the nature and the aim of the study.

Exclusion Criteria: Subjects who have history of peptic ulcer or previous H.pylori eradication, evidence of connective tissue or haematological diseases, severe cardiac decompensation, known history of myocardial infarction, cerebrovascular stroke, severe uncontrolled hypertension, end stage liver or renal diseases, inflammatory and neoplastic disorder, and who have urinary tract infection or any other infectious diseases are excluded from the study.

Clinical Examination: All patients were subjected to full history taking including (age in years, duration of diabetes in years and smoking habits), clinical examination, blood pressure measurement and anthropometric parameters were recorded as Body mass index **(BMI)** according to the **Quetelet equation: BMI=Body weight in (kg) / Height in (m^2)**. Waist hip ratio **(WHR)** was calculated according to the formula **WHR=Waist circumference / Hip circumference**.

Laboratory Investigation: done to each patient were **FBG** by hexokinase method, **HbA1c**, serum **TG**, serum **Cholesterol**, **HDL-c** by automated Hitach 912 autoanalyzer, [13,14] **LDL-c** was calculated by Friedewaled equation,[15] **hsCRP** by automated nephlometery,[16] **WBCs** by using System XT-1500, **ESR** by automated analyzer, Fasting insulin by IMMUNLITE immunoassay analyzer,[17] **HOMA-IR** was calculated according to the equation **HOMA-IR = (Fasting glucose in mg/dl×fasting insulin in mu/L)/405.**[17] **HOMA-B** was calculated by equation **HOMA-B= (Fasting insulin in mu/L×360)/(fasting glucose in mg/dl-63)**.[17]

Serological Diagnosis: Detection of specific **IgG** antibodies against H.pylori **(CagA)** was performed for all patients using ELISA (EUROIMMUN kits. UK).[18]

Statistical analysis:[19] Data were statistically analyzed using IBM SPSS (Statistical Package

for the Social Science) software package version 20.[20] Data were expressed as range, mean, standard deviation, and median. Chi-square test, t-test, and ANOVA tests were used for comparison between groups. Pearson correlation co-efficient used for comparison between variables.

Results:

The duration of diabetes was significantly longer in Cag-A seropositive diabetic patients than Cag-A seronegative diabetic patients (P=0.026*), BMI was also higher in Cag-A seropositive patients(P<0.001*), inflammatory markers including (WBCs, hsCRP, ESR) were higher in Cag-A seropositive diabetic patients (P<0.001*, P=0.025*, P<0.001*) than Cag-A seronegative diabetic patients. HbA1c, Fasting insulin, HOMA-IR, HOMA-B were significantly higher in Cag-A seropositive patients (P<0.001*, P=0.039*, P=0.027, P=0.013*) respectively. Regarding to lipid profile serum TG was higher in Cag-A seropositive patients (P=0.035*) while than HDL-c was significantly lower (P<0.001*).

Table I: Comparison between 2 groups in all parameters:

Parameter	Seropositive Cag-A (48.4%)	Seronegative Cag-A(51.6%)	p-value
Age (Y) **Mean± SD** **Median**	42.0-76.0 57.31 ± 8.79 56.50	41.0 – 72.0 58.06 ± 8.26 59.0	0.673
Duration of DM (Y) **Mean± SD** **Median**	2.0 – 21.0 6.97 ± 4.99 5.75	0.50 – 15.0 4.97 ± 3.61 4.0	0.026*
Smoking **No** **Yes** **Ex-smoker**	21 (47.7%) 19 (43.2%) 4 (9.1%)	20 (42.6%) 19 (40.4%) 8 (17.0%)	0.533
BMI Kg/m^2 **Mean± SD** **Median**	34.02 – 60.10 41.18 ± 5.42 40.35	22.80 – 44.20 30.67 ± 4.15 30.40	<0.001*
WHR **Mean± SD** **Median**	1.10 – 1.93 1.39 ± 0.18 1.36	1.04 – 1.90 1.38 ± 0.19 1.33	0.757
Systolic Bp(mmHg) **Mean± SD** **Median**	90.0 – 160.0 128.64 ± 18.75 130.0	100.0 – 160.0 128.51 ± 14.74 130.0	0.972
Diastolic BP(mmHg) **Mean± SD** **Median**	60.0 – 100.0 82.05 ± 10.47 80.0	60.0 – 100.0 78.94 ± 12.20 80.0	0.197
WBCs×10^3/µl **Mean± SD** **Median**	4.0 – 19.90 14.48 ± 3.37 14.05	2.10 – 12.0 6.70 ± 2.64 6.30	<0.001*
hsCRP mg/dl **Mean± SD** **Median**	1.10 – 39.40 7.35 ± 6.98 5.20	1.0 – 17.20 4.56 ± 3.80 3.20	0.025*
ESR1 **Mean± SD** **Median**	4.0 – 65.0 17.45 ± 15.59 11.50	2.0 – 65.0 13.62 ± 17.80 7.0	<0.001*
ESR2 **Mean± SD** **Median**	6.0 – 117.0 35.66 ± 25.37 27.0	5.0 – 110.0 28.43 ± 30.43 17.0	<0.001*
FBG (mg/dl) **Mean± SD** **Median**	70.0 – 315.0 179.30 ± 66.52 184.0	70.0 – 300.0 177.60 ± 61.99 172.0	0.900

HbA1c(%) **Mean± SD** **Median**	3.80 – 12.60 9.87 ± 2.38 10.30	3.90 – 12.50 8.01 ± 2.18 8.0	<0.001*
Fasting insulin(µu/ml) **Mean± SD** **Median**	4.06 – 57.50 19.96 ± 13.03 14.60	2.90 – 57.50 15.60 ± 11.92 11.50	0.039*
$HOMA_2$-IR **Mean± SD** **Median**	0.50 – 8.10 2.95 ± 1.88 2.15	0.40 – 10.20 2.40 ± 2.05 1.60	0.027*
$HOMA_2$-B **Mean± SD** **Median**	10.20 – 283.80 74.33 ± 63.32 51.55	5.10 – 236.10 48.57 ± 48.83 33.80	0.013*
Serum TG (mg/dl) **Mean± SD** **Median**	145.0 – 358.0 222.91 ± 53.0 224.0	120.0 – 265.0 202.62 ± 34.66 212.0	0.035*
Serum cholest (mg/dl) **Mean± SD** **Median**	210.0 – 400.0 296.98 ± 45.02 301.50	180.0 – 370.0 296.19 ± 39.56 302.0	0.930
HDL-c (mg/dl) **Mean± SD** **Median**	10.0 – 35.0 21.09 ± 8.04 23.0	14.0-48.0 30.45 ± 8.51 33.0	< 0.001*
LDL-c (mg/dl) **Mean± SD** **Median**	115.0 – 441.0 199.18 ± 53.13 198.0	90.0 – 255.0 193.28 ± 34.30 198.0	0.733

P-values ≤ 0.05 was significant and ≤ 0.01 highly significant.

Discussion:

In the present study, the duration of diabetes was significantly longer in seropositive Cag-A patients which show agreement with Roga et al.[21] and disagreement with Pietroiusti et al.[22] and Christie et al.[23]

In our study, BMI was significantly higher in seropositive Cag-A patients which is in agreement with Chen et al,[24] Arslan et al,[25] and Isomoto et al. The gastric colonization with Cag-A positive strains reduces gastric motility which would explain increase BMI in those patients.[26,27] However the studies of Jamshid et al,[28] Pietroiusti et al,[22] and Christie et al,[23] not report this difference in BMI.

In our study, inflammatory markers were significantly higher in seropositive Cag-A patients which is in accordance with Siddiqui et al,[29] Diomedi M et al,[30] Roga et al.[21] This would be attributed to production of hsCRP which is a hepatic protein produced in acute phase of inflammation, and its synthesis is regulated by various cytokines, predominantly IL-6.[31] which is released during H.pylori Cag-A infection.

In the present study, HbA1c and Fasting insulin were higher in seropositive Cag-A patients in agreement with Fernandini et al,[32] and Bener A et al.[33] This association may be through Cag-A strains containing type 4 secretory apparatus which allows translocation of Cag-A protein in to the cells and induces pro-inflammatory cytokines release and proliferation of the cells. This mechanism is considered an important contributor in metabolic syndrome.[34] However Jamshid et al,[28] Tanriverdi et al,[35] and Pietroiusti et al,[22] did not report this any significant difference in HbA1c and Fasting insulin.

In our study, HOMA-IR and HOMA-B were significantly higher in seropositive Cag-A patients which shows agreement with Wong F et al,[36] Franceschi F et al,[37] Afzalur et al.[38] This association between seropositivity and HOMA-IR and HOMA-B is due to that Cag-A infection stimulate the release of insulin counter regulatory hormones also induce hyperinsulinemia by decrease serum concentration of somatostatin which has inhibitory effect on insulin secretion which

lead to IR and ISD.[39] In contrast, Gillum et al, Park et al,[40,41] Naja et al, and Lu et al. [42,43] did not report any significant difference in HOMA indices (IR and B).

In our study, serum TG was higher and HDL-c was lower significantly in seropositive Cag-A pa tients. This show agreement with Hoffmeister A et al, and Roga et al. studies which report that significant difference in TG and HDL-c.[44,21] In contrast a meta-analyses of 18 studies involving 10000 seropositive Cag-A diabetic patients found no strong correlation between the infection and serum concentrations of TG and HDL-c.[45]

Conclusion: Cag-A seropositivity is associated with development of Obesity through increase body mass index also the inflammatory mechanism of the Cag-A infection may play a role in development of IR, and ISD, the infection also increase HbA1c leading to poor glycemic control.

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Original Article

Assessment of Thyroid Function During the Three Trimesters of Pregnancy.

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Abstract:

Background: Undetected and untreated thyroid disorders are associated with adverse maternal and fetal outcomes. There are limited data on the prevalence of newly diagnosed thyroid disease during pregnancy from Egypt Therefore; this study was designed to evaluate the prevalence of thyroid dysfunction during the three trimesters of pregnancy. Pregnancy is associated with significant but reversible changes in thyroid function due to the effect of HCG and estrogen. That might cause maternal and fetal complications so screening is important. **Patient and Method:** The present cross-sectional study was conducted at antenatal clinic of El Chatby Maternity Hospital in Alexandria University. The total sample population comprised of of 90 pregnant women divided into 30 women for each trimester compared with 30 non pregnant healthy women regarding thyroid function parameters and anti-TPO by using COBAS analyzer measured by the electrochemiluminescence immunoassay "ECLIA" employs monoclonal antibodies specifically directed against human TSH,FT4,FT3 and anti TPO. **Results:** 120 ladies were enrolled for this study aged between 20-45years excluding ladies with previous endocrinal anomalies showed significant difference between pregnant and non- pregnant females regarding TSH and FT4 and no significant difference regarding FT3 and anti TPO in all trimesters. **Conclusion:** There is discrepancy between FT4 &TSH in pregnancy due to presence of other stimulatory and inhibitory factors in pregnancy and thyroid anomalies increased with advance in pregnancy so screening of TSH and anti TPO is important. Considering the immense impact that maternal thyroid dysfunction has on maternal and fetal outcomes, prompt identification of thyroid dysfunction and its timely treatment is essential.

Keywords: Thyroid function, Trimesters, Pregnancy.

Introduction:

The thyroid gland is one of the largest endocrine glands in the body, weighing 2-3 grams in neonates and 18-60 grams in adults, and is increased in pregnancy. This gland is found in the neck inferior to the thyroid cartilage, produces the hormones T4, T3, and calcitonin. Up to 80% of the T4 is converted to T3 by peripheral organs such as the liver, kidney and spleen. T3 is about ten times more active than T4. It plays a vital role in the overall body function during all stages of life; it produces hormones that regulate the body's overall metabolism, the rate at which the body produces energy from nutrients. Pregnancy is associated with significant but reversible changes in thyroid function. These are a result of normal physiologic state and hormonal changes that alter thyroid function.[1] Therefore, some experts strongly recommend universal screening either before conception or at least when pregnancy is confirmed. [2]The high circulating estrogen levels during pregnancy change the pattern of glycosylation of TBG at the time of hepatic synthesis, leading to a longer plasma half-life and, consequently, an increase in the plasma TBG concentration[3] This lead to increased serum T4 -binding globulin and T4 concentrations.[4,5]

Although a transient decrease in serum free T4, followed by a rise in TSH to a new equilibrium, may occur,[6] this is usually not appreciated with routine thyroid testing. A high

circulating HCG level in the first trimester leads to HCG cross reactivity with the TSH receptor, prompting a temporary increase in free T4 and partial suppression of TSH. The final physiologic change results from placental deiodination of maternal T4, which increases T4 turnover. Fetal thyroid function begins from the end of the first trimester. Prior to that, there is evidence that normal development of the fetal brain is dependent upon maternally derived T4, which is converted intracellular to T3. Such T4 has been detected from 5-8 weeks' gestation and by 11 weeks it is at 100 time greater concentration than in the maternal circulation. Maternal hyothyroxinaemia at this stage may have adverse effects on subsequent fetal brain development. Fetal FT4 and total T4 reach adult levels by 36 weeks gestation. Fetal TSH is greater than adult TSH and fetal T3 remains low. The relatively high levels of T4 allow intracellular conversion to T3 in the fetal brain. The aim of this work was to assess thyroid functions in a group of pregnant women attending the Pregnancy Outpatient Clinic at the Chatby Maternity Hospital in Alexandria University, and to compare the results with matching non-pregnant women.

Patients and Methods:

The present cross-sectional study was conducted at antenatal clinic Department of the Obstetrics and Gynecology. The total sample population comprised of 90 pregnant ladies of the three trimesters of gestation without any history of thyroid disease or intake of any thyroid medication and 30non-pregnant healthy ladies as control. On the enrollment of participants, an informed written consent was taken, detailed history was enquired, participants were subjected to relevant general physical examination and findings were recorded on a predesigned preform. 3 mL blood was sampled from the participants under aseptic conditions; stable for 7 days at 2-8 °C, 1 month at -20 °, they were frozen only once, the sample types listed were tested with a selection of sample collection tubes, and we centrifuged samples containing precipitates before performing the assay heat-inactivated samples do not used. The samples, calibrators, and controls were at ambient temperature (20-25 °C) before measurement. They were analyzed for thyroid function tests, which included FT3, FT4, TSH and anti-thyroid peroxidase antibodies (TPO) by using the electrochemiluminescence technique. The analyzer was Cobas with immunoassay analyzer The Eleusis TSH assay employs monoclonal antibodies specifically directed against human TSH. The antibodies labeled with ruthenium complex consist of a chimeric construct from human and mouse-specific components. As a result, interfering effects due to HAMA (human anti-mouse antibodies) are largely eliminate also FT4 and FT3 was measured by the same principle through Competition principle: 1st incubation: 15 µL of sample and a T3 or T4-specific antibody labeled with a ruthenium compel,. 2nd incubation: After addition of biotinylated T4 and streptavidin-coated 2microparticles, the still-free binding sites of the labeled antibody become occupied, with formation of an antibody-hapten complex. The entire complex becomes bound to the solid phase via interaction of biotin and streptavidin. The reaction mixture is aspirated into the measuring cell where the micro particles are magnetically captured onto the surface of the electrode. Unbound substances are then removed with Propel/Propel M. Application of a voltage to the electrode then induces chemiluminescent emission which is measured by a photomultiplier. Results were determined via a calibration curve which is instrument-specifically generated by 2-point calibration and a master curve provided via the reagent barcode. Regarding anti TPO the Elecsys Anti-TPO Cal Check calibration verification solutions comprise three levels - low, mid and high - each with a defined Anti-TPO concentration. The low solution concentration is near the lower detection limit of the assay. The mid solution is in the middle or at a clinically critical point of the measuring range. The high solution is near the upper limit of the measuring range.

Results:

A total of 120 women were enrolled for this study: 30 ladies in each trimester of the three trimesters and 30 non- pregnant healthy ladies as a control, from El Chatby Alexandria university hospital. The mean age among ladies in 1st trimester was 26.10 years, in

2nd trimester was 27.33 years, and in 3rd trimester was 27.83 years and in non- pregnant ladies was 27.37 years. No significant difference between the ages in the four groups. Regarding thyroid dysfunction symptoms; all cases of thyroid disorders were asymptomatic compared to 3cases in non-pregnant women having palpitation, hair loss and menses disturbance (10%). This referred to the magnitude of the problem that most cases of thyroid disorders were asymptomatic and if there were symptoms these were closely similar to physiological symptoms of pregnancy so screening is important. By examination of the ladies 3 women in pregnant women group were have goitre (3.3%): 2 women were found with goiter (by examination) in 3rd trimester and 1 woman in 2nd trimester.The study showed significant difference between pregnant women and non-pregnant control group according to mean of TSH and FT4 but it showed no significant difference between pregnant women and non-pregnant control group regarding FT3 and anti TPO (Table I & II).

Regarding anti TPO, it showed significant difference between 3rd trimester and non-pregnant control group, no significant difference between 2nd trimester and non-pregnant control group and there was no significant difference between 1st trimester and non-pregnant control group however; it shows no significant different between pregnant and non-pregnant ladies.

Euthyroid state was in 76.7 % of pregnant in 1st trimester compared to control 73.3% with least percentage in 3rd trimester 46.7%, hypothyroidism was more prevalent in 3rd trimester that was 40% and that associated with Anti TPO in 13.3%, subclinical hypothyroidism is more prevalent at 1st trimester 10% of women compared to control women which is 6.7% and that associated with anti TPO prevalent in 1st trimester 6.7% of women. Hyperthyroidism is more prevalent in 3rd trimester 10% compared to non-pregnant which is 6.7% &Anti TPO appeared more prevalent in euthyroid state in the 2nd trimester with prevalence 6.7% but appeared more with hypothyroidism in 3rd trimester 13.3% (Table III)

Table (I): Comparison between the ladies groups according to TSH (uIU/ml).

	3rd thermistor (n=30)		2nd trimester (n=30)		1st trimester (n=30)		Non Pregnant (n=30)		Test of Sig.	p
	No.	%	No.	%	No.	%	No.	%		
TSH (uiu/ml)										
Normal (0.27 – 4.2)	23	76.7	24	80.0	24	80.0	24	80.0		
Abnormal low	0	0.0	0	0.0	2	6.7	3	10.0	χ^2= 7.432	0.329
Abnormal high	7	23.3	6	20.0	4	13.3	3	10.0		
Min. – Max.	0.75 – 6.77		0.59 – 38.30		0.01 – 7.10		0.0 – 7.19			
Mean ± SD.	2.90 ± 1.73		4.08 ± 6.66		2.51 ± 1.78		1.75 ± 1.66		$^{KW}\chi^2$=14.584*	0.002*
Median	2.56		2.76		2.14		1.13			
Sig. bet. Grps	p_1= 0.745, p_2=0.363, p_3=0.001*, p_4=0.211, p_5=0.002*, p_6=0.026*									

χ^2: Chi square test KWχ2: Chi square value for Kruskal Wallis test

p_1: p value for Mann Whitney test for comparing between 3rd trimester and 2nd trimester

p_2: p value for Mann Whitney test for comparing between 3rd trimester and 1st trimester

p_3: p value for Mann Whitney test for comparing between 3rd trimester and Non Pregnant

p_4: p value for Mann Whitney test for comparing between 2nd trimester and 1st trimester

p_5: p value for Mann Whitney test for comparing between 2nd trimester and Non Pregnant

p_6: p value for Mann Whitney test for comparing between 1st trimester and Non Pregnant

*: Statistically significant at $p \leq 0.05$

Table II: Comparison between the studied groups according to FT4 (ng/dl).

	3rd trimester (n=30)		2nd trimester (n=30)		1st trimester (n=30)		Non Pregnant (n=30)		Test of Sig.	p
	No.	%	No.	%	No.	%	No.	%		
FT4 (ng/dl)										
Normal (0.9 – 1.8)	14	46.7	23	76.7	25	83.3	26	86.7		
Abnormal low	16	53.3	3	10.0	4	13.3	0	0.0	χ^2= 31.509*	<0.001*
Abnormal high	0	0.0	3	10.3	1	3.3	4	13.3		
Sig. bet. Grps	p_1=0.001* , p_2 = 0.003*, p_3 <0.001* , p_4 = 0.703, p_5 = 0.273 , p_6 = 0.085									
Min. – Max.	0.58 – 1.70		0.10 – 3.36		0.35 – 2.50		0.92 – 5.20			
Mean ± SD.	0.92 ± 0.24		1.21 ± 0.56		1.29 ± 0.48		1.40 ± 0.78		KWχ^2=25.892*	<0.001*
Median	0.83		1.10		1.23		1.20			
Sig. bet. Grps	p_1= 0.001*, p_2<0.001*, p_3<0.001*, p_4=0.166, p_5=0.084, p_6=0.807									

Table (III): The difference between thyroid functions in the four groups.

	3rd trimester (n=30)		2nd trimester (n=30)		1st trimester (n=30)		Total in pregnancy		Non Pregnant (n=30)	
	No.	%	No.	%	No.	%	No.	%	No.	%
Thyroid function										
Normal	14	46.7	21	70.0	23	76.7	58	64.4	22	73.3
Normal with anti TPO	1	3.3	2	6.7	1	3.3	4	4.4	1	3.3
Hypothyroidism	6	20.0	1	3.3	0	0.0	7	7.7	0	0.0
Hyperthyroidism	3	10.0	1	3.3	1	3.3	5	5.5	2	6.7
Hypothyroidism with +ve anti TPO	4	13.3	3	10	0	0.0	7	7.7	0	0.0
SUBCLINICAL Hypothyroidism	2	6.7	1	3.3	3	10.0	6	6.6	2	6.7
Subclinical hypo with anti TPO	0	0.0	1	3.3	2	6.7	3	3.3	1	3.3
Subclinical hyperthyroidism	0	0.0	0	0.0	0	0.0	0	0	2	6.7

Discussion:

Specific reference intervals for FT4, T4, and T3 in addition to TSH during pregnancy may be particularly important for several reasons. First, it would be important to know why the FT4 levels are in the first trimester and 2nd trimester higher than in the 3rd trimester in a euthyroid pregnancy, and this is the time when the fetus is wholly dependent on T4 from the mother. Accurate reference intervals for FT4 and FT3 would then provide the ability to detect a deficiency at this critical time and provide a subtle indication of maternal hypothyroidism. In our study there was significant difference between pregnant group and non-pregnant group in mean TSH, some cases may be subclinical or mild with normal FT4. In the pregnant ladies group; 28 ladies with abnormal thyroid function (31%): hyperthyroidism in 5 ladies (5.5%), hypothyroidism in 23 ladies (25.5%) and 19 ladies with anti -TPO +VE (21%).On the other hand, the present results were not concordant with some other studies in this field, in the Tunisian study done by Feki and his colleges;

the incidence of thyroid disorder in Tunisian determined TSH and TPO-Abs in 1519 pregnant ladies aged 17 to 47 years. Thyroid disorder was defined as hyperthyroidism (TSH≤0.10) or hypothyroidism (TSH >4.5 mIU/L), and/or positive TPO Abs (>12 IU/L). Thyroid disorders were observed in 147 pregnant ladies (9.7%). Positive TPO-Abs was noted in 99 ladies (6.5%), hypothyroidism in 48 ladies (3.2%) and hyperthyroidism in 10 women (1.3%).[7] In our study 1st trimester showed: prevalence of hypothyroidism 16.7% (5of 30) but all are subclinical cases due the stimulatory effect of HCG Compared to other study in USA reported by Cleary-Goldman where a total of 10,990 first- and second-trim ester serum assayed for TSH, FT4), and ant thyroglobulin and ant thyroid peroxidase antibodies. Subclinical hypothyroidism was documented in 2.2% (240 of 10,990) in the first trimester. Overt hypothyroidism was documented in 2.1% (232 of 10,990).[8] The wide gap observed in our study than other Tunisian and USA one is due to iodine deficiency that proved with increase demand for iodine with the advance in pregnancy. Hypothyroidism in our study is more in 3rd trimester may be due to lack of program that ensures proper nutrition for pregnant women and misuse of Pesticides by farmers, this leads to contamination of vegetables and fruits, which ultimately affects human health.

This gap reflects the magnitude of the problem that we discuss that major maternal and fetal complications will occur. So ministry of health should do proper situation analysis regarding diet and life style of pregnant women for detection of major causes for iodine deficiency and find cost effective plan to solve this major problem through collaboration of efforts of health and agriculture ministries and whole population to prevent further fetal and maternal complications. The prevalence of hyperthyroidism in pregnant women was 5.5% .more prevalent in 3rd trimester 10% of women compared with Tunisian study done by Feki and his colleges (1.3%), and (1%) in Gaza study. [7] also this study showed significant difference between TSH parameter in pregnant and non-pregnant women, mean TSH was higher in pregnant than non-pregnant it was initially increased in 1st trimester, increased more in 2nd trimester then decreased again in 3rd trimester. FT4 in the studied group showed significant difference between pregnant and non pregnant group, mean FT4 was lower in pregnant than non pregnant women and also it showed more decline with advance in pregnancy.

However FT3 parameter there was no significance between pregnant women and non- pregnant women, but it showed decline from 1st to 3rd trimester in mean of FT3. also there was no significant difference between pregnant and non-pregnant regarding anti TPO however it appeared more significantly in 3nd trimester (n=10) that maybe associated with hypothyroid hashimoto thyroiditis. Compared to other studies that measured the same parameters a Study in Tabriz-Iran (2005) (by Zarghami) was carried out to find out alterations in thyroid function tests in each trimester in normal pregnant women as compared to non-pregnant women. A case-control study designed with 229 normal pregnant and randomly selected non-pregnant healthy female controls. Mean age groups was 16-40 years. Thyroid function tests were carried out by measuring serum level s of TSH, FT4, and FT3. They found that mean FT4 was strongly decreased during the third trimester. Free T3 showed declining in the second and third trimesters that was similar to our study. But TSH did not show significant difference in each trimester compared with non-pregnant women that was different from our study. [9] On the other hand, the present results were not concordant with some other studies in this field. In India a case-control study designed by Pasupathi with two groups of women: 75 normal pregnant ladies randomly selected from the first trimester and randomly selected non-pregnant healthy ladies control that thyroid function tests were carried out by measuring the serum level s of (TSH), (FT4), and (FT3). The mean FT4 levels in the first trimester were non-significantly lower than that of the non-pregnant subjects. The pregnant groups' mean FT3 non-significantly higher than that of the non-pregnant ladies. The mean TSH levels of pregnant women were lower than the mean level of

non pregnant but was not significant, the same as we found in our study.[10] Also Kurioka and his colleagues reported significantly reduced levels of free T3 and free T4,[11] Kumar reported that TSH values were increased steadily with each trimester.[12] Anti TPO measured in three trimesters showed significant increasing with advance in pregnancy and cause hypothyroidism (hashimoto thyroiditis) and may be prolonged to postpartum thyroiditis.

Conclusion:

From the results of this study we can conclude the followings: In our study 21.6% of the studied ladies were having hypothyroidism; 88.42 % of them were pregnant, 7.5% of the studied ladies were having hyperthyroidism; 55.5 % of them were pregnant. No correlation between age & thyroid parameter, significant difference between pregnant women and non pregnant regarding TSH & FT4 and no significant difference between pregnant and non pregnant regarding FT3 but the mean FT3 showed decline in pregnancy. There was discrepancy between FT4 &TSH in pregnancy due to presence of other stimulatory and inhibitory factors in pregnancy, however the increase in anti TPO titer in pregnancy was not significant in relation to non-pregnant; it showed significant increase during 3rd trimester.

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Original Article

Use of Nitric Oxide Donor Isosorbide Mononitrate for Cervical Ripening at 41 Weeks' gestation: a Double Blind, Randomized, Controlled Trial.

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Abstract:

Background: The ideal agent for cervical ripening would induce adequate cervical ripening with minimal adverse effects to the mother and the fetus, the most favorable method for cervical ripening is not fully agreed till now, however, vaginal administration of isosorbide mononitrate (IMN) is considered a low-risk method of labor induction for post term. Our study was designed to assess the effect of IMN on cervical ripening and labor induction among 41week's pregnant women. **Materials and Methods:** This study will be conducted on 100 pregnant women recruited from the outpatient clinic at El Shatby Maternity University-Hospital, all cases pregnant at 41 weeks gestational age, uncomplicated singleton pregnancy, cephalic presentation, intact membranes and not in labor. Cases divided into 2 groups in first group 40 mg isosorbide mononitrate (IMN) tablet applied vaginally in posterior fornix, and in second group placebo applied vaginally in posterior fornix. Follow up the cervical status after 24 hours of administration, the patient were asked about new symptoms especially headache, palpitation, dizziness or abdominal pain and the mode of delivery was be assessed. **Aim of the Work:** The aim of this study is to assess the efficacy of the nitric oxide donor isosorbide mononitrate on cervical ripening at 41 weeks' gestation. **Results:** There was a significant difference between the IMN group and the controls with respect to the Bishop score (4. 40 vs. 3. 68, *P* = 0. 031) table (1), but there was no significant differences in the mode of delivery between the two groups table (2) The major side effect of IMN was headache, about 70% of cases complain from headache, which responded to a mild analgesia. **Conclusions:** Vaginal administration of IMN reduces the cervical resistance and induces cervical ripening without inducing uterine hyperstimulation or abnormal fetal heart rate. So IMN can be used in outpatient clinic for cervical ripening with no need for admission or close fetal monitoring.

Keywords: Cervical ripening, Nitric oxide donor.

Introduction:

Post term pregnancy refers to any baby born after 42 weeks gestation or 294 days past the first day of the mother's last menstrual period. [1] Post term may be in itself be high risk The placenta, which supplies the fetus with nutrients and oxygen from the mother start aging and will eventually fail,[2] The etiology of post-term pregnancy is not unknown; however miscalculation of last menstrual period (LMP) may be common cause of post term.[3] The use of ultrasound in early pregnancy for precise dating significantly reduces the number of post-term pregnancies compared to dating based on the LMP. [4]

Post-mature baby are larger than average size baby, so increase the incidence of cephalopelvic disproportion, shoulder dystocia, operative vaginal delivery[5], postpartum hemorrhage and caesarean sections rate[6]. Meconium aspiration.[7] fetal macrosomia increase incidence of birth injury, [8] neonatal encephalopathy and[9] Sudden infant death syndrome. [10]

Once fetus is diagnosed post-mature, the mother should be offered additional monitoring as this can provide valuable clues that the fetus's health is being maintained. Compared with waiting indefinitely or waiting at least one week for labor to occur spontaneously, labor induction after 41 weeks of gestation is associated with fewer perinatal deaths.[11]

Physiological cervical ripening is an active biochemical process; it has been described as an inflammatory process.[12] The Bishop Score is a common measure used to assess the cervical repining[13]. Non pharmacologic approaches to cervical ripening included herbal compounds, castor oil, hot baths, enemas, sexual intercourse, and breast stimulation till now have not proven efficacy for cervical ripening or induction of labor.[14] Surgical methods for cervical repining (stripping of the Membranes, amniotomy, balloon catheter insertion in cervix), have been recommended for cervical ripening, however risks of this techniques including infection, bleeding, accidental rupture of the membranes, and patient discomfort. [15, 16]

Pharmacological cervical ripening, Prostaglandins E2 (Misoprostol) are the current method used for cervical ripening and labor induction. [17] But risks associated with it, s use include uterine hyperstimulation, nausea, vomiting, diarrhea, fever and accompanying FHR changes. [18] Uterine rupture in women with previous cesarean section limiting its use to women who have a uterine scar.[19] There is insufficient information to support the use of mifepristone and relaxin for cervical ripening.[20] Oxytocin is the preferred pharmacologic agent for inducing labor when the cervix is favorable or ripe.[21]

Nitric oxide (NO) is a small, highly reactive, free radical gas with a half-life time of a few seconds, [22] expressed in three isoforms, all of these isoforms are present in the various cells of the uterine cervix.[23] Cervical nitric oxide production is very low in post term pregnancy. Thus, it has been suggested that reduced cervical nitric oxide release may contribute to prolonged pregnancy.[24] In cervical ripening immunological mediators play a crucial role in this process. NO is involved in the acute inflammatory response and amplifies the cytokine cascade stimulated during this response,[25] via interactions either with prostaglandin biosynthesis or with lytic enzymes. It stimulates cyclooxygenase to increase the production of pro-inflammatory prostaglandins.[26] Its action accomplished by effects on connective tissue and smooth muscle cells.[27] Isosorbide mononitrate (IMN) is a drug used principally in the treatment of angina pectoris. Vaginal administration IMN reduces the cervical resistance without inducing uterine hyperstimulation,or abnormal fetal heart rate.[28]

Participants:

Participants in this study consisted of 100 post term pregnant women at 41 weeks gestational age; the participants were selected according to the inclusion/ exclusion. Inclusion criteria were: women gestational age of 41weeks, not in labor, cephalic presentation, singleton fetus, having a normal non-stress test and biophysical test. Exclusion criteria: pregnancy associated diseases as preeclampsia, placenta previa or unexplained vaginal bleeding during pregnancy, cardiac, pulmonary, renal or hepatic disease, history of severe persistent headache, polyhydramnios, placenta previa, probability of placenta abruption, or any contraindication for induction of labor were excluded.

Intervention:

At the first visit each woman will be subjected to: complete history taking, routine investigations: complete blood picture, fasting blood sugar and complete urine analysis, general examination, obstetric examination (vaginal examination to assess the pelvis, Bishop score and the presenting part), obstetric ultrasonography, biophysical profile and doppler to assess fetal condition.

Cases divided into 2 groups, first group received 40 mg IMN tablet applied vaginally in posterior fornix, second group received placebo applied vaginally at the same site. Women were permitted to go home and instructed to come to the hospital immediately if they had any sign of dangerous symptoms (leakage of amniotic fluid, vaginal bleeding or reduction in fetal movements), otherwise they were asked to come back to the hospital after 24. The women were examined vaginally by the same physician to assess the cervical status, fetal monitoring and follow up the case till delivery.

Results:

There was a significant difference between the IMN group and the controls with respect to the Bishop score (4. 40 vs. 3. 68, P = 0. 031) table (I), but there was no significant differences in the mode of delivery between the two groups table (II) The major side effect of IMN was headache, about 70% of cases complain from headache, which responded to a mild analgesia.

Table (I): Comparison between the two studied groups regarding the Bishope base score before and after 24 hours of administration

	Study group	**Control group**
Bishope base score before administration		
Min. – Max.	0. 0 – 6. 0	0. 0 – 6. 0
Mean ± SD.	3. 72 ± 1. 29	3. 60 ± 1. 54
Median	4. 0	3. 0
p	0. 471	
Bishope score after administration		
Min. – Max.	0. 0 – 7. 0	0. 0 – 7. 0
Mean ± SD.	4. 40 ± 1. 81	3. 68 ± 1. 73
Median	5. 0	3. 0
p	0. 031*	

Table (II): Comparison between the two studied groups regarding the mode of delivery.

	Study group		**Control group**	
	No.	**%**	**No.**	**%**
Mode of delivery				
Normal vaginal delivery	32	64. 0	26	52. 0
C. S.	18	36. 0	24	48. 0
p	0. 224			

Discussion:

Our study demonstrated that outpatient use of IMN has a significant effect in cervical repining and Bishop Score after 24 hours of administration in posterior cervix. In our study 30 women (60%) in the IMN group in contrast to 10 women (20%) in the placebo group had positive cervical repining after 24 hours (p=0. 001*). These results are consistent with previous studies were done by Erling Ekerhovd study in 2003.[28], Maria Bullarbo, [29] and Rameez study in 2007, which concluded that outpatient cervical ripening followed by labor induction with isosorbide mononitrate seems to be an effective, safe and well tolerated procedure. [30]Another study done by Eddama et al, demonstrated

that the proportion of women with an unripe cervix after 24 h of outpatient treatment was significantly lower in the IMN group as compared with the placebo group (64% vs. 77%, *P* = 0. 02). [31]In our study there was a significant difference between the IMN group and the controls with respect to the Bishop score (4. 40 vs. 3. 68, *P* = 0. 031*), which was consistent with Hamideh Yazdizadeh et al, study in which There was a significant difference between the IMN group and the control group with respect to the Bishop score (4. 92 vs. 4. 03, *P* = 0. 0. 01). [32] In another study done by Kavita Agarwal et al, the Bishop score was significantly improved 24 hours after initiation of the outpatient IMN treatment (*P*<0. 001) and the needs for further cervical ripening and oxytocin infusion were less in the study than in the control group (*P*<0. 001 and *P*=0. 008). [33]

In our study there was no significant differences in the mode of delivery between the two groups, In our study 32 women treated with isosorbide mononitrate went into normal labor compared to 26 women in the placebo group (p>0. 05), there was no significant differences as regard to normal labor. In Mohamed Furukan study reported that there was marked increase in the proportion establishing spontaneous labor (28% *vs* 7. 5%, *P* < 0. 01) and cervix being favorable for oxytocin infusion (40% vs 9% *P* < 0. 001) 2 days after therapy, in the same study the cesarean section rates were similar in both groups. [34]

In our study there was no significant differences in cesarean delivery rate (36. 0%vs 48%, p>0. 05), neonatal outcomes and apgar score between the two groups, which was consistent with Sherif M. Habib et al, study. [35]

The most common side effect in women treated with (IMN) was headache, experienced by 35 women (70%) compared to 4 women (8%) in placebo group ((p< 0. 05) the intensity of headache was be from mild to moderate headache. However in Maria Bullarbo study reported more higher incidence (88%) of women treated with isosorbide mononitrite was complain from headache compared to (4%)in placebo group. [29]

Conclusions:

Vaginal administration of IMN reduces the cervical resistance and induces cervical ripening without indu cing uterine hyperstimulation or abnormal fetal heart rate. So IMN can be used in outpatient clinic for cervical ripening with no need for admission or close fetal monitoring.

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Original Article

Evaluation of Neurokinin B in Severe Preeclamptic Pregnancies & Their Impact on Pregnancy Outcomes.

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Abstract:

Aim of the work: The aim of the present study is to evaluate serum neurokinin b level in third trimester of pregnancy in women with severe pre-eclampsia in comparison to a control group of pregnant females and its impact on pregnancy outcomes. **Methods & Patients** The study was done on 78 pregnant females in Main University Hospital in Alexandria. The patients were categorized into two groups 38 females each: Group 1: 38 female as control, Group 2:38 female with severe preeclampsia, both are in their third trimester >34 weeks of gestation, primigravidas, didn't receive antihypertensive medications before. Obstetric, menstrual & medical histories were taken from all patients. Complete general examination was done & investigations were done to both mothers and to their foetuses pre & postoperative. Preoperative investigations & assessments related to the mother include :mean blood pressure, urine output per day, complete blood picture, serum glucose level, liver function tests & kidney function tests, whereas for postoperative investigations & assessments were the same as preoperative plus monitoring blood loss, postpartum complications, mood of delivery & need for icu. Preoperative investigations & assessments related to the foetus was ultrasonography to assess viability, gestational age & foetal weight; whereas for postoperative investigations Apgar scoring, foetal weight & need for icu were assessed. For Neurokinin B assay method used was Enzyme-Linked Immunosorbent Assay Kit where samples were aspirated on a serum separator vacutainers and allowed to clot for 2 hours at room temperature. Centrifugation was done for each sample for 20 minutes at approximately 1000xg and samples were stored at -20 degree Celsius till time of assay. **Results & Conclusions:** From the present study we concluded that Neurokinin b serum levels are higher in preeclamptic females in comparison to normotensive ones. Neurokinin b levels play a role in the pathophysiology of preeclampsia. Preeclamptic females with high levels of neurokinin b had good pregnancy outcomes with fewer complications.

Introduction:

Preeclampsia is a major cause of maternal mortality & morbidity, perinatal deaths, preterm births, & intrauterine growth retardation, especially in developing countries.[1,2] It occurs in 3 - 4% of all pregnancies worldwide, and in 10% before 34 weeks of gestation.[3]

The precise origin of preeclampsia is multifactorial and includes a scenario of required steps.[4] It often affects young & nulliparous women, whereas older women are at greater risk for chronic hypertension with superimposed hypertension. Also, the incidence is markedly influenced by race, ethnicity & by genetic predisposition. Other factors include environmental, socioeconomic & even seasonal influences.[5]

Preeclampsia was defined as systolic blood pressure ≥ 140 mm hg &/or diastolic blood pressure ≥ 90 mm hg, measured on at least 2 consecutive occasions, at least 4 to 6 hours apart. Furthermore, first presentation had to be after 20th week of gestation, in a previously normotensive women, & coupled with significant proteinuria (>300 mg/dl with 24 hour urinary collection; +1, +2 on a qualitative dipstick examination).[6]

Mild preeclampsia is defined as the presence of 1 of the following symptoms or signs in the presence of preeclampsia.

AS for severe preeclampsia, it is defined as the presence of 1 of the following symptoms & signs in the presence of preeclampsia:

* Systolic blood pressure of 160 mm hg or higher or Diastolic blood pressure of 110 mm hg or higher on 2 occasions at least 6 hours apart.
* Proteinuria of more than 5 gm in a 24 hour collection or more than +3 on 2 random urine samples at least 4 hours apart.
* Pulmonary edema or cyanosis.
* Oliguria (<400 ml in 24 hour).
* Persistent headaches.
* Epigastric pain &/or impaired liver functions.
* Thrombocytopenia.

Preeclampsia affects approximately 3 to 10 percent in nulliparous populations while it's less than that in multiparous females,[7] However the risk for stillbirth was more likely in multiparous compared to nulliparous females.[8]

Risk factors for preeclampsia include: Gestational age between 32 & 36 weeks of gestation increased the risk from 1.1 to 1.8 %,[9] Parity where nulliparity triples the risk for preeclampsia.[10] Multiple pregnancy where pregnant women with twins triples the risk, [11] Maternal Age ;women aged >40 years had approaching twice the risk,[12] Race; the incidence of preeclampsia is 1.8 % among white females & 3 % among black ones,[13] women with previous preeclampsia have seven times increased risk for recurrence in subsequent pregnancies.[14] Obesity increase the risk from 4.3% in women with BMI <20 kg/m^2 to 13.3% in women with BMI >35kg/m^2 ,[15] a Family history nearly triples the risk,[16] Vitamin D deficiency increase the risk of preeclampsia & foetal growth restriction[17] & Smoking where current cigarette smokers during pregnancy seem to have a decreased risk. [18]

Numerous factors currently are considered to be implicated in the development of preeclampsia which are maternal immunologic intolerance, abnormal placental implantation, genetic factors, angiogenic factors, oxidative stress & inflammation, renin angiotensin system, nutritional & environmental factors & additional factors in preeclampsia. [19]

Pathophysiology of preeclampsia include various body systems including; Cardiovascular system where hypertension present due to marked vasoconstriction causing both cardiac output & arterial compliance to be reduced.[20] The Kidney where renal plasma flow & glomerular filtration rate are both decreased by about 25%.[21] The placenta where there is failure of normal trophoblastic invasion of spiral arteries leading to hypoxia & release of angiogenic proteins that initiate preeclampsia.[22] The Brain where its effect is referred to as the posterior reversible encephalopathy syndrome [23] & the Liver with the Coagulation abnormalities where there is usually mild thrombocytopenia as the most commonly detected abnormality, increased platelet activation & size leading to hypercoagulability,[19] aspartate aminotransferase & lactate dehydrogenase are also elevated [24] & gross hepatic changes were reported to be in the form of hepatic hemorrhage from areas of infarction.[24, 25]

Prognosis of preeclampsia & eclampsia tend to worsen with the following conditions: [26]

* Systemic endothelial dysfunction.
* Vasospasm & small vessel thrombosis leading to tissue & organ ischemia.
* CNS events, such as seizures, strokes, and hemorrhage.
* Acute tubular necrosis.
* Coagulopathies.
* Placental abruption in the mother.

AS for the, Prevention of preeclampsia; low dose aspirin may reduce incidence by 10 %.[27] For treatment of preeclampsia many drugs can be used including;[28,29] Methyldopa which is considered the drug of choice & safety after first trimester is well documented, Labetalol given in 200-1200mg/day in 2-3 divided doses, Nifidipine which is a calcium channel blocker given in 30-120 mg/day of a slow release preparation & has synergistic effect with magnesium sulfate, Hydralazine which is useful only in combination with sympatholytic agents given in 50-300 mg/day in 2-4 divided doses, Beta blockers but may

cause growth restriction when given in first or second trimester especially atenolol & ACE inhibitors with AT 1 receptor antagonists,[30] are both contraindicated cause of the major anomalies associated which may be fatal.

Magnesium Sulphate is the anticonvulsant of choice for both prevention & treatment of eclampsia. [31] The exact mechanism of action is the blockade of N-Methyl D-Aspartate receptors involved in seizure genesis or calcium channel blocking preventing cerebral vasospasm.[32] Side effects include[33]; hypotension, facial flushing, visual disturbances, chest pain, nasal stiffness, circulatory collapse, gastrointestinal upset, urine retention, magnesium toxicity. Contraindications & precautions include; [33] as it is excreted by the kidney should be used with caution in those with impaired renal functions, with those using digitalis/cardiac glycosides & with CNS depressants as it may result in an enhanced CNS depression too. Prophylactic dose; loading dose at first using 10 ml vial, prepare 4 gram of 50 % magnesium sulfate given at rate 32 ml per hour for 15 minutes, where the maintenance dose prepare 50 ml of 50 % magnesium sulfate & administer at a rate of 1 gm/hr until at least 24 hours post birth/delivery. [32]

As it is now believed, preeclampsia is hidden in the placenta; researchers are directed to novel peptide genes in this organ. Using mRNA fingerprinting[34] & human genomic database,[35] they found nine matches that showed high similarity to the bovine neurokinin b precursor.[36] Neurokinin b mRNA expression was found to be restricted to the outer syncytiotrophoblast of the human placenta, in an ideal position to be secreted into the maternal serum.[37]

From all candidates, neurokinin b which is a member of the tachykinin family,[38] appeared to be the most promising as a potential marker & factor to cause pre-eclampsia. The placenta was found to express unusually high levels of TAC3 that encodes neurokinin b,[39] a gene previously believed not to be expressed in any peripheral tissue,[40] but to the outer syncytiotrophoblast.[41] Elevated expression levels of TAC3 are found to be significantly higher in preeclamptic placenta at term when compared to controls.[42, 43]

IN terms of neurokinin b binds & activated the neurokinin 3 receptor, evoking vasoconstriction in the mesenteric & portal veins, & increasing heart rate; indicative of pressor activity. Theses cardiovascular effects of neurokinin b culminate in the clinical manifestations of preeclampsia. Also it causes increase in the blood pressure & damage to the kidneys & liver observed in preeclampsia. At very elevated concentrations NK1 receptors on platlets may be affected, eventually causing thrombocytopenia.[44]

Other studies showed that neurokinin b acted as a dilator in the placental vasculature.[45,46] Where this effect was found to be initiated solely through the neurokinin 1 receptor. The NK1 receptor is associated with vasodilatory responses,[41] and evidence has shown that the vasoconstrictive NK3 receptor is either absent or expressed at extremely low levels in the human placenta at term when compared to NK1 & NK2 receptors.[42,45] This would advocate a mechanism in the human placenta, whereby high NKB levels induce placental vasodilatation via NK1 receptor to maintain low placental resistance. Nonetheless, preeclampsia is typically associated with maternal vasoconstrictive responses and we proposed that activation of NK3 receptors on the venous side of the maternal circulation could be responsible for hypertension that develops during pre-ecalampsia.[39,41] It was also found that there's a link between elevated NKB level in women with preeclampsia in the third trimester and haemodynamic adaptation via nitric oxide production. [47]

Results:

As regard to patient's SBP, in the control group it ranged between (110 – 120) mmHg

with mean ± S.D (117.37±4.463) mmHg while in severe preeclampsia group it ranged between (160 – 200) mmHg with mean ± S.D (174±11.503) mmHg. There was statistically significant difference between the studied groups where P=0.000 (P significant as P<0.05). **(Table I)**

As regard to patient's DBP, in the control Group it ranged between (70 – 80) mmHg with mean ± S.D (73.68±4.889) mmHg while in severe preeclampsia Group it ranged between (110–120) with mean±S.D (110.75±2.667) mmHg. There was statistically significant difference between the studied groups where P< 0.001.

As regard to patient's MAP, in the control Group it ranged (83.33 – 93.33) with mean ± S.D. 88.246 ± 3.171 while in severe preeclampsia Group it ranged (126.67 – 140) with mean ± S.D. 131.833±3.77. There was statistically significant difference between the studied groups where P=0.000 (P significant as P<0.05). **(Table III)**

As regard to patient's MAP, in the control Group it ranged (83.33 – 93.33) with mean ± S.D. 88.246±3.171 while in severe preeclampsia Group it ranged (126.67 – 140) with mean ± S.D. 131.833±3.77. There was statistically significant difference between the studied groups where P=0.000 (P significant as P<0.05). **(Table IV)**

As regard to patient's EFW, in the control Group it ranged between (2800 – 3400) with mean ± S.D. (3131.58 ± 141.034) while in severe preeclampsia Group it ranged between (2000 – 2910) with mean ± S.D (2444.38 ± 254.718). There was statistically significant difference between the studied groups where P=0.000 (P significant as P<0.05). **(Table V)**

As regard to patient's AFI, in the control Group it ranged between (9.4 – 14) with mean ± S.D. 11.668 ± 1.149 while in severe preeclampsia Group it ranged between 7 – 12 with mean ± S.D. 10.385 ± 1.638. There was statistically significant difference between the studied groups where P=0.000 (P significant as P<0.05). **(Table VI)**

As regard to patient's Neurokinin B, in the control Group it ranged between (0.8 – 50) with mean ± S.D. (20.388 ± 17.417) while in severe preeclampsia Group it ranged between (0.1 – 150) with mean ± S.D. (50.699 ± 55.215). There was statistically significant difference between the studied groups where P<0.001 (P significant as P<0.05). **(Table VII)**

As regard to patient's complication, in the control Group all patients had no complication while in severe preeclampsia Group 31(77.5%) out of the patients had no complication and 9(22.5) out of the patients had complication (all of them had NICU while 6 of the patients had IUGR and ICU and 3 patients had eclampsia, finally 1 of the patients had HEIIP). There was statistically significant difference between the studied groups where P=0.000 (P significant as P<0.05). **(Table VIII)**

Table (I): Comparison between the two groups regarded to SBP

SBP	Control Group (n=38)	Severe Preeclampsia Group (n=38)	Total
Min.	110	160	110
Max.	120	200	200
Mean	117.37	174.00	146.41
S.D	4.463	11.503	29.803
P Value	<0.001*		

Table (II): Comparison between the two groups regarded to DBP

DBP (mmHg)	Control Group (n=38)	Severe Preeclampsia Group (n=38)	Total
Min.	70	110	70
Max.	80	120	120
Mean	73.68	110.75	92.69
S.D	4.889	2.667	19.047
P Value	<0.001*		

Table (III): Comparison between the two groups regarded to MAP

MAP	Control Group (n=38)	Severe Preeclampsia Group(n=38)	Total
Min.	83.33	126.67	83.33
Max.	93.33	140.00	140.00
Mean	88.246	131.833	110.598
S.D	3.171	3.770	22.200
P Value	<0.001*		

Table (IV): Comparison between the two groups regarded to MAP

MAP	Control Group (n=38)	Severe Preeclampsia Group (n=38)	Total
Min.	83.33	126.67	83.33
Max.	93.33	140.00	140.00
Mean	88.246	131.833	110.598
S.D	3.171	3.770	22.200
P Value	<0.001*		

Table (V): Comparison between the two groups regarded to EFW

EFW	Control Group(n=38)	Severe Preeclampsia Group (n=38)	Total
Min.	2800	2000	2000
Max.	3400	2910	3400
Mean	3131.58	2444.38	2779.17
S.D	141.034	254.718	402.414
P Value	<0.001*		

Table (VI): Comparison between the two groups regarded to AFI

AFI	Control Group(n=38)	Severe Preeclampsia Group(n=38)	Total
Min.	9.400	7.000	7.000
Max.	14.000	12.000	14.000
Mean	11.668	10.385	11.010
S.D	1.149	1.638	1.553
P Value	< 0.001*		

Table (VII): Comparison between the two groups regarded to Neurokinin B

NKB	Control Group (n=38)	Severe Preeclampsia Group (n=38)	Total
Min.	0.800	0.100	0.800
Max.	50.000	150.000	150.000
Mean	20.388	50.699	35.932
S.D	17.417	55.215	43.845
P Value	<0.001*		

Table (VIII): Comparison between the two groups regarded to complication

Complications	Control Group (n=38)		Severe Preeclampsia Group (n=38)		Total	
	No	%	No	%	No	%
No	38	100	31	77.5	69	88.5
Yes	0	0	9	22.5	9	11.5
IUGR	0	0	6	15	6	7.7
NICU	0	0	9	22.5	9	11.5
ICU	0	0	6	15	6	7.7
HEIIP	0	0	1	2.5	1	1.3
Eclampsia	0	0	3	7.5	3	3.8
P Value	<0.001*					

Discussion:

Preeclampsia & eclampsia may occur in as many as 8 % of pregnancies and remain a leading cause of maternal and foetal morbidity and mortality.[48] The most recent official report from the UK Center for Maternal and Child Enquiries (CMACE) ranked preeclampsia as the second most common direct cause of maternal death.[49] Several theories have been advocated and by far the most compelling evidence that placenta holds the key.[50,51]

Neurokinin B is amongst these markers, which is a neuropeptide of the tachykinin family, and is expressed in the outer syncytiotrophoblast of the placenta. Detectable plasma concentrations in pregnant women were observed as early as 9 weeks. It is postulated that neurokinin B binds & activates the neurokinin 3 receptor, evoking vasoconstriction in the mesenteric & portal veins & heart rate: all factors indicative of pressor activity. It now seems probable that the vasoactive properties of neurokinin B are involved in the clinical manifestations of preeclampsia. [52]

In the presenting study we investigated the serum level of neurokinin b in 38 normal pregnant females (Group 1) in comparison to 38 pregnant females with severe preeclampsia (Group 2).

The aim of the present study was to evaluate serum neurokinin B level in pregnant females with severe preeclampsia in third trimester and to see its impact on pregnancy outcomes.

Both groups of pregnant females included in the study were investigated through analyzed detailed patient history and clinical systemic and local examination.

AS regards the laboratory investigations (AST, ALT and Haemoglobin level) there was no statistically significant difference between the 2 groups.

The results of our study showed that there was a significant increase in the serum level of neurokinin b in the preeclamptic group compared to control group by using

Enzyme-Linked immunosorbent (ELISA) Assay kit for neurokinin b (P<0.001)

Results of our study coincides with D'Anna et al[53] in a prospective randomized study that involved a total of 90 pregnant women. Thirty had a gestation complicated by preeclampsia and 30 by isolated IUGR; the other 30 were controls. In all patients neurokinin B plasma levels was measured. Neurokinin B blood samples were taken at 35 weeks of gestation and at term. Results showed that neurokinin B plasma levels in the preeclamptic and IUGR groups were significantly higher than controls.

Moreover, Geissbuehler V et al [54] in a study determined the levels of neurokinin B in the plasma of South African colored pregnant women with and without preeclampsia and correlated the results with clinical data. Additionally, the peptid radioimmunoassay (RIA) and the peptid enzyme immunoassay (EIA) methods were compared in the determination of neurokinin B levels, using 58 samples from patients with preeclampsia and 62 healthy pregnant women. Clinical data were gathered using questionnaires were 58 patient samples were tested by both RIA and EIA. The mean neurokinin B concentration in the preeclamptic group was significantly higher than in the control group.

Also, our results were in agreement with Geissbuehler V et al. [55] who in a prospective randomized study measured neurokinin B levels in pregnant females with and without preeclampsia in the third trimester. Third trimester plasma neurokinin B levels were determined by enzyme-linked immunosorbent assay technique (EIA) in 72 pregnant females with preeclampsia and in 94 healthy female. The EIA results were then correlated with clinical data. Results showed that the mean neurokinin B concentration in the preeclamptic group was significantly higher than in control group (P<0.001)

Furthermore, our results were in accordance with Zulfikaroglu E et al.[56] who in a prospective randomized study involved 22 preeclamptic and normotensive female, measured the peripheral and umbilical cord blood neurokinin B levels by radioimmunoassay. The neurokinin B levels in women with preeclampsia were 0.70 nmol/L in peripheral blood and 1.92 nmol/L in umbilical cord blood. In normotensive pregnant women, neurokinin B levels were 0.43 nmol/L and 0.14 respectively. Significantly higher levels of neurokinin B in preeclamptic females compared to normotensive ones in umbilical cord blood suggested that neurokinin B enters both fetal and maternal circulation and may modulate fetoplacental hemodynamics.

Again, Li ZM et al.[57] in a study that involved 22 women, who received antenatal examination in the Department of Obstetrics and Gynecology of Union Hospital of Tongji Medical College in Huazhong University of Science and Technology from March to July in 2005, including 12 gestational hypertension (gestational hypertension group) and 10 preeclamptic female (preeclamptic group); 22 normal pregnant women in the same period were served as control. At different gestational weeks, maternal plasma levels of neurokinin B in the three groups were detected by enzyme-linked immunoassay technique, the expression and location of neurokinin B in placenta were examined by immunohistochemical SP, and mRNA expressions of neurokinin B in placenta were measured with RT-PCR method.

There was significant difference between preeclamptic group and control group (P<0.05), while there was no significant difference between gestational hypertension group and control group (P>0.05).The expressions of neurokinin B in placenta of preeclamptic group were significantly higher than that in control group, with a significant difference between the two groups (P<0.05).

Also, Liu Y et al.[58] conducted a prospective randomized study that involved a total of 60 women in the third trimester of pregnancy, 40 women with preeclampsia (study patients) and 20 normotensive women (healthy controls). They were divided into three groups: the 20 normotensive pregnant women (Group 1); 20 women with mild preeclampsia (Group 2); 20 women with severe preeclampsia (Group 3). The plasma levels of neurokinin B were significantly higher in women with mild or severe preeclampsia (p50.01 for both groups) compared with controls.

Our results were in agreement with D'Anna et al[53] who in a randomized study,

showed a positive correlation between neurokinin B plasma levels and systolic blood pressure (r=0.719, p=0.029) and with diastolic blood pressure (r=0.058, p=0.94)

Our study was done using a sample size of 76 pregnant primigravidae females. First group is composed of 38 female (control group); their age range was from 18 to 30 years old , gestational age from 38 to 40 weeks of gestation, their systolic blood pressure range from 110 to 120 mmHg, their diastolic blood pressure range from 70 to 80 mmHg , with ultrasound follow up their mean estimated foetal weight was around 3130 gms while their mean amniotic fluid index was around 11.5 ml which indicates good pregnancy outcomes where no complications were found in these 38 pregnant females who had normal vaginal delivery with healthy babies. The mean value of serum neurokinin B measured before labour was around 20.4 pg/ml in this group.

While the other group of 38 preeclamptic pregnant females ; have their age range from 24 to 32 years old, at 35 to 39 weeks of gestation , their systolic blood pressure range from 160 to 200 mmHg with mean value around 174 mmHg , their diastolic blood pressure range from 110 to 120 mmHg with mean value around 111 mmHg, with ultrasound follow up the estimated foetal weight of their fetuses range from 2000 to 2910 grams with mean value around 2444 grams and the mean value of amniotic fluid index was around 10.39 ml according to gestational age. The mean value of serum neurokinin B of this group was around 50.7 pg/ml, where 31 patients of them suffered no complications with follow up done before and after delivery with their serum level of neurokinin B high or around 50.7 pg/ml.

While 7 patients out of these 38 preeclamptic females had their babies admitted to neonatal intensive care unit with their apgar scoring at 5 minutes range from 6-7 while at 10 minutes were all above 8 and in good condition, 6 out of which had intrauterine growth restriction with low birth weight and their estimated foetal weight and amniotic fluid index below normal value for their gestational age detected during their follow up.

Three females out of these 7 preeclamptic females had eclampsia and one had HELLP syndrome which was detected after investigations was done. Serum level of neurokinin B of these 7 females was found to be low or away from the mean value for this group which was equal 50.7 pg/ml.

After our study, we concluded that there is positive significant correlation between the serum level of neurokinin B with the age, systolic blood pressure and diastolic blood pressure, while there is negative correlation between serum level of neurokinin B with the gestational age and the risk of complications including (HELLP, eclampsia, IUGR, need for NICU and ICU admission).

Conclusions:

1. Neurokinin B blood levels are higher in preeclamptic women compared to normotensive women.
2. There is positive significant correlation between neurokinin B and age,SBP,DBP while there is negative correlation between neurokinin B and gestational age & complications.
3. Neurokinin B may play a role in the pathophysiology of preeclampsia.
4. Preeclamptic patients with high levels of neurokinin B had good pregnancy outcomes.
5. Preeclamptic patients with high neurokinin B serum levels have less risk of having intrauterine gross restricted foetuses and need for neonatal intensive care unit.
6. Preeclamptic patients with high neurokinin B serum levels have less risk for developing HELLP syndrome, eclampsia and other systemic complications.

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Original Article

Study of Serum Zinc Level and Its Effect on CD4/CD8 Ratio in the Elderly before and After Zinc Supplementation.

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Abstract:

Objective: The interaction between immunity, infection, and mortality in older people is of considerable scientific and clinical significance. Proper nutrition, including adequate dietary zinc intake or supplementation, could play an important role in the prevention or reduction of infectious diseases in this population. The aim of this work is to estimate the plasma zinc level in a group of healthy elderly and to find out its effect on CD4/CD8 ratio before and after zinc supplementation. **Methods:** This study was carried out on 20 healthy subjects of old people and 10 healthy young adults as a control group. In the elderly group serum zinc and CD4/CD8 ratio were measured before and after zinc supplementation. **Results:** Significant statistical decrease in serum zinc and CD4/CD8 ratio in old compared to young before zinc supplementation ($p1<0.001$, $p1=0.005$). After one month of zinc supplementation, there was significant statistical increase in serum zinc and CD4/CD8 ratio in old subjects ($p2<0.001$, $p2<0.001$). Conclusion: Ageing is associated with decrease in serum zinc and CD4/CD8 ratio. These conditions can be reversed by zinc supplementation.

Keywords: zinc, CD4/CD8, Elderly.

Introduction:

Many micronutrients affect immunity and suboptimal nutritional supply can cause an impaired immune response.(1) This is especially true for zinc, given its essential role in many immunological processes. In many elderly, the required supply of zinc is not met.(2) A multitude of influencing factors has been suggested, which include physiological, social, psychological, and economic factors. For example, reduced mobility leads to a decrease in energy requirements. The resulting consumption of smaller quantities of food also means consuming lower amounts of trace elements, including zinc. In addition, decreased intestinal absorption, which in part depends on the composition of the food, and medication like diuretics, could cause a negative zinc balance, even if there is sufficient intake. All these factors together can result in insufficient nutritional supply with trace metals in the elderly.(3) Finally, some diseases that occur with increased frequency in older people, such as diabetes, are also accompanied by zinc deficiency.(3-5)

One major mechanism by which zinc affects immunity is its role as a signaling ion (figure 1). The intracellular concentration of free zinc is regulated by three mechanisms. One is transport through the plasma membrane.(6) Another mechanism involves storage in and release from vesicles, so-called zincosomes, in which zinc is stored as a complex with multiple ligands.(7) Finally, zinc binds to metallothionein (MT). Through its 7 binding sites with different affinities, MT buffers zinc in the pico- to nanomolar range, and can additionally be controlled by release of zinc by oxidation of zinc-binding cysteine thiol residues.(8)

Zinc signals, i.e. changes in the intracellular concentration of free zinc mediated by these three mechanisms, act on immune cell signal

transduction.[9] The first example was protein kinase C (PKC), which has been identified as a molecular interaction partner for zinc in T cells.[10] Its N-terminal regulatory domain contains four Cys3His zinc binding motifs. Zinc treatment stimulates PKC kinase activity, its affinity to phorbol esters, and binding to the plasma membrane and cytoskeleton. Furthermore, zinc chelators inhibit the induction of these events by physiological activators of PKC.[9] The lymphocyte protein tyrosine kinase (Lck), a Src-family tyrosine kinase, is an example for a different mechanism by which zinc acts on signal transduction. Zinc ions promote activation of Lck and its recruitment to the T cell receptor complex by linking two protein interface sites. The N-terminal region of Lck is recruited to the intracellular domains of the membrane proteins CD4 or CD8 by a'zinc clasp' structure.[11-13]

Aging of the immune system, also referred to as immunosenescence, describes the age-related changes in immune function that lead to increased susceptibility of older people to infectious diseases, autoimmunity, and cancer. The capacity of the immune system to mount an adequate response decreases with age, starting around 60, but several factors such as lifestyle and underlying disease can significantly affect the onset in each individual.[14] As it could be expected from the decline in immune function, aged patients suffer from an augmented incidence and mortality of infectious diseases such as pneumonia [15] and tuberculosis [16], and re-infections with herpes zoster.[17] The frequency of autoimmune diseases is augmented with age, too, accompanied by an increase in autoantibodies, which is, interestingly, not observed in centenarians.[18,19] A prominent feature of immunosenescence is thymic involution. This leads to a decrease in the generation of new T cells, finally resulting in a lower number of naïve (CD45RA+) and a higher number of memory (CD45R0+) T cells.[20]

The main changes of aging affect the T cell system. T cells from elderly subjects show decreased proliferation in response to T cell receptor (TCR) stimulation or mitogens [21], an altered CD4/CD8 ratio, and higher expression of CD95 and the pro-apoptotic BAX combined with a decrease in BCL-2 and p53, which leads to increased apoptosis.[22] The expanded subsets were primarily CD8 positive whereas CD4 populations remained unchanged. Monoclonal expansion has been found for T cells from elderly subjects. The expanded subsets can make up a large fraction of T cells, but no signs of malignant transformation have been reported.[23] However, T helper cells are also affected by aging, showing a decreased TH1/TH2 ratio in the elderly, measured by CCR4/CCR5 surface expression.[24] The TH1 cytokines IFN-γ, IL-2, and sIL-2R are reduced. In contrast, TH2 cytokines IL-4 and IL-10 are increased, resulting in a shift toward TH2 cytokines.[25,26] The effects of ageing on T cells are summarized in figure 2.

Subjects and Methods:

After approval of local ethics committee, this study was conducted on thirty subjects who were divided into two groups, the first was the elderly group which was divided into before and after zinc supplementation. The second group was 10 healthy young adults as a control.

Subjects with the following criteria were excluded from the study; patients who had diabetes mellitus, chronic hepatic diseases, collagenic diseases, subjects under immunosuppressive therapy or drugs affecting immunity and subjects taking vitamin and/or mineral supplements in the last 6 months. All subjects were subjected to thorough history taking and clinical examination, routine laboratory investigations including: CBC, fasting postprandial blood sugar, tests to asses lipid profile, tests to asses renal functions: Creatinine clearance (modified equation) and complete urine analysis, tests to asses liver functions: Aspartate aminotransferase (AST), Alanine aminotransferase (ALT), Serum albumin.

Specific investigations: Estimation of serum zinc and CD4/CD8 ratio before and after 1 month of zinc supplementation (zinc sulphate 50 mg/day) in elderly. Estimation of serum zinc and CD4/CD8 ratio was done to the young control group.

Results:

Table I shows that among cases, the mean lymphocytes count was 26.84±4.42 (ranged between 18-34) and 32.52±2.45 (ranged between 29.6-37) in the control group with significant statistical decrease in lymphocyte count in old compared to young ($p<0.001$).

Table II shows that the mean serum zinc among cases before zinc supplementation was 60.4±13.73 (ranged between 43 and 90) and after zinc supplementation was 92.1±9.4 (ranged between 78 and 107). Among subjects of the control group, the mean serum zinc was 111.8±21.05 (ranged between 85 and 149). Significant statistical decrease in serum zinc in old compared to young before zinc supplementation ($p1<0.001$). After one month of zinc supplementation, there was significant statistical increase in serum zinc in old subjects ($p2<0.001$).

Table III shows that among cases, the mean CD4% was 33.05±7.01% (ranged between 20 and 50) before zinc supplementation and after zinc supplementation was 51.55±6.02% (ranged between 40 and 61). Among subjects of the control group, the mean CD4% was 46.9±12.03 (ranged between 30 and 68). Significant statistical decrease in CD4% in old compared to young before zinc supplementation ($p1<0.006$). After one month of zinc supplementation, there was significant statistical increase in CD4% in old subjects ($p2<0.001$).

Table IV shows that among cases, the mean CD8% was 42.7±7.83 (ranged between 34 and 60) before zinc supplementation and 37.35±5.73 (ranged between 30 and 50) after zinc supplementation. Among subjects of the control group, the mean CD8% was 28.4±6.57 (ranged between 19 and 40). Significant statistical increase in CD8% in old subjects compared to young before zinc supplementation ($p1<0.001$). After one month of zinc supplementation, there was significant statistical decrease in CD8% in old subjects ($p2=0.002$).

Table V shows that among cases, the mean CD4/CD8 ratio was 0.8±0.17 (ranged between 0.4-1.2) before zinc supplementation and 1.39±0.2 (ranged between 1.1 and 2) after zinc supplementation. Among the subjects of the control group, the mean CD4/CD8 ratio was 1.79±0.86 (ranged between1-3.6) before zinc supplementation. Significant statistical decrease in CD4/CD8 ratio in old compared to young before zinc supplementation ($p1=0.005$). After one month of zinc supplementation, there was significant statistical increase CD4/CD8 ratio in old subjects ($p2<0.001$).

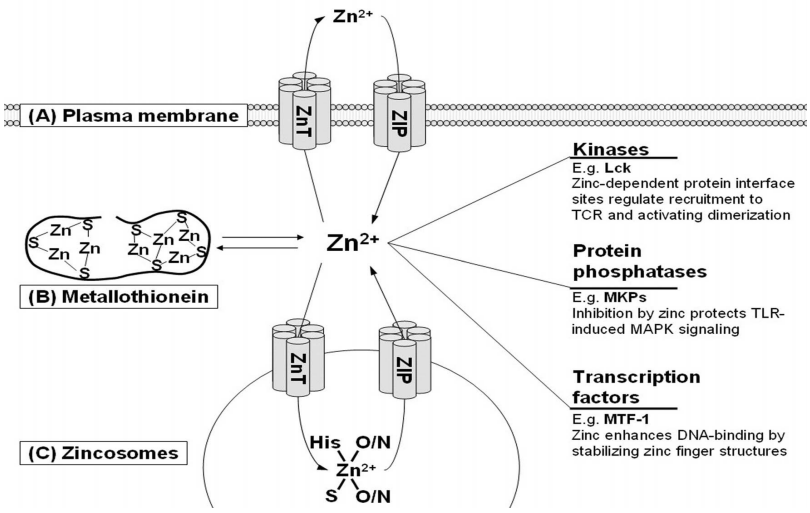

Figure 1: Zinc as a signal molecule for immune cells.

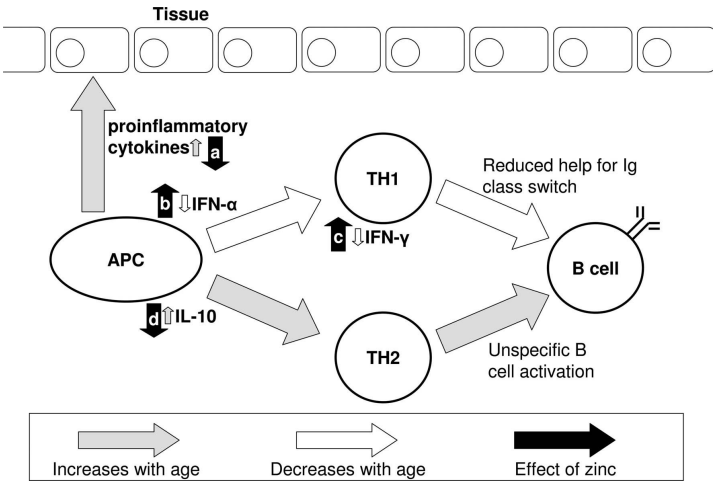

Figure 2: Influence of zinc on age-related changes of immune function.

Table (I): Comparison between the two studied groups according to WBCs picture differential count

	Control (n = 10)	Cases (n = 20)	t	p
Neutrophils				
Min. - Max.	49.0 – 61.50	49.0 – 68.0		
Mean ± SD.	57.34 ± 3.80	58.42 ± 4.75	0.621	0.540
Median	58.15	58.50		
Lymphocytes				
Min. - Max.	29.60 – 37.0	18.0 – 34.0		
Mean ± SD.	32.52 ± 2.45	26.84 ± 4.42	4.531*	<0.001*
Median	31.80	27.95		
Monocytes				
Min. - Max.	3.50 – 6.50	2.50 – 8.0		
Mean ± SD.	5.10 ± 1.04	4.81 ± 1.64	0.507	0.616
Median	5.20	4.45		

Table (II): Comparison between the two studied groups according to serum zinc before and after zinc supplementation

	Control (n = 10)	Cases (n= 20)	
		Before	After
Serum zinc			
Min. - Max.	85.0 – 149.0	43.0 – 90.0	78.0 – 107.0
Mean ± SD.	111.80 ± 21.05	60.40 ± 13.73	92.10 ± 9.40
Median	108.0	56.50	91.50
p_1		<0.001*	0.017*
p_2		<0.001*	

Table (III): Comparison between the two studied groups according to CD4 before and after zinc supplementation

	Control (n = 10)	Cases (n= 20)	
		Before	After
CD4			
Min. - Max.	30.0 – 68.0	20.0 – 50.0	40.0 – 61.0
Mean ± SD.	46.90 ± 12.03	33.05 ± 7.01	51.55 ± 6.02
Median	45.50	33.0	51.0
p_1		0.006*	0.273
p_2		<0.001*	

Table (IV): Comparison between the two studied groups according to CD8% before and after

	Control (n = 10)	Cases (n= 20)	
		Before	After
CD8%			
Min. - Max.	19.0 – 40.0	34.0 – 60.0	30.0 – 50.0
Mean ± SD.	28.40 ± 6.57	42.70 ± 7.83	37.35 ± 5.73
Median	29.0	40.50	38.0
p_1		<0.001*	0.001*
p_2		0.002*	

Table (V): Comparison between the two studied groups according to CD4/CD8 ratio before and after

	Control (n = 10)	Cases (n= 20)	
		Before	After
CD4/CD8 ratio			
Min. - Max.	1.0 – 3.60	0.40 – 1.20	1.10 – 2.0
Mean ± SD.	1.79 ± 0.86	0.8 ± 0.17	1.39 ± 0.20
Median	1.55	0.80	1.35
p_1		0.005*	0.177
p_2		<0.001*	

Discussion:

In the current study plasma zinc was measured in a sample of elderly individuals. plasma zinc was found to be lower than normal in 60% of the cases in comparison to a sample of young adults suggesting age-related decrease in plasma zinc concentration (p1<0.001). After zinc supplementation (50 mg of zinc sulfate for one month) to elderly there was increase in plasma zinc (p2<0.001). This is summarized in table 2 and figure 4. Haase et al[27] reported that, in general, plasma zinc levels decrease with advancing age. Hotz et al [28] in the second NHANES revealed that serum zinc levels increase into the third decade of life and decline from that age. Andriollo-Sanchez et al [29] found that the decrease of zinc level in healthy elderly is detected in both men and women in comparison to mean concentrations in young adults. Such results substantiate the evidence that, in a healthy ageing body zinc content progressively declines, leading to a consistent loss of plasma zinc. For physiological, social, economic and psychological reasons, elderly subjects are at risk of zinc deficiency. This occurs in agreement with the results of previous studies as Blumberg et al,[30] McClain et al.[31] Sowers et al[32] suggested that the decrease in the serum zinc with ageing may be related to the nutritional status of the elderly people, because the principle sources of bioavailable zinc include more expensive food items e.g. meat and seafood. In addition, the increase intake of prescription and over-counter medications may contribute to a compromised zinc status as they decrease the absorption of zinc. Coudry et al [33] reported that after 3 months of zinc supplementation (at 15 or 30 mg/day), a beneficial effect on plasma zinc concentration was obtained in subjects aged over 55 years old.

In the present study, we have found statistically significant decrease in CD4/CD8 ratio in old compared to young (p1=0.005) and after one month of zinc supplementation (50 mg of zinc sulfate), there was significant statistical increase in CD4/CD8 ratio in old subjects (p2<0.001). This is summarized in table 5 and figure 7. Olsson et tal [34] and Happert Fa et tal [35] reported that CD4/CD8 ratio decline with age. Hodkinson et al [36], the Zenith study, which aimed to investigate the effect of zinc supplementation on immune function in a total of 147 individuals, aged 55-70 years(77 women, 70 men). They describe no effect on some markers of immunity (natural killer cells) or inflammation (C-reactive protein), but only increased the ratio of CD4/CD8 T lymphocytes post zinc supplementation at month 6, suggesting that longer term Zn supplementation at a moderate dose (15 mg Zn/d) may result in maintenance of these lymphocyte subpopulations. Fortes et tal [37] in a study conducted on 209 healthy residents from Casa di Risopo Roma III, a home for older people (65 years of age or older) in Rome, Italy, reported that with zinc supplementation (25 mg as zinc sulfate for 3 months) there was statistically significant increase in the absolute number of CD4 cells and CD8 cells. Beck FWJ et al [38] and Prasad AS et al[39], in T cell subpopulation studies revealed that the CD4 to CD8 ratio was significantly related to zinc status. A decrease in this ratio was observed during zinc deficiency (for 6 weeks) but was corrected by zinc supplementation. Mocchegiani et al[40], Franceschi etal[41], Kahmann et al[42,43] and Chiricolo et al[44], in different studies reported that following zinc treatment (even if as zinc sulphate form) at the dose of 15 mg

Zn++/day for 1 month at alternating cycles in Down's syndrome subjects, in elderly and in old infected patients restores thymic endocrine activity, lymphocyte mitogen proliferative response, CD4(+) cell number, NK cell cytotoxicity, pro-inflammatory cytokine production and DNA repair. Bogden JD et al[45], Provinciali M et al[46] and Stewart-Knox et al[47] report that no effects on cell mediated and humoral immunity were observed when zinc was used at high doses and as zinc sulfate (90 mg/day for 3–6 months).

In the current study there was significant increase of CD8% in old subjects ($p1<0.001$) that decreases after zinc supplementation. ($p2=0.002$) as table 4 and figure 6 show. We also discover that there is a decrease in lymphocyte % in old subjects compared to young (table 1, figure 3). Chandra RK et al [48] found that the number of circulating T lymphocytes is slightly decreased. The number of CD4 is decreased, where as the number of CD8 cells is variously reported as normal, decreased, or increased. Wikby A et al[49] ,Strendhall J et al[50], Wikby A et al[51] and Pawelec G et al[52] in another recent sweedish studies, OCTO and NONA studies which aimed at identifying factors predicting 2, 4 and 6 year mortality rates and have resulted in the emerging concept of an Immune Risk Profile (IRP). The IRP defined from healthy octogenarians and nonagenarians, characteristically display (i) high levels of CD8+ and low level of CD4+ T-cells (an inverted CD4+:CD8+ ratio), (ii) an increase in the number of dysfunctional terminally differentiated memory T-cells (CD8+CD28-).

From all these studies, a physiological dose of zinc applied for a long period or high doses of zinc for short periods might induce limited effects on the immune response, perhaps due to zinc accumulation in various organs and tissues with subsequent toxic effect of zinc upon the immune functions.[53] In this context, it is also useful to remember that high doses of zinc trigger apoptosis of the immune cells in the presence of high-oxidative stress and inflammation.[54] Therefore, caution is advised for the management of zinc supplementation with the suggestion to perform the trial for short periods and on alternate cycles only.[53]

Conclusion:

Zinc ions are indispensable for immune function, especially for T cell mediated events, which are primarily affected in immunosenescence. The high prevalence of zinc deficiency in old subjects and the correlation between zinc status and immune function surely justifies zinc supplementation to these subjects to normalize zinc levels, and hereby restore important functions of the immune system. One central question remains: Should the decrease of zinc status with age be seen as a marginal zinc deficiency, which, in combination with multiple other factors, increases the susceptibility for infectious diseases and cancer, and should zinc be given to those with no clinical symptoms? From the results published so far, it looks like a moderate zinc supplementation that stays well below the limits for adverse effects could have substantial benefits. However, a rapid and reliable method for the assessment of zinc status would be helpful to identify those who would benefit most from zinc supplementation.

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Original Article

Bone Densitometry in the Elderly Female and Possible Relation to Serum Copper.

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Abstract:

Background: Osteoporosis is a skeletal disorder characterized by compromised bone strength predisposing to an increased risk of fracture. It is a major public health problem throughout the world. The social and economic burden of osteoporosis is increasing steadily because of the aging of the world population. Osteop orosis is frequently only diagnosed after the first clinical fracture has occurred. Consequently, therapy is often aimed at preventing further fractures. It is therefore important to assess individual osteoporosis risk early enough to prevent the first fracture. Trace elements, including copper, are essential for normal growth and development of the skeleton in humans and animals. The role of copper in bone metabolism can be linked primarily to the Cu-dependent enzyme lysyl oxidase, for which copper acts as a cofactor. Lysyl oxidase is required for the formation of lysine-derived cross-links in collagen and elastin. The aim of this study was to estimate the plasma copper level in a group of healthy elderly female, and to compare it with bone mineral density BMD of the same group, using dual energy X-ray absorptiometry DEXA to assess if serum copper could potentially be developed into a simple, cheap, and radiation-free screening method for osteoporosis. **Subjects & Methods:** This study was conducted on thirty female subjects divided into two groups; the first consisted of ten healthy control female subjects below the age of 60 years. The second group consisted of twenty healthy female subjects 60 years old and older. Serum copper, serum albumin and total serum calcium levels were assessed in all subjects. Bone mineral density using DEXA scan was done for all subjects. **Results:** The serum copper showed significant increased level in the elderly female subjects compared with the younger subjects ($p = 0.035$). This study also showed no statistically significant correlation between BMD and serum copper ($p = 0.072$), while, a statistically significant positive correlation between BMD and serum albumin was found ($p = 0.017$). **Conclusion:** In this study serum copper did not seem to correlate with BMD and so cannot be used as a screening method for osteoporosis. We also can conclude that, serum albumin may have a beneficial role in bone health.

Keyword: Bone Densitometry, Elderly Female, Serum Copper.

Introduction:

Osteoporosis is a major health problem that significantly affects the aging population. Catastrophic effects on disability and mortality accompany the increase in the incidence of osteoporotic fractures in patients aged 65 and older.[1] A combination of factors, including genetics, nutrition, physical activity, and bone turnover, determine bone mass and ultimately bone strength.[2] After the acquisition of peak bone mass during the third decade of life, there is a progressive decline of approximately 0.5% a year, which is considered a physiological age-related change.[3]

Age-related bone loss is the consequence of changes in bone cellularity as well as hormones. Increasing osteoclast formation and activity and diminished osteoclast apoptosis, follow the declining estrogen levels in women although more significantly in women during peri-menopausal years cellular changes in aging bone reduce the number of osteoblasts available for bone remodeling and formation.[4] A proportion of subjects lose their bone mass only at a physiological basis, whereas a proportion of subjects will suffer pathological bone loss leading to osteoporosis. Although diet, physical activity, and genetics play a role

in accelerated bone loss, it is likely that there are additional hormonal and molecular factors that remain to be elucidated.[5]

Osteoporosis is a silent disease until it is complicated by fractures that can occur following minimal trauma. These fractures are common and place an enormous medical and personal burden on aging individuals and a major economic toll on the nation. Osteoporosis can be prevented and can be diagnosed and treated before any fracture occurs. Prevention, detection and treatment of osteoporosis should be a mandate of primary care providers.

The National Osteoporosis Foundation NOF recommends a comprehensive approach to the diagnosis and management of osteoporosis. A detailed history and physical examination together with BMD assessment and the WHO 10-year estimated fracture probability are utilized to establish the individual patient's fracture risk.[6]

Dual-energy x-ray absorptiometry (DXA) measurement of the hip and spine is the technology now used to establish or confirm a diagnosis of osteoporosis, predict future fracture risk and monitor patients by performing serial assessments.[7]

Nutrients such as calcium, phosphorus, zinc, copper, protein, and vitamin D have a relationship with bone mass[8,9]. Proper nutrient intake has a crucial role in both prevention and treatment of osteoporosis [10].

Copper (Cu) is an essential trace element for humans and animals. The ability of copper to easily accept and donate electrons explains its important role in oxidation-reduction reactions and in scavenging free radicals[11]. Copper is essential in collagen-crosslink formation as a component of the metalloenzyme, lysyl oxidase. The action of lysyl oxidase helps maintain the integrity of connective tissue in the heart, blood vessels and also plays a role in bone formation [12]. Collagen cross links provide tensile strength to bone and copper deficiency in several animal species produces skeletal abnormalities [13, 14].

Subjects and Methods:

After approval of local ethics committee, this study was conducted on thirty female subjects divided into two groups, the first consisted of ten healthy control female subjects below the age of 60 years. The second group consisted of twenty healthy female subjects 60 years old and older. Subjects under hormonal replacement therapy or drugs affecting bone density, smokers, those with chronic liver disease, diabetic patients, and patients with metabolic bone disease or connective tissue disease were all excluded from the study.

All subjects were subjected to thorough history taking and clinical examination, height and weight measurement for BMI assessment, routine laboratory investigations including: CBC, fasting blood sugar, blood urea, serum creatinine, serum transaminases (ALT, AST), and assessment of serum albumin and total serum calcium levels.

For serum copper; blood samples were collected in serum separator tubes, centrifuged separating serum samples which were frozen at - 20°C. Serum copper concentrations were measured by using an induction coupled serum atomic emission spectrometer.

A standard questionnaires concerning smoking, physical activity, sun exposure, diet, medications use and disease history was done.

The diagnosis of osteoporosis was based on BMD measurements. BMD was measured at the lumbar spine (L1–L4) by dual energy X-ray absorptiometry (Hologic QDR-4500, USA). The diagnosis of osteoporosis was based on the WHO criteria[15] in which a loss of bone mass ≤1 standard deviation (SD) was considered as normal, loss of bone mass >1 SD , ≤2.5 SD was diagnosed as osteopenia and loss of bone mass >2.5 SD was diagnosed as osteoporosis.

Results:

T-score results in the studied groups showed that, in group I, 5 subjects (50%) were normal, 4 subjects (40%) were osteopenic and only one subject (10%) was osteoporotic. While in group II, 7 subjects (35%) were normal, 6 subjects (30%) were osteopenic and 7 subjects (35%) were osteoporotic.(table I)

Regarding serum copper level, in group I it ranged between 7 and 199 ug/dl with a mean of 62.70 ± 68.88 ug/dl. While in group II, it ranged between 11 and 199 ug/dl with a mean of 120.74 ± 65.83 ug/dl. The serum copper showed

significant increased level in the elderly female subjects compared with the younger subjects (p = 0.035).(table II)

Regarding serum calcium level, in group I, it ranged between 8.5 and 9.9 mg\dl with a mean of 9.11 ± 0.5 mg\dl. In group II, it ranged between 7.2 and 9.4 mg\dl with a mean of 8.82 ± 0.5 mg\dl.(table II)

Serum albumin level in group I ranged between 3.5 and 5.2 g\dl with mean of 4.27 ± 0.53 g\dl in group I. While in group II, it ranged between 3.6 and 4.9 g\dl with a mean of 4.0 ± 0.42 g\dl. There was no significant statistical difference between the studied groups regarding serum calcium or serum albumin levels.(table II)

The current study found no statistically significant correlation between T-score and serum copper (p = 0.072), while a statistically significant positive correlation between T-score and serum albumin (p = 0.017) was found. (table III, figures 1,2)

There was no statistically significant correlation between T-score and age, BMI or serum calcium level. (Table III)

Table (I): Comparison between the two groups according to T-score

	Age (years)				Total (n = 30)	
	<60 (n = 10)		≥60 (n = 20)			
	No.	%	No.	%	No.	%
Total BMD T-score						
Normal (≥-1)	5	50.0	7	35.0	12	40.0
Osteopenia (-2.5 - -1)	4	40.0	6	30.0	10	33.3
Osteoporosis (≤ -2.5)	1	10.0	7	35.0	8	26.7
χ^2(^{MC}p)	2.101 (0.386)					

χ^2: value for Chi square MC: Monte Carlo test

Table (II): Serum copper, calcium, and albumin levels in the studied groups:

	Age (years)		Total (n = 30)
	Group I (n = 10)	Group II (n = 20)	
Serum copper (ug\dl)			
Min. – Max.	7.0 – 199.0	11.0 – 199.0	7.0 – 199.0
Mean ± SD.	62.70 ± 68.88	120.74 ± 65.83	101.39 ± 71.32
Median	28.0	142.50	97.30
Z (p)	2.112* (0.035*)		
Serum calcium (mg\dl)			
Min. – Max.	8.50 – 9.90	7.20 – 9.40	7.20 – 9.90
Mean ± SD.	9.11 ± 0.50	8.82 ± 0.50	8.91 ± 0.51
Median	9.05	8.90	8.90
t (p)	1.526 (0.138)		
Serum albumin (g\dl)			
Min. – Max.	3.50 – 5.20	3.60 – 4.90	3.50 – 5.20
Mean ± SD.	4.27 ± 0.53	4.0 ± 0.42	4.09 ± 0.47
Median	4.15	3.88	3.90
t (p)	1.528 (0.138)		

t: Student t-test

Z: Z for Mann Whitney test

*: Statistically significant at p ≤ 0.05

Table (III): Correlation between T-score with age, BMI, serum copper, serum calcium and serum albumin.

	T-score	
	r_s	p
Age	-0.269	0.151
BMI	0.058	0.762
Serum copper (ug\dl)	-0.333	0.072
Serum calcium (mg\dl)	-0.032	0.868
Serum albumin (g\dl)	0.431*	0.017*

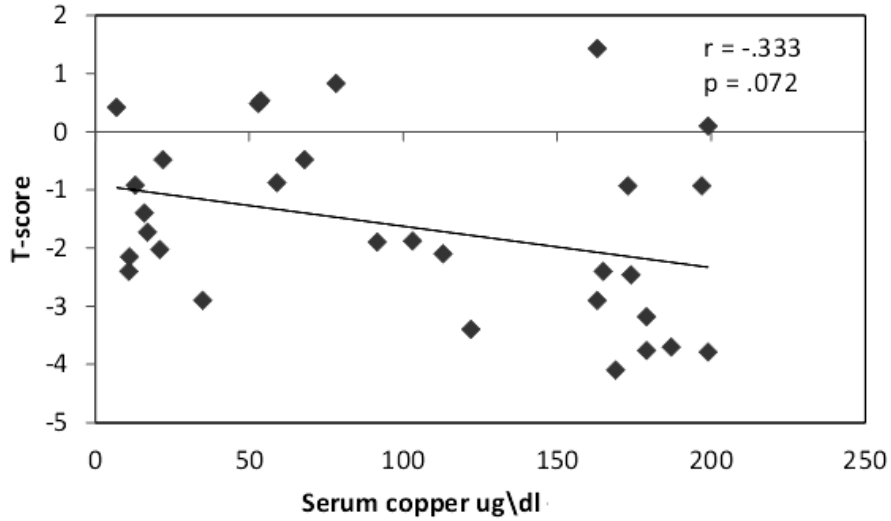

Figure (1): Correlations between serum copper and T-score.

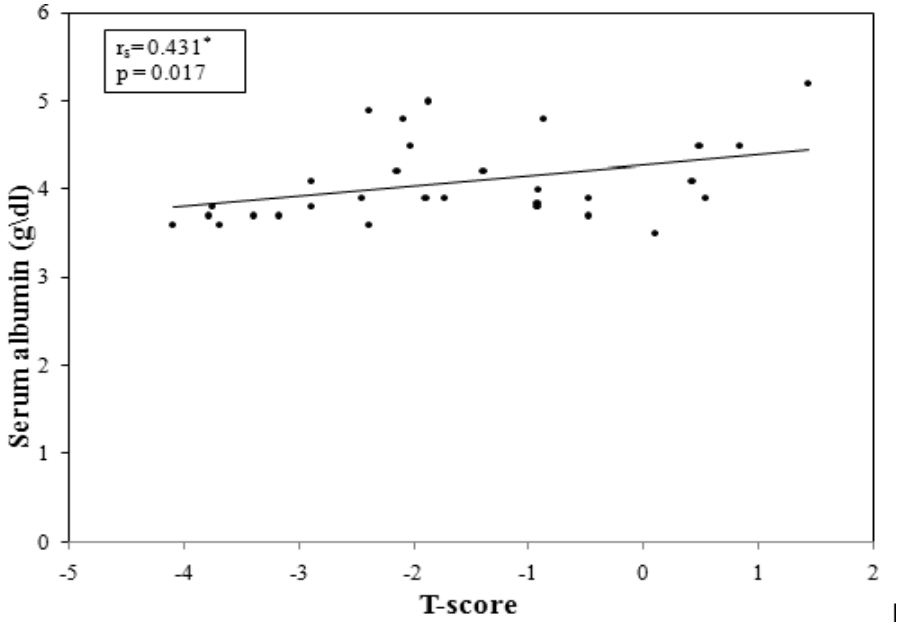

Figure (2): Correlation between T-score with Serum albumin (g\dl)

Discussion:

In the current study, serum copper level was found to be significantly higher in elderly women compared to younger healthy women (p = 0.035) (table 2). This agree with Madaric et al.[16] who found that serum copper concentrations and the copper/zinc ratio correlated positively with age (p< 0.0001). Tietz et al[17] and Bunker et al.[18] also found that serum copper values increase with age.

Similar results were found in a study conducted in the internal Medicine Department, Alexandria University on 50 subjects, aged 65 years old or older on 2012 which showed that plasma copper level was higher than normal in 40% of the cases suggesting an age related increase in plasma copper concentration .[19]

This increase of copper level with age in our population may be related to environmental factor, pollution or dietary habits.

On the other hand, Gamez et al,[20] Mir et al.[21] and Lowe et al,[22] found that there was no significant difference in plasma Cu concentration between elderly and young healthy women.

In the current study there was no significant statistical correlation between serum copper and T-score (BMD), with (p = 0.072). (Table 3).

Similarly, Odabasi et al. observed that there was no significant difference between healthy and osteoporotic elderly women, both in plasma and in red blood concentrations, for copper.[23] Similar results were found by Mutlu et al[24] and Arikan et al.[25], who reported that there was no significant difference in serum Cu levels between elderly women with osteoporosis and the non-osteoporotic control subjects. Razmandeh et al.[26] in a case–control study published on 2014 reported that there was no significant difference in serum copper level between osteoporotic and normal control female subjects.

Other studies showed that high copper level was associated with osteoporosis. Massie et al. have suggested that excess copper was associated with decreasing bone size and density.[27] Koulourides observed an inhibition of enamel remineralization by Cu.[28] Menerey described that the excessive toxic copper in the skeletal system may mediate oxygen free radical release and thus damage tissue.[29]

On the other hand, Gur et al. reported that Zn and Cu levels in serum were lower among patients with osteoporosis than the controls. [30] Sierpinska et al. reported that severe tooth wear is associated with reduced spinal BMD and that enamel in adult individuals with severe tooth wear is low in copper content.[31]

Many previous clinical studies provide evidence of beneficial role of copper and zinc in improvement of bone density and quality in both osteoporotic and healthy individuals, particularly found in cancellous bone, i.e., lumbar spine vertebrae [32-34].

Regarding serum calcium, no significant statistical difference between serum calcium levels with age was found (Table 2), also, no statistically significant correlation between BMD and serum calcium level was found. (Table 3).

Nieves et al.[35,36], Arikan et al.[25]and liu et al.[37] in agreement with our results did not find differences in serum calcium by age group or when compared between osteoporotic, osteopenic and normal subjects.

The current study showed a statistically significant positive correlation between T-score and serum albumin (p=0.017). (Table 3) Similarly, serum albumin has been reported to be positively associated with bone mineral content[38,39] and negatively associated with the risk of hip fracture, both in a prospective study[40] and in a case–control study[41,42], supporting the view that severe protein depletion plays a role in causing hip fracture[43]. Underweight hypoalbuminemic subjects had a lower T-score than underweight

cases with normal serum albumin levels[44] findings consistent with the positive correlation demonstrated by other authors in both genders[40] and in women alone [43].

Coin et al. studied the relationship between serum albumin and hip bone mineral density (BMD) in 352 elderly outpatients (216 women aged 73.57±5.3 years and 136 men aged 73.97±5.6 years) and found that albumin was significantly associated with BMD in both genders.[45]

However two large cross-sectional studies failed to confirm this correlation between albumin and BMD[46,47]. Lunde et al. in a cross-sectional study, examined the relation between serum albumin and bone mineral density (BMD) in 1593 white, community dwelling men and women aged 50–95 years. In both sexes there was positive correlation between serum albumin and BMD in the unadjusted model ($p<0.005$). After age adjustment, however, the relationship was no longer significant ($p >0.18$).[48]

Conclusion:

From the previous results and from the opposing studies in the literature we can conclude that; although the beneficial role of copper in improvement of bone density and quality, it does not seem to be correlated with BMD and so cannot be used as a screening method for osteoporosis. Serum albumin has been found to be positively correlated with bone mineral content and may have a beneficial role in bone health that needs more studying.

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Original Article

Association between Vascular Endothelial Growth Factor (VEGF) and Thrombosis of Native Arteriovenous Fistula in Patients on Maintenance Hemodialysis (HD)

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Abstract:

Introduction: Vascular access continues to be a leading cause for hospitalization and morbidity in patients with chronic kidney disease (CKD) stage 5. Appropriate care of hemodialysis (HD) patients with CKD stage 5 requires constant attention to the maintenance of vascular access patency and function. The main aim for vascular access monitoring and surveillance is that stenosis develops over variable intervals in the great majority of vascular accesses and, if early detected and corrected, under dialysis can be minimized or avoided and the rate of thrombosis can be reduced. Vascular endothelial growth factor (VEGF) is a highly specific mitogen for vascular endothelial cells. Factors that can potentiate VEGF production include fibroblast growth factor 4 (FGF-4), PDGF , tumor necrosis factor α , transforming growth factor β (TGF-β), keratinocyte growth factor (KGF) , IGF-I, interleukin 1β (IL-1β), and IL-6. Other cytokines such as IL-10 and IL-13 can inhibit the release of VEGF. **Subjects and Methods:** This study was carried on 40 patients divided into 2 groups, group I contain 20 patients with thrombosed AVF proved by Doppler ultrasound and group II 20 patients with normally function AVF for at least 6 months with no previous vascular access thrombosis. All patients will be investigated for CBC, BUN, Cr, FBS, fasting lipid profile, liver function tests, CRP and VEGF. **Results** There was statistically significant difference between the 2 groups where CRP and VEGF values were higher in cases than those of control group (P= 0.002). There were no statistical significant differences between the 2 groups regarding total cholesterol, LDL-cholesterol, HDL-cholesterol, VLDL-cholesterol and TGs **Conclusion:** Detection of elevated concentrations of circulating VEGF could be useful in prediction of native AVF thrombosis in ESRD on maintenance hemodialysis. Elevated CRP level is a significant risk factor for native AVF thrombosis in ESRD on maintenance hemodialysis.

Keywords: vascular endothelial growth factor (VEGF), thrombosis, arteriovenous fistula, hemodialysis (HD)

Introduction:

End stage renal (ESRD) is defined as irreversible decline in a person's own kidney function, in which initiation of renal replacement therapy (RRT) either dialysis or transplantation is essential for life. The three primary treatment options for patients with ESRD are hemodialysis (HD), peritoneal dialysis (PD) and kidney transplantation[(1)] The principle of hemodialysis is the same as other methods of dialysis; it involves diffusion of solutes across a semipermeable membrane. Hemodialysis utilizes counter current flow, where the dialysate is flowing in the opposite direction to blood flow in the extracorporeal circuit. Counter-current flow maintains the concentration gradient across the membrane at a maximum and increases the efficiency of the dialysis.

Vascular access for patients on maintenance hemodialysis:

Vascular access continues to be a leading cause for hospitalization and morbidity in patients with chronic kidney disease (CKD) stage 5. Appropriate care of hemodialysis (HD) patients with CKD stage 5 requires constant attention to the maintenance of vascular access patency and function.[(2)] An ideal access delivers a flow rate to the dialyzer

adequate for the dialysis prescription, has a long use-life, and has a low rate of complications (eg, infection, stenosis, thrombosis, aneurysm, and limb ischemia), painless and available for use immediately upon placement. Unfortunately this ideal access is not yet available, but of the available accesses, the surgically created arteriovenous fistula (AVF) comes closest to fulfilling these criteria. Studies over several decades consistently demonstrate that native fistula accesses have the best 4- to 5-year patency rates and require the fewest interventions compared with other access types.[3,4,5]

There are three main forms of permanent HD vascular access: The arteriovenous fistula (AVF), the arteriovenous graft (AVG), and the tunneled central venous catheter.

Access monitoring and surveillance:

Vascular access function and patency are essential for optimal management of HD patients.

Low blood flow rate and loss of patency limit HD delivery and extend treatment times, this result in under dialysis that leads to increased morbidity and mortality [6] The main aim for vascular access monitoring and surveillance is that stenosis develops over variable intervals in the great majority of vascular accesses and, if early detected and corrected, underdialysis can be minimized or avoided and the rate of thrombosis can be reduced.

A number of monitoring and surveillance methods are available: sequential access flow, sequential dynamic or static pressures, recirculation measurements, and physical examination.[7]

Asymptomatic, but hemodynamically significant, stenosis usually are detected through a systematic monitoring and surveillance program. Detection of such stenosis is important to prevent progression to a functionally significant stenosis, which is currently defined as a decrease of greater than 50% of normal vessel diameter, accompanied by hemodynamic or clinical abnormality, such as abnormal recirculation values, elevated venous pressures, decreased blood flow, swollen extremity, unexplained reduction in Kt/V, or elevated negative arterial pre-pump pressures, that prevent increasing to acceptable blood flow

Physical examination include:

- Simple inspection can reveal the presence of aneurysms. A fistula that does not at least partially collapse with arm elevation is likely to have an outflow stenosis. This logic applies to the case in which a tourniquet does not appear necessary for optimal cannulation.
- Strictures can be palpated and the intensity and character of the bruits can suggest the location of stenosis.
- Presence of edema maybe an indicator of local infection or venous outflow impairment.
- Absence of auscultatory thrill can be found in AVF thrombosis.

Doppler ultrasound (DU)[8] and MRA[9,10] are direct techniques for assessing flow in vascular accesses. Duplex Doppler ultrasound (DDU) requires an accurate measurement of the cross-sectional diameter of the access. The method is operator dependent and subjected to error caused by variation in cross-sectional area[11]

AVF steno-thrombotic complications:

Failure of dialysis access can result from either inadequate blood flow on account of stenosis of the venous outflow tract or complete occlusion due to thrombosis.[12]

A- Venous stenosis:

Venous stenosis constitutes the major causae of AVF failure. Three types of venous stenosis can be identified: juxta-anastomotic, proximal and lesions affecting central veins. Juxta-anastomotic lesion implies stenosis within 5 cms of the anastomosis and it's the most commonly observed form. Proximal stenosis is located between the juxta-anastomotic region and central veins. The most frequent sites of venipuncture are the most commonly affected.[12]

B- Arterial stenosis:

The lesion here is generally manifest in the pre-anastomotic tract, although potentially any portion of the afferent artery can be affected.

C- AVF thrombosis:

About 80–85% of arteriovenous (AV) access failures come from AV access thrombosis, more than 80% of which result from AVF stenosis. Decreased access flow is associated with an increased risk of access thrombosis. Access flow (Qa) < 500mL/ min was demonstrated to be predictive of poorer unassisted patency of native AVF. (13,14,15)

Thrombosis however may also be manifested in the absence of stenosis in this case other hemodynamic factors are usually associated such as marked hypotension or hypercoaguable states.

In addition to access flow, some mechanical factors influence AVF patency, such as the surgical skill, the puncture technique, the shear stress on the vascular endothelia and various medical factors all contribute to AVF thrombosis (16)

Factors affecting patency of the AVF:

A- Medical factors:

Hypotension, Hypoalbuminemia, DM and cardiovascular risk factors, Red blood cell mass, Hypercoagulable states

B- Endothelial cell injury:

Many factors lead to endothelial cell injury or dysfunction and they are listed as follows.

1- Preexisting intimal hyperplasia

2- Tumor necrosis factor-α (TNF-α)

Leukocytes release TNF-α, which could induce proliferation of vascular smooth muscles leading to subsequent intimal hyperplasia. (17)

3- Oxidative stress

Oxidative hyperactivity in the uremic status usually leads to an increased amount of circulating and tissue inflammatory molecules. Interaction with dialysis membranes have also been reported as an important cause leading to oxidative stress, resulting in an increased expression of endothelin-1.

4- Calcium phosphate deposition

Stenosis of AVFs were associated with calcium phosphate deposition, which is mainly in the form of calcium apatite.

5- Activated platelets

Injury to endothelial cells exposes the basement membrane and extracellular matrix leading to activation of platelets. It has been shown that higher levels of circulating activated platelets are associated with shorter survival of AV access

Vascular endothelial growth factor (VEGF):

is a highly specific mitogen for vascular endothelial cells. Five VEGF isoforms are generated as a result of alternative splicing from a single VEGF gene. These isoforms differ in their molecular mass and in biological properties such as their ability to bind to cell-surface heparan-sulfate proteoglycans.(18) The expression of VEGF is potentiated in response to hypoxia, by activated oncogenes, and by a variety of cytokines.

The vascular endothelial growth factor (VEGF) family members VEGF-A, VEGF-B, placental growth factor (PlGF), VEGF-C and VEGF-D, they bind their cognate receptors VEGFR-1, VEGFR-2 and VEGFR-3 found on the vascular endothelium. VEGF is a proinflammatory cytokine acts by increasing endothelial permeability and inducing adhesion molecules that bind leukocytes to endothelial cells.

VEGF production:

VEGF expression and secretion by a variety of cell types, including human vascular smooth muscle cells (HSMC), are well known. More recently, release of VEGF from circulating blood cells such as T lymphocytes), mononuclear cells, polymorphonuclear neutrophils (PMN), and platelets has been described. Platelets release VEGF during platelet aggregation together with the release of β-thromboglobulin, suggesting that VEGF resides in the α-granules of platelets

VEGF induces endothelial cell proliferation, promotes cell migration, and inhibits apoptosis. *In vivo* VEGF induces angiogenesis as well as permeabilization of blood vessels, and plays a central role in the regulation of vasculogenesis. It supports trans endothelial migration of monocytes and is chemotactic for mast cells and monocytes. Inflammatory cytokines such as IL-1α and IL-6 induce expression of VEGF in several cell types, including synovial fibroblasts.

Factors affecting VEGF production:

Factors that can potentiate VEGF production include fibroblast growth factor 4 (FGF-4), PDGF, tumor necrosis factor α [19], transforming growth factor β (TGF-β)[20], keratinocyte growth factor (KGF) [21], IGF-I, interleukin 1β (IL-1β), and IL-6 .Other cytokines such as IL-10 and IL-13 can inhibit the release of VEGF .

Subjects and Methods:

The present study was conducted on 20 patients with ESRD on maintenance hemodialysis 3 times weekly of average of 4 hours each session, with a previously thrombosed AVF, assessed clinically and confirmed by duplex, 9 females and 11 males, their age ranged from 33- 60 years. And compared to a control group of 20 patients with ESRD on maintenance hemodialysis 3 times weekly, but with no history of previously thrombosed vascular access, confirmed clinically and with duplex study of neck veins and AVF. The control group included 8 females and 12 males, their age ranged from 32-59 years.

In both groups, thorough history taking was done including: demographic data, original kidney disease, duration of CKD, time of diagnosis of ESRD, time to start HD therapy, complications that occur during HD session, other co-morbidities as DM and HTN, current drug history and smoking habits. Special concern about vascular access history was taken, previous HD catheter insertions, previous complications, time of AVF creation, complications that occurred to previous or current AVFs. Also full clinical examination was done, and clinical examination was done for the previous and current AVFs. A mid-week fasting pre-dialysis blood sample was withdrawn from each patient. Also Duplex US was done for jugular, subclavian veins and AVFs

Laboratory investigations:

Complete blood picture.[22] Lipid profile (total cholesterol (TC), Low density lipoprotein cholesterol (LDL-C), high density lipoprotein cholesterol (HDL-C), very low density lipoprotein cholesterol (VLDL-C) and triglycerides (TGs).[23] Fasting blood sugar level.[24] Liver function tests (SGOT, SGPT, Prothrombin time and activity, Plasma proteins).[25] BUN, creatinine, serum calcium and serum phosphorus.[26] C- reactive protein level.[27] VEGF level measurement with ELISA technique.[28,29]

Doppler US study of both subclavian and both internal jugular veins and the native AVF assessing access flow, presence and location of stenosis or thrombosis or other complications.[30,31]

The following patients were excluded from the study:

Patients with AVF of less than one month duration. Patients with hypotension, hypoalbuminemia, or have systemic infection within one month before entrance in the study. Patients with known chronic inflammatory disease including systemic lupus erythematosis (SLE) and vasculitis, Smokers.

Results:

There was no statistical significant differences between the two groups regarding age, sex, CBC, S Cr, Ca, Ph, FBS, Lipid profile. There was a statistically significant difference between the 2 groups, BUN level was higher in group I (P=0.04). There were no statistically significant differences between the 2 groups regarding total protein (p=0.27), S. Albumin (P=0.333), SGPT (P=0.445), SGOT (P=0.383), PT (P=0.429) and INR (P=0.529).

In group I: CRP levels ranged from 3-66 mg/L with a mean of 19.16± 17.02 mg/L

In group II: CRP levels ranged from 3-32 mg/L with a mean of 7.07±6.98 mg/L There was statistically significant difference between the 2 groups where CRP values were higher in cases than those of control group (P= 0.002).

In group I: VEGF levels ranged from 42–1300pg/ml with a mean of 358.50±348.44 pg/ml

In group II: VEGF levels ranged from 48–500 pg/ml with a mean of 174.20±128.54 pg/ml There was statistically significant difference between the VEGF levels between the 2 groups, VEGF was higher in group I than group II. (P=0.033).

In group I: 25% of the cases were diabetic, while 75% of cases were non-diabetic.

In control group II: 10% were diabetic while 90% were non-diabetic. There was no statistically significant difference between the 2 groups regarding DM. (P=0.407)

Table (I): Comparison between the two studied groups according to CRP and VEGF

	Group I	Group II	p
CRP(mg/L)			
Min. – Max.	3.0 – 66.0	3.0 – 32.0	
Mean ± SD	19.16 ± 17.02	7.07 ± 6.98	0.002*
Median	13.90	4.15	
VEGF (pg/ml)			
Min. – Max.	42.0 – 1300.0	48.0 – 500.0	
Mean ± SD	358.50 ± 348.44	174.20 ± 128.54	0.033*
Median	195.0	130.0	

p: p value for Mann Whitney test for comparing between the two studied groups
*: Statistically significant at p ≤ 0.05

Table (II): Comparison between the two studied groups according to DM

	Group I		Group II		FEp
	No	%	No	%	
DM					
No	15	75.0	18	90.0	0.407
Yes	5	25.0	2	10.0	

FEp: p value for Fisher Exact test for comparing between the two studied group

Table (III): Distribution of the studied cases according to site of the thrombosed AVF

	No.	%
Radio cephalic	10	50.0
Brachio cephalic	5	25.0
Both	5	25.0

Discussion:

In our study, there was no statistical significance between the 2 studied groups regarding the age (P= 0.517) and sex (P=0.749). This data coincides with the study of Da Silva et al. 2003[32], which was unable to report significant difference in AVF thrombosis regarding age and sex. and also coincides with the study of Weiss et al 2001[33]

In our study we found no statistical significance between the 2 studied groups regarding regarding total cholesterol (P=0.606), LDL-cholesterol (P=0.75), HDL-cholesterol (P=0.557), VLDL-cholesterol (p=0.583) and TGs (P=0.445).

These results mismatch those of De Marchi et al 1996[34] which found significant association between AVF thrombosis and higher values of TGs, total cholesterol, LDL-C and lower values of HDL-C.

Mohrshaldt et al 2000[35], reported that the high LDL-C levels are associated with a pro-inflammatory cytokine production capacity.

Cokreill et al[36] found that Low HDL-C levels were associated with increased AVF thrombosis risk in maintenance HD patients, HDL-C decreases the cytokine induced expression of adhesion molecules by vascular endothelial cells.

Hypoalbuminemia is a significant risk factor for AVF thrombosis in HD patients, Baron et al 2010[37] reported strong association between hypoalbuminemia and AVF failure

due to thrombosis. Those results can't be correlated with our study as we excluded patients with hypoalbuminemia from both groups.

In our study there was a stastistical significant difference between the 2 studied groups regarding the CRP levels (P=0.002). This result coincides with the study of Chou et al[38] which showed that CRP strongly predicts AVF thrombosis in maintenance hemodialysis patients, this predictive effect might be possibly because CRP reflects intimal hyperplasia in native AVF.

Owing to the fact that CRP is produced by smooth muscle cells of atherosclerotic lesions, serum CRP has been reported to be effective in predicting cardiovascular events and mortality in HD patients, Schillinger et al[39] suggested that baseline CRP predicts restenosis in the femoropopliteal segment after angioplasty and shows a directive measurement of neointimal hyperplasia.

Regarding VEGF levels, in our study there was a statistical significant difference between the 2 studied groups, it was higher in group I, (P= 0.03), our result coincides with the results of Zohny et al[40] which found a significantly increased levels of VEGF in ESRD patients on maintenance hemodialysis.

Regarding DM, in our study there was no statistically significant difference between the 2 studied groups (P= 0.4), this result coincides with the study of Ghorbani et al[41] which failed to find an association between diabetes and AVF thrombosis in the studied group.But mismatches the study of Windus et al[42] which found significant association between AVF thrombosis and diabetes. Further studies with large samples is needed to prove the relation between different disease (diabetes, hyperlipideamia, vasculitis) and AVFy thrombosis.

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Original Article

Study of the Role of Urinary Tweak as a Biomarker of Lupus Nephritis.

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Abstract:

Background: Systemic lupus erythromatosis (SLE) is an auto immune disease characterized by overproduction of various auto-antibodies affecting almost all body organs. Renal involvement is common in SLE and often determines the course of the disease. The cytokine TWEAK is one of the novel biomarkers of renal lupus activity. **Objectives:** The aim of the work was to study the role of uTWEAK in lupus patients, its relation to clinical manifestations of the disease especially lupus nephritis. Furthermore, its correlation with the conventional measures of renal lupus activity. **Subjects:** The present study was conducted on 30 patients with biopsy-proven lupus nephritis, 20 SLE patients without nephritis and 20 healthy subjects of matched age and sex as a control. The diagnosis of patients with SLE was based on fulfilling at least four of the American College of Rheumatology Classification Criteria. **Methods:** All patients were subjected to thorough history taking and complete clinical examination to detect various organs involvement by SLE. Laboratory investigations done included: CBC, ESR, FBG, blood urea, serum creatinine, complete urine analysis, 24 hours urinary protein ,urinary protein/creatinine ratio, ANA, Anti-ds DNA, C3, C4, urinary TWEAK by ELISA, SLEDAI for all lupus patients and renal SLEDAI for lupus nephritis**.** **Results:** Among all cases of SLE, the highest mean of uTWEAK was detected in patients with renal manifestations (lupus nephritis) ($p<0.001$). There is a statistically significant increase in uTWEAK level in patients with lupus nephritis than patients with SLE without nephritis ($p<0.001$) as well as than controls ($p<0.001$).In patients with lupus nephritis, there is a statistically significant positive correlation between uTWEAK level and ANA($p=0.034$), Anti-ds DNA($p=0.005$), 24 hours Urinary protein($p=0.001$), urinary protein/creatinine ratio ($p<0.001$), hematuria ($p<0.001$), pyuria ($p=0.016$), proteinuria ($p=0.014$), urinary casts ($p=0.001$), Total SLEDAI ($p=0.018$), Renal SLEDAI($p<0.001$) as well as pathological activity index of renal biopsy($p=0.004$).While there is a significant negative correlation between uTWEAK and C3($p<0.001$) as well as C4($p<0.001$).On the other hand, there is no statistically significant correlation between uTWEAK level and routine lab tests, blood urea, serum creatinine, extra-renal SLEDAI, SLE damage index, histological class of renal biopsy or pathological chronicity index of renal biopsy. **Conclusion:** Our results suggest that uTWEAK can be used as a non-invasive biomarker in adjunction with conventional laboratory measures for detection of LN activity.

Keywords: Systemic Lupus Erythromatosis, Lupus Nephritis, uTWEAK.

Introduction:

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease characterized by overproduction of various auto-antibodies. The great majority of SLE patients are women in their childbearing years. SLE is a complex disease with variable presentations, course and prognosis that is characterized by remissions and flares. [(1)]Renal involvement is common in SLE and often determines the course of the disease. Nearly 70-80% of all cases of SLE have some clinical manifestations of lupus nephritis, mostly glomerulonephritis. [(1)]

Lupus nephritis (LN), is a common and serious complication, with reports of 5-year renal survival with treatment ranging from 46 to 95%.[(1)] LN is characterized by a relapsing-remitting course, requiring constant follow-up and surveillance and often entailing changing treatments. [(1)]Earlier treatment has a beneficial effect on the prognosis of lupus

nephritis, and it has been shown that late diagnosis of lupus nephritis is correlated with a higher frequency of renal insufficiency. Moreover, delayed diagnosis is associated with an increased incidence of end stage renal disease, underlining the importance of early diagnosis in this disease.[2]

Histological analysis of kidney tissue is a valuable tool for diagnosis, assessment, and prognosis in lupus patients. However, kidney biopsy can be accompanied by significant morbidity and, therefore, is not usually performed serially. Furthermore, there can be a question of how representative are the limited number of glomeruli usually obtained of nephritis activity and chronicity. [3]

A noninvasive, easily obtainable, and accurate marker that can be followed serially may therefore be of great value in monitoring lupus patients. Laboratory markers in current use, which include serological determination of serum anti-double-stranded (ds) DNA antibodies and complement levels, can be helpful clinically, but the correlation between those and lupus renal disease is imperfect and their utility in reflecting disease activity and in predicting outcome remains controversial.[3] Thus, novel biomarkers that are able to discriminate lupus renal activity and its severity, predict renal flares, and monitor treatment response and disease progress are clearly necessary.

A number of these biomarkers has been of recent interest including Neutrophil Gelatinase-Associated Lipocalin (NGAL)[4], Urine Proteomics, Monocyte Chemoattractant protein-1(MCP-1), and TNF-like Weak Inducer of Apoptosis (TWEAK). [5,6]

The cytokine TWEAK was first discovered in 1997[7] and assigned to the TNF superfamily. The TWEAK receptor (TWEAK-R), a TNF receptor superfamily member more commonly known as Fn14. Fn14 is expressed on endothelial cells, vascular smooth muscle cells, kidney, heart, lung, spleen, brain, monocytes/macrophages, and NK cells, but not B or T cells[8], and is upregulated under conditions of tissue stress and inflammation.[9] In kidney cells, TWEAK mediates important biological effects, including modulation of cell survival and upregulation of proinflammatory mediators. In human mesangial cells, podocytes, and tubular cells, TWEAK induces the expression of multiple inflammatory mediators, including Regulated on Activation Normal T-cell Expressed and Secreted(RANTES), (MCP)-1,Interferon gamma-induced Protein-10 (IP-10), Macrophage Inflammatory Protein-1alpha (MIP-1α), Intercellular Adhesion Molecule-1 (ICAM-1), Vascular Cell Adhesion Molecule-1 (VCAM-1), Matrix MetalloProteinase-1 (MMP-1), and Matrix MetalloProteinase-9 (MMP-9). Thereby causing glomerular and tubular injury, which might play an important role in the pathogenesis of lupus nephritis.[10-13]

Aim of the Work:

To study the role of uTWEAK in lupus patients, its relation to clinical manifestations of the disease especially lupus nephritis. Furthermore, its correlation with the conventional measures of renal lupus activity.

Subjects:

The present study was conducted on two groups of SLE patients fulfilling at least four of the American College of Rheumatology classification criteria:[14]

Group A: Thirty SLE patients with biopsy-proven lupus nephritis.

Group B: twenty SLE patients without lupus nephritis.

They were compared with twenty healthy controls of matched age and sex. The setting of the present study was the Alexandria Main University Hospital.

Exclusion Criteria:

1. SLE patients with proteinuria due to other conditions than lupus nephritis as pregnancy and fever or patients with impaired renal functions due to any other cause than lupus nephritis as diabetes mellitus.
2. Patients with lupus nephritis on hemodialysis or with history of renal transplantation.

Methods:

All subjects were subjected to:

- Thorough history taking and complete clinical examination to detect various organs involvement by SLE.
- Laboratory investigations:

1- Routine laboratory tests:
   a. Complete blood Count (CBC).[15]
   b. Erythrocyte sedimentation rate (ESR).[16]
   c. Fasting blood sugar (FBS).[17]
2- Renal function tests:
   a. Blood urea.[18]
   b. Serum creatinine.[19]
   c. Complete urine analysis.[20]
   d. 24 Hs urinary protein.[21]
   e. Urinary protein to creatinine ratio.[21]
3- Immunological profiles:
   a. Anti-nuclear antibodies (ANA).[22]
   b. Anti-ds DNA.[23]
   c. Complement level determination (C3, C4).[24]
4- Urine TWEAK by ELISA.[25,26]
5- SLEDAI[27] for all lupus patients, and renal DAI (rDAI) for lupus nephritis.

Statistical Analysis of the Data:

Data were fed to the computer and analyzed using IBM SPSS software package version 20.0. Comparison between different groups regarding categorical variables was tested using Chi-square test. When more than 20% of the cells have expected count less than 5, correction for chi-square was conducted using Fisher's Exact test or Monte Carlo correction. Histogram and QQ plot were used for vision test. If it reveals normal data distribution, parametric tests was applied. If the data were abnormally distributed, non-parametric tests were used. Agreement of the different predictives with the outcome was used and was expressed in sensitivity, specificity, positive predictive value, negative predictive value and accuracy. Receiver operating characteristic curve (ROC) was plotted to analyze a recommended cutoff, the area under the ROC curve denotes the diagnostic performance of the test. Significance of the obtained results was judged at the 5% level.[28,29]

Results:

There was no statistically significant difference between the three studied groups as regard the age and sex (p=1 and 0.444 respectively). (table I)

There was no statistically significant difference between the three studied groups as regard the disease duration (p=0.819).

The level of uTWEAK was significantly higher in LN than non-LNSLE group (p<0.001) and than controls (p<0.001). There was also a significantly increased level of uTWEAK in non-LN SLE group than controls (p<0.001) (table II). The percentage of LN patients with high TWEAK level was significantly higher than that of non-LN SLE patients with high TWEAK level (93.3% versus 10%) (p<0.001). Where as none of the control group had a high TWEAK level (0%).

The highest mean level of uTWEAK was found in SLE patients with renal manifestations (Lupus Nephritis) with (p<0.001) (table II).

Table (III) shows: Regarding immunological profile; in LN group there was a significant positive correlation between uTWEAK and ANA (p=0.034), Anti ds DNA (p=0.005) and a significant negative correlation between uTWEAK and C3 (p<0.001) and C4 (p<0.001). Regarding renal function tests; In the LN group there was a significant positive correlation between uTWEAK and urinary 24 hs. Protein (p=0.001) as well as urinary protein /creatinine ratio (p<0.001).

In LN group, there was also a significant positive correlation between uTWEAK and hematuria (p<0.001), pyuria (p<0.016), proteinuria (p=0.014) and presence of urinary cast (p=0.001) as well as granular type of cast (p=0.025). Whereas, no correlation was found between urinary TWEAK and casts, pyuria and hematuria or proteinuria in non LN SLE group. As for total SLEDAI, a significant positive correlation was detected with uTWEAK in LN group (p=0.018) but there was no correlation in non LN SLE group (p=0.763).

As regard the correlation between renal SLEDAI and uTWEAK; there was a positive significant correlation between renal SLEDAI and uTWEAK (p<0.001) where as there was no correlation with extra-renal SLEDAI (p=0.622). As for pathological activity index; a positive significant correlation with uTWEAK was found (p=0.004) where as no correlation was found between uTWEAK and pathological chronicity index (p=0.447) or between uTWEAK and SLE Damage index (p=0.251).

Urinary TWEAK level was elevated in all classes of LN. The highest mean level was in class IV+V+III (16.8 pg/ml). This was followed by class IV +V (16.43 pg/ml) then class IV (15.98 pg/ml) then class III (12.63 pg/ml) then class IV+IIIc (11.92 pg/ml) and lastly class V+IIIa (6.9 pg/ml).

No significant statistical difference in mean uTWEAK level was detected among different histological classes of renal biopsy [p=0.539] (table 3).

Relative to renal involvement, uTWEAK showed a sensitivity of 93.33%, specificity of 90%, a positive predictive value of 93.33% and a negative predictive value of 90% with an area under the curve of 0.953 (p<0.001). (Table IV)

Table (I): Comparison between the three studied groups according to demographic data

	Lupus Nephritis (n = 30)		**Lupus only without nephritis (n = 20)**		**Control (n = 20)**		**Test of sig.**	**p**
	No.	**%**	**No.**	**%**	**No.**	**%**		
Sex								
Male	2	6.7	1	5.0	1	5.0	$\chi^2 = 0.346$	$^{MC}p= 1.000$
Female	28	93.3	19	95.0	19	95.0		
Age (years)								
Min. – Max.	13.0 - 44.0		13.0 - 46.0		18.0 - 46.0			
Mean ± SD	28.97 ± 8.19		30.60 ± 11.0		32.30 ± 8.09		F = 0.822	0.444
Median	26.50		30.0		32.0			

χ^2: Chi square test
F: F test (ANOVA)

Table (II): Comparison between the three studied groups according to urinary TWEAK level (pg/ ml)

	Lupus Nephritis (n = 30)	**Lupus only without nephritis (n = 20)**	**Control (n = 20)**	**p_1**	**p_2**	**p_3**
Urinary TWEAK level (pg/ml)						
Min. – Max.	5.50 – 30.10	2.0 – 8.40	1.10 – 6.30			
Mean ± SD	13.86 ± 7.55	4.85 ± 1.86	2.75 ± 1.28	<0.001*	<0.001*	<0.001*
Median	10.10	5.15	2.50			
$^{KW}\chi^2$(p)	50.251* (<0.001*)					

$^{KW}\chi^2$: Chi square for Kruskal Wallis test
Sig. bet. grps was done using Mann Whitney test
p_1: p value for comparing between Lupus Nephritis and Lupus only without nephritis
p_2: p value for comparing between Lupus Nephritis and control
p_3: p value for comparing between Lupus only without nephritis and control
***:** Statistically significant at p ≤ 0.05

Table (III): Correlation between Urinary TWEAK level and different studied parameters

	Lupus Nephritis		Lupus Without Nephritis	
	r_s	p	r_s	p
Personal characteristics				
Age	-0.214	0.256	-0.016	0.947
Sex (male/female)	-0.170	0.369	-0.020	0.934
Disease Duration	-0.199	0.291	0.061	0.798
Immunological profile				
ANA	0.388*	0.034*	0.057	0.810
Anti-ds DNA	0.498*	0.005*	-0.258	0.272
C3	-0.915*	<0.001*	0.048	0.841
C4	-0.729*	<0.001*	0.067	0.778
Routine lab test				
Hb	-0.253	0.178	0.361	0.118
PLT	-0.133	0.482	0.189	0.424
WBcs	-0.129	0.498	-0.088	0.712
ESR	0.074	0.697	0.135	0.569
FBG	-0.142	0.454	0.123	0.606
Renal function test				
Blood urea	0.326	0.078	0.010	0.967
S. creatinine	0.139	0.465	-0.170	0.474
24hr urinary Protein	0.574*	0.001*	-.0038	0.874
Urinary Protein /Creatinine ratio	0.599*	<0.001*	0.062	0.797
Complete urine analysis				
Casts (absent/ present)	0.579*	0.001*	-	-
Granular (absent/ present)	0.409*	0.025*	-	-
Hyaline (absent/ present)	0.268	0.152	-	-
Pyuria (absent/ present)	0.437*	0.016*	-0.191	0.421
Hematuria (absent/ present)	0.704*	<0.001*	0.339	0.144
Proteinuria (absent/ present)	0.442*	0.014*	-	-
Total SLEDAI	0.428*	0.018*	0.072	0.763
Renal SLEDAI	0.900*	<0.001*	-0.019	0.937
Extra Renal SLEDAI	-0.094	0.622	0.144	0.545
SLE Damage Index	0.216	0.251	0.324	0.163
Pathological Activity index	0.512*	0.004*	-	-
Pathological chronicity index	0.144	0.447	-	-

r_s: Spearman coefficient

***:** Statistically significant at $p \leq 0.05$

Table (IV): Agreement (sensitivity, specificity and accuracy) for Urinary TWEAK level (pg/ml) of the whole group of SLE patients (n=50)

		Lupus only without nephritis (n = 20)	Lupus Nephritis (n = 30)	Sensitivity	Specificity	PPV	NPV	Accuracy
Urinary TWEAK level (pg/ml)	**≤6.5**	18	2	93.33	90.0	93.33	90.0	92.0
	>6.5	2	28					

Discussion:

Due to the unpredictable nature of LN, it would be clinically valuable to discover a reliable biomarker for disease activity and progression. TWEAK has been established as a pro-inflammatory cytokine that by binding to its receptor Fn14 induces the secretion of chemokines known to play a major role in the pathogenesis of LN.

On studying the different SLE manifestations, there was no significant difference between LN and non-LN SLE patients in the occurrence of constitutional, mucocutaneous, joint, occular, cardiac, pulmonary, neurological or hematological manifestations.

Regarding the immunological tests, ANA was positive in all (100%) SLE patients. The mean ANA titre in LN group was higher than in the non-LN SLE group yet with no significant statistical difference between the two groups (p=0.377). Anti-ds DNA was also positive in 100% of SLE patients, with higher mean anti-ds DNA level in LN than in non LN SLE patients but again without reaching statistical significance (p=0.063). It has been shown that patients with active lupus nephritis often have raised levels of anti-ds DNA antibodies with evidence supporting their pathogenic role in LN.[30,31]

This comes in accordance with Farid et al[32] who studied 88 SLE patients divided as 44 patients with biopsy-proven LN and 44 patients without LN and they found that all patients of SLE with and without nephritis had positive ANA (100%). He also found that Anti-ds-DNA was higher in the LN group (84.09%) compared with the non-LN group (70.45%), but the difference was not statistically significant (P = 0.082).

In our study, the mean value of C3 in LN patients was lower than that in non-LN SLE patients (0.52 ± 0.42 versus 1.19 ± 0.41g/L) with a statistically significant difference between the two groups (p< 0.001). Similarly, the mean value of C4 was lower in LN patients than that in non-LN SLE patients (0.18 ± 0.15 versus 0.29 ± 0.16g/L) with a significant statistical difference between the two groups (p=0.014). Low levels of C3 and C4 in LN usually indicate disease activity or flare, this can be explained by consumption of the complement system to clear immune complexes from the blood.[33-35] In contrast to our results, Farid et al found no statistically significant difference between LN and non-LN SLE patients regarding C3 and C4.

As regard the renal functions, LN patients had significantly higher levels of serum creatinine (p=0.001), blood urea (p<0.001), 24 hs. urinary proteins(p<0.001) and PCR (p<0.001) than non-LN SLE patients. These results come in agreement with El-shehabi et al. who found a statistically significant difference between the LN and non-LN SLE patients regarding serum creatinine(p=0.001) as well as 24 hs. urinary proteins (p<0.001). Similarly, Pitashny et al found a statistically significant difference between the two groups regarding urinary PCR (p<0.0001).

In the current study, LN patients had more prevalent urinary changes than SLE patients without LN, which is an expected finding. Pyuria was detected in 76.7% of LN patients and 25% of non-LN SLE patients. Hematuria was present in 43.3% of LN cases and 5% in non-LN SLE patients. Urinary casts were present in 26.7% of LN cases while no casts were detected in non LN SLE patients.

Regarding the type of urinary cast, 20% of LN cases had granular cast while 3.3% only of them had hyaline cast. Similarly, Proteinuria was found in 86.7% of LN patients while no proteinuria was found in non LN SLE group. This comes in agreement with Pitashny et al who found a statistically significant difference between the two groups regarding proteinuria (p<0.0001).

In our study, assessment of the disease activity in SLE patients was done by applying SLEDAI. Every patient had got a score which is the sum of scores applied to certain symptoms and some selected laboratory parameters. We found that the mean total SLEDAI in LN group was significantly higher than non LN SLE group (24.53 ± 8.24 versus 16.2 ± 7.25) [p=0.001]. As regards the renal activity, it was assessed by the renal SLEDAI (rSLEDAI) score that consists of the 4 kidney-related items of the SLE Disease Activity Index 2000 (SLEDAI-2K) (hematuria, pyuria, proteinuria and urinary casts).[36] The presence of each one of the 4 parameters gives a score of 4 points thus, the rSLEDAI score can range from 0 (non-active renal disease) to a maximal score of 16. The mean valueof rSLEDAI was found to be also significantly elevated in the LN than the non-LN SLE group (9.33 ± 4.25 versus 1.2 ± 1.88) [p < 0.001]. On the other hand, extra-renal SLEDAI which is calculated by substracting the renal SLEDAI score from the total SLEDAI had no significant statistical difference between the two groups [p=0.818]. This can be explained by the fact that almost all patients of LN group were in activity raising the score of rSLEDAI hence, raising the score of total SLEDAI.

El-shehaby et al.[37] who studied 73 SLE patients divided as 50 patients with active LN and 23 patients with inactive LN or non-renal patients similarly found a statistically significant difference between LN and non-LN SLE patients regarding total SLEDAI (p=0.0002) as well as rSLEDAI (p=0.0002). Also, similar to our results, Pitashny et al. found a statistically significant difference between the two groups regarding total SLEDAI (p<0.0001) as well as rSLEDAI.

Meanwhile, there was no statistically significant difference between the two groups regarding SLE Damage index in the current study (p=0.155).

In the current study, the level of uTWEAK was significantly higher in LN group (ranging from 5.5 to 30.1 pg/mL with a mean of 13.86 ± 7.55 pg/mL) than non-LN SLE group (ranging from 2 to 8.4 pg/mL with a mean of 4.85 ± 1.86 pg/mL) [p<0.001]. uTWEAK was also significantly higher in LN than the control group (ranging from 1.1 to 6.3pg/mL with a mean of 2.75 ± 1.28 pg/mL) [p<0.001]. On comparing uTWEAK levels between non LN SLE patients and controls, there was also a significant statistical difference between the two groups [p<0.001]

This runs in accordance with Schwartz N. et al.[38] Who performed a cross-sectional study of a large, multi-center cohort of 66 SLE patients (divided into 23 LN patients and 43 non-LN SLE patients). He reported that the levels of uTWEAK were significantly higher in the LN group when compared to those with inactive or no nephritis (ranging from 9.9 to 23 with a mean of 16.3 versus a range from 2.3 to 16.8 with a mean of 5.5) pg/mg creatinine (p=0.001). Similarly, In a further multicenter longitudinal study done later by the same author[39] involving 30 biopsy-proven LN patients and five control groups (normal, nonrenal SLE, rheumatoid arthritis, osteoarthritis and non-SLE renal disease) he found that uTWEAK levels were significantly higher in LN patients than non LN SLE patients (p=0.005) and were also higher in LN patients than normal controls (p=0.003). On the other hand, El-shehaby et al. found that significantly higher levels of uTWEAK were observed in LN compared with non-LN SLE patients (p<0.001) and in LN patients compared with control subjects (p<0.001) while there was no statistically significant difference between non-LN SLE and control group.

On comparing the prevalence of high versus low uTWEAK levels in the studied groups, we found that 93.3% of LN group had high uTWEAK levels where as only 10% of non- LN SLE group had high uTWEAK levels with a statistically significant difference between the two groups (p<0.001). None of the control group had a high uTWEAK level. The cut off value applied was suggested by the statistical package for social sciences (SPSS) programme.[29] This suggests that uTWEAK is related to the renal involvement

rather than other system involvement in SLE patients.

On studying the correlation between uTWEAK and SLE manifestations, a significant positive correlation was found between patients with renal involvement and the level of uTWEAK ($p<0.001$) while no significant correlation was found between any of the extra-renal manifestations and uTWEAK. This implies that uTWEAK was primarily dependent on the renal component of the disease and it is unrelated to the systemic (non-renal) SLE manifestations. Yet, this assumption still needs to be confirmed by further larger studies.

No correlation was found between uTWEAK and the different demographic data; age, sex or disease duration. This comes in agreement with El-shehaby et al.

Regarding the correlation between uTWEAK and conventional markers of lupus activity, a significant positive correlation was found between uTWEAK and anti-ds DNA ($r=0.498$, $p=0.005$). There was also a significant negative correlation between uTWEAK and C3($r=-0.915$, $p<0.001$) as well as between uTWEAK and C4 ($r=-0.729$, $p<0.001$). This runs in agreement with Schwartz N. et al who found that uTWEAK was correlated with common serologic indicators of SLE renal activity; positively with anti-ds DNA antibodies ($r=0.459$, $p=0.008$) and negatively with C3 ($r=-0.262$, $p=0.019$) as well as C4 ($r=-0.269$, $p=0.016$). Similarly, El-shehaby et al who found a significant negative correlation between uTWEAK and C3 ($P<0.001$) as well as C4($P<0.001$).

Concerning the laboratory measures, no correlation was found between uTWEAK and Hb level, WBC count, platelet count or ESR.

Regarding renal function tests; no correlation was found between uTWEAK and blood urea or between uTWEAK and serum creatinine. This runs in accordance with Schwartz N. et al who also did not find a correlation between uTWEAK and BUN or serum creatinine. The authors suggested that uTWEAK levels did not correlate well with serum BUN and creatinine as this cytokine is situated very proximally in the pathogenesis of LN. If this is true, uTWEAK may turn out to be helpful as a forecaster of flares, with higher diagnostic accuracy in the pre-flare period rather than following flare onset. Longitudinal studies with more patients are needed to study this in detail.[39]Similarly, El-shehaby et al found no correlation between uTWEAK and serum creatinine($p=0.217$).

In the current study, there was a significant positive correlation between uTWEAK and 24 hs. urinary proteins ($r=0.574$, $p=0.001$) as well as urinary PCR ($r= 0.599$, $p<0.001$)), the latter being a simple, reliable and valuable tool to monitor LN progression.[40] In agreement with this result, El-shehaby et al found also a significant correlation between uTWEAK and 24 hs. Urinary protein ($p=0.03$).

There was a significant positive correlation between uTWEAK and hematuria ($r=0.704$, $p<0.001$), pyuria ($r=0.437$, $p=0.016$), presence of urinary casts ($r=0.579$, $p=0.001$) as well as granular type of cast ($r=0.409$, $p=0.025$). This runs in agreement with El-shehaby et al as they found a significant correlation between uTWEAK and hematuria ($p=0.03$), pyuria ($p=0.01$) as well as urinary casts($p<0.001$).

As regard the correlation between uTWEAK and proteinuria, we found a significant positive correlation ($r=0.442$, $p=0.014$).This was in contrast to Schwartz N. et al who did not find a correlation between uTWEAK and proteinuria ($r=0.073$, $p=0.562$). He suggested that This finding indicated that high urinary TWEAK levels are not a result of damage to the glomerular filtration barrier and non-specific protein loss into the urine, but rather the source of uTWEAK is the kidneys themselves, reflecting local inflammatory activity.

The level of uTWEAK Was found to be strongly correlated with total SLEDAI ($r=0.428$, $p=0.018$) as well as renal SLEDAI ($r=0.900$, $p<0.001$). However, this correlation was no longer significant when only the extra-renal component of the index was correlated with uTWEAK in LN ($r=-0.094$, $p=0.622$). These results are consistent with the findings by Schwartz N. et al. who proved also that uTWEAK was significantly correlated with the total SLEDAI as well as renal SLEDAI but not with the extra-renal SLEDAI. These results were confirmed in an other study by the same author[39,40] in which they found a significant correlation between uTWEAK and total SLEDAI ($p<0.001$) as well as between

uTWEAK and rSLEDAI (r=0.388, p=0.047) while no correlation was found with extra-renal SLEDAI (p=0.426). This comes also in accordance with El-shehaby et al. who found a significant correlation between uTWEAK and tSLEDAI (p<0.001) as well as rSLEDAI (p<0.001). The finding of significant correlation of uTWEAK with total SLEDAI and rSLEDAI but not extra-renal SLEDAI suggests that elevation in uTWEAK is an indicator of renal activity, not extra-renal activity.

Moreover,we compared the mean level of uTWEAK of LN patients with rSLEDAI ≤ 8 (8.24 ± 1.71) with that of LN patients with rSLEDAI > 8 (21.21± 5.52) and we found a significant statistical difference between these two sub groups (p<0.001). This furtherly indicates that higher levels of uTWEAK are present in patients with higher grades of renal activity.

Regarding the disease damage, no correlation was found between uTWEAK and SLEDDI (p=0.251). This indicates that the elevation of uTWEAK level may be related to disease activity rather than damage.

In this study, renal biopsy was done in all the 30 patients of LN group. Biopsies were classified according to International Society of Nephrology/Renal Pathology Society (ISN/RPS) 2003 Classification of Lupus Nephritis[41]. We found that the highest percentage of patients had class III LN (36.7%) followed by class IV (26.7%) then class IV+IIIc (16.7%) then class IV+V (13.3%) then class V+IIIa and class IV+V+III (3.3% each), while no patients were found in classI, II or VI.

On studying the correlation of uTWEAK and the different histological classes of LN, we found that there was no significant difference in the levels of uTWEAK among the different classes of renal biopsy. Accordingly, uTWEAK could not discriminate between the histological classes of renal biopsy in the current study. This was confirmed by the results of other studies such as that done by Xuejing Z. et al. (who studied 46 patients with LN divided as 12 of nonactive LN and 34 of active LN)[42] as well as studies done by Schwartz {38,39} as they also did not find a significant difference in uTWEAK levels among different classes of LN. This indicates that uTWEAK can not replace renal biopsy for detection of renal involvement in SLE patients. The authors suggested that This may be due to the fact that TWEAK is predominantly a pro-inflammatory cytokine that contributes to active renal inflammation, while additional factors and mediators are necessary to induce progression into chronicity. Genetic background and/or environmental triggers may influence disease progression and perhaps even contribute to the evolution of the different WHO classes. Yet, they also suggested that this finding still remained to be confirmed in larger numbers of patients displaying each of these histological subtypes. This also applies for other studied biomarkers in LN as none of them up till now proved to replace renal biopsy.[37,43]

Regarding the correlation between uTWEAK and renal biopsy indices; in the current study, a significant positive correlation was found between uTWEAK and pathological activity index (r=0.512, p=0.004) while no correlation was found between uTWEAK and pathological chronicity index (r=0.144, p=0.447). This runs in agreement with Xuejing Z. et al who found that uTWEAK levels of patients with LN had significantly positive correlation with activity index (r=0.825, p<0.01) but had no significant correlation with chronicity index (p>0.05). They concluded that uTWEAK levels could reflect the level of histological activity in LNpatients so, the expression of TWEAK may be relevantto glomerular and tubulointerstitial lesions. They suggested that the elevation of uTWEAK level may be related to the increased expression of TWEAK in kidney. This was shown in a cross sectional cohort study by Albert Einestein College of Medicine of 66 SLE patients divided as 23 LN patients and 43 non-LN SLE patients compared with 19 healthy controls, they found that there was a statistically significant difference in serum TWEAK between SLE patients and controls[p=0.034] where as there was no statistically significant difference in sTWEAK between LN and non-LN SLE patients [p=0.747]).[39] However, it is still uncertain whether the kidney is the only organ expressing TWEAK, which needs to be confirmed by further studies in the future.[42]

It is well known that the relapsing-remitting course of LN, among the most serious complications of SLE thus it requires close monitoring and often frequent treatment

adjustments throughout patients' lives. Trend towards re biopsy upon renal flares is increasing.[44] as some authors in their studies found that histological transformations were common, and they occurred when the previous biopsy had non-proliferative lesions as well as when lesions were proliferative. Treatments were modified after repeat renal biopsy in the majority of patients. In this experience, kidney repeat biopsies were useful in guiding treatment of LN flares.[44] However, is impractical as a clinical tool to be repeatedly used upon frequent relapses as it is considered as an invasive maneuver with possible complications. Moreover, contra indications for this procedure may be encountered in some patients such as thrombocytopenia, bleeding tendency and severe hypertension.[45] A dependable biomarker that can reflect the patient's renal disease activity is therefore highly desirable. This may eventually enable clinicians to institute treatment of flares earlier, and hopefully improve the significant short- and long-term morbidity associated with lupus renal disease.

In the current study, we concluded that uTWEAK excretion correlated strongly with renal disease progression and activity and not with extra renal disease activity score in LN. More over TWEAK was also correlated with pathological activity index of renal biopsy.

In addition, uTWEAK served as a biomarker of LN activity and was correlated with conventional markers of lupus renal activity (anti-dsDNA Abs, C3 and C4).

Moreover, In the current study, uTWEAK showed a sensitivity of 93.33%, specificity of 90%, a positive predictive value of 93.33% and a negative predictive value of 90% with an area under the curve of 0.953 ($p<0.001$). This comes in agreement with El-shehaby et al. who found that uTWEAK showed a sensitivity of 89%, specificity of 56%, a positive predictive value of 93% and a negative predictive value of 66.7% with an area under the curve of 0.816 .

But as none of the biomarkers studied till now proved to replace the valuable role of renal biopsy,[37,43] future directions in SLE biomarker research should focus on combination of novel markers with conventional clinical and laboratory parameters to enhance the sensitivity and specificity for the prediction of renal flares and to improve prognosis in LN.

Conclusions:

We found that, the mean level of uTWEAK was strongly correlated with renal SLEDAI as well as the pathological activity index of renal biopsy. In addition, it showed a high sensitivity, specificity and positive predictive value for renal involvement and activity so, it can be used as a non invasive biomarker for early detection of LN activity yet it can not replace the need for renal biopsy. Accordingly, uTWEAK can be used in association with other conventional markers to detect renal flare early in SLE patients.

Recommendations:

Further longitudinal studies on larger sample size are promptly needed to test the value of urinary TWEAK in early detection of renal involvement in lupus patients as well as its prognostic value in follow up of SLE patients.

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Original Article

Renal Doppler Ultrasound Assessment in Diagnosis of Lupus Nephritis in Comparison to Biopsy Findings and Disease Activity Markers.

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Abstract:

Background: Nephritis is one of the most common complication of systemic lupus erythematosus (SLE). Studies are going on to find a non-invasive alternate to renal biopsy. Renal Doppler US derived resistive index (RRI) has been studied before in patients with lupus nephritis, but its role is still a matter of controversies. **Objectives:** To study the role of RRI in the diagnosis of lupus nephritis and correlate it to the biopsy findings**,** laboratory, and serology markers of the disease. **Methods:** We evaluated 46 lupus nephritis patients in comparison to 24 SLE patients with no nephritis and 18 healthy control subjects. The study protocol included ultrasonographic assessment to measure the RRI and comparing it to renal biopsy (International Society of Nephrology/Renal Pathology Society classification), clinical, laboratory and serology markers of the disease. **Results:** RRI was significantly higher in LN group than LNN and healthy groups (p= 0.007, and 0.026) respectively. No difference of RRI values was seen among ISN/RPS renal biopsy classes. It was correlated with serum creatinine and eGFR (r=0.447, p=0.002) and (r= -0.435, p= 0.002) respectively, but it didn't correlate with any of the serology markers of the disease activity. Also it was found to be highly significantly correlated with both chronicity (CI) index and degree of tubulointerstitial fibrosis (r=0.625, p<0.0001) and (r=0.629, p<0.0001) respectively, but not correlated with activity index (AI) in renal biopsy. Regression analysis showed RRI to be the most significant predictor to both CI and tubulointerstial fibrosis. **Conclusion:** Although RRR has a limited ability to differentiate between renal biopsy classes, we suggest its possible role as a marker of chronicity and interstitial fibrosis in patients with lupus nephritis and may be a useful noninvasive tool that help decision making as regard patient selection for aggressive immunosuppressant.

Introduction:

Systemic lupus erythematosus (SLE) is the systemic inflammatory autoimmune disease that affect almost all body organs with a wide variety of manifestations and an unpredictable course. One of the victims of this aggressive disease is the kidney. The kidney is the most common visceral organ affected by SLE. Renal involvement is a leading cause of mortality and morbidity from SLE.[1] The clinical presentations of the lupus nephritis vary a lot from mild asymptomatic active urinary sediment to rapidly progressive kidney failure and complete loss of kidney function.[2]

Many authors have illustrated the low reliability of clinical feature alone in the diagnosis of lupus nephritis,[3,4] making the diagnosis difficult and risky without renal biopsy.

Although renal biopsy is an invasive method of investigation that carries a lot of risks and limitations, it is still considered the standard of care procedure in management of LN,[5] which any new tool is compared. It provides not only a diagnostic information but also help to predict the course of the disease.

Over the past decades and up till now, a lot of researchers have tried to find an alternate noninvasive tool that can substitute the renal biopsy. Some have tried several urinary and serum biomarkers.[6-8] Others have tried the radiological imaging techniques. Platt et al, have tried the use conventional grey scale US and Doppler US for the diagnosis of lupus nephritis.[9]

Renal Doppler US parameters specially the renal resistive index (RRI) have been studied for many years in a variety of clinical situations such as, the detection of renal allograft rejection,[10] diagnosis and management of renal artery stenosis[11] evaluation of progression risk in chronic kidney disease (CKD),[12] differential diagnosis of acute and chronic obstructive kidney disease,[13] and more recently used as a predictor of renal and also the overall outcome in the critically ill patient.[14]

However renal Doppler derived resistive index (RI) has been used before to assess kidneys of patients with lupus nephritis, it still remains a matter of controversy whether it can substitute or give much information as always given by renal biopsy. Some authors found no role of renal Doppler US in diagnosis of lupus nephritis,[15] while others suggested its role in prediction of degree of chronicity and tubulointerstial fibrosis,[16,17] and hence prediction of poor outcome[9] and response to therapy. [18]

To best of our knowledge, only one study tried to correlate RRI to renal biopsy class obtained by renal biopsy, they found a significant correlation between pathologic RI and class IV nephritis and suggest the role of RRI in prediction of lupus nephritis activity.[19]

Aim of the work:

The aim of the current study is to evaluate the role of renal Doppler ultrasound derived resistive index in the diagnosis of lupus nephritis and compare it to biopsy findings (specially activity and chronicity index, degree of tubulointerstial fibrosis as well as ISN/RPS renal biopsy classes), clinical, laboratorial, and serology markers of the disease.

Subjects and Methods:

This is a case control cross sectional study that was conducted at Mansoura University Hospital in the department of Internal Medicine. It was carried on 70 cases of SLE patients who were attending Rheumatology, Immunology and Nephrology unit (inpatient and outpatient), and 18 normal individuals. The study was carried over the period of two years from July 2012 to June 2014.

Subjects were divided into:

Group 1 "Lupus nephritis": This group included 46 lupus patients with evidence of mephitis. **Group 2** "Lupus non nephritis": This group included 24 lupus patient with no nephritis. **Group 3** "healthy control": This group included 18 normal individuals of matched age and sex.

Exclusion criteria: overlap syndrome, other systemic autoimmune disorder, diabetic or hypertensive nephropathy, hepatic, cardiac, primary glomerulopathy, and Pregnant SLE female, extreme age, renal vascular disorders, obstructive renal disease, post transplantation.

All patients were subjected to the following:

After explanation of the study and taking an informed written consent. Diagnosis of SLE was confirmed according to 1997 update of ACR criteria for diagnosis of systemic lupus erythematosus.[20] They underwent detailed medical history (SLE manifestation specially that suggest nephritis activity), detailed clinical examination, laboratory test (ANA, anti-ds DNA, C3,C4, antiC1q, serum creatinine, CBC, urinary albumin/creatinine ratio, and ,urine analysis) Estimated glomerular filtration rate (eGFR) was calculated by the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation (EPI) equation.[21] radiological assessment includes (renal grey scale ultrasound and renal doppler sonography).

Doppler signals were taken by using a MEDISON (Medison CO-LTD), model-Sonoace X6, Korea, power 100-120/ 200-240 V- 0.8/ 5A, 50/60 HZ. equipped with 2.5 – 5 MHz. A 3.5-MHz transducer "curved "working at 2.5 MHz was used for Doppler analysis. Placing the sample volume at the edge of the medullary pyramids from the interlobar arteries or at corticomedullery junction from arcuate arteries. The operator was blinded to the study design. It was performed within days of renal biopsy. Oblique Approach. Angling 45 degrees to the renal arteries or rolling the patient in a semi left or right decubitus position to avoid the bowel gas and improve the Doppler angle. At least three reproducible waveforms have been obtained. The RI [(Peak systolic velocity - End-diastolic velocity)/Peak systolic velocity] was taken by using six measurements (three from each of the two kidneys) taken for each patient then the mean RI was calculated.

Renal biopsy was taken by 'biopsy gun' (single-use, disposable device). 16G needles were used. Two cores of renal biopsy was taken, fixed by formalin for conventional light microscopy. The two cores were processed for paraffin sections and stained with H&E, Massion trichrome stain, PAS and silver stains. The slides were examined by an expert pathologist who were blinded to the study design. The following data were obtained from the biopsy results Biopsy class according to ISN/RPS (2004), activity (AI) and chronicity (CI) indices according to the following parameters (AI of maximum24 point, CI of maximum 12 point)

Point System in Calculating Lupus Nephritis Biopsy Activity and Chronicity

	Lesions Contributing to Activity Index						Lesions Contributing to Chronicity Index			
Semiquantitative Lesion Score*	*Cellular Crescents*	*Glomerular Necrosis-Karyorrhexis*	*Glomerular Neutrophils*		*Large Subendothelial Immune Deposits*	*Interstitial Inflammation*	*Glomerular Sclerosis*	*Fibrous Crescent*	*Tubular Atrophy*	*Interstitial Fibrosis*
None	0	0	0	0	0	0	0	0	0	0
Mild	2	2	1	1	1	1	1	1	1	1
Moderate	4	4	2	2	2	2	2	2	2	2
Severe	6	6	3	3	3	3	3	3	3	3

Statistical Analysis:

Data were collected, revised, verified, then analyzed using the Statistical Package of Social Sciences (SPSS) version 17 for Windows (SPSS, Inc., Chicago, IL, USA).The significance of differences between variables was determined using independent samples t- test, Mann-Whitney U-test , and Chi- square ($\chi 2$) or Fisher exact when appropriate if two groups are compared . One-way analysis of variance (ANOVA) and kruskal-wallis test were used if more than 2 groups are compared. Pearson's correlation or Spearman correlation were used to examine correlation between continuous variables. Regression analysis was done using general linear model (GLM). P values <0.05 were considered significant for all statistical analyses in this study.

Results:

This study evaluated 88 individuals, seventy of them (13 males and 57females) and 18 of them (6 males and 12 females) were healthy control subjects. Forty six of lupus patients (10 male, 36 female), 24 lupus non nephritis patient (3 male, 21 female). The mean age values in the 3 groups (LN, LNN, healthy control) were 27± 8.3, 30± 9.21, and 29.8±6.7 respectively with no statistical difference.

Both groups "nephritis and non-nephritis were enrolled in a comparison as regard demographic, clinical, and laboratory data Table 1.

According to the ISN/RPS classification, 1(2%) patients had minimal mesangial nephritis 3 (6%) patients had mesangial proliferative nephritis (class II), 40 (88%) patients had proliferative nephritis (11 in class III and 29 in class IV), 2 (4 %) had membranous nephritis (class V), and no case was detected with glomerulosclerosis (class VI), mean and IQ values of activity index(AI) 8 (5-11), mean and IQ values of chronicity index(AI) 4 (2-6).

Renal resistive index (RRI) was compared among the 3 studied group (LN, LNN, and healthy control). LN patient showed a higher values LNN and healthy individuals (p= 0.007, and 0.026) respectively Table2. The highest levels were detected in class IV (Fig 1), although when compared to class III, no statistical significant difference was detected (P=0.3). Limited number of cases in class I, II, V made the comparison between all groups inappropriate.

Table3 lists the main demographic and clinical features of LN patients grouped with respect to the RRI value "lower 50 % percentile (n=23, RRI<0.6) and upper 50 % percentile (n=23, RRI>=0.6)".The LN patient grouped as upper 50% percentile of RRI showed a higher levels of serum creatinine and hence lower eGFR (p=0.024, and 0.029) respectively, also the chronicity index and tubulointerstitial fibrosis were higher in this

group (p=0.001, and 0.01) respectively than LN patients with lower 50% percentile of RRI.

In lupus nephritis group, RRI was highly significantly correlated with serum creatinine and eGFR (r=0.447, p=0.002) and (r= -0.435, p= 0.002) respectively, but it didn't correlate with any of the serology markers of the disease activity. Also it was found to be highly significantly correlated with both chronicity index and degree of tubulointerstitial fibrosis (r=0.625, p<0.0001) and (r=0.629, p<0.0001), but not correlated to activity index. Fig2.

In univariate regression analysis using a general linear model (GLM) we found serum creatinine and RRI was the most significant predictor to both chronicity index and tubulointerstial fibrosis after adjustment of MABP, eGFR. Table 4, 5.

Table 1: Comparison between nephritis and non-nephritis SLE patients.

Factor	Lupus nephritis (n=46)	Lupus non nephritis (n=24)	P
Age	27±8.3	30±9.21	0.208***
Sex **Male n (%)** **Female n (%)**	 10 (21.7%) 36 (78.3%)	 3 (12.5%) 21 (87.5%)	0.520**
BMI (kg/m2)	26 (23.5-31)	25.8 (24-30)	0.853*
Disease duration(m)	15 (3.7-36)	11 (3.3-24)	0.0535*
Mean BP(mm hg)	110 (100-120)	92 (87-96)	<0.0001*
ANA(IU/ml)	3.5 (2.7-4.3)	2.4 (1.5-5.7)	0.171*
Anti-ds DNA (IU/ml)	69 (40-101)	42 (33.3-76.4)	0.04*
C3(mg/dL)	64 (46-84)	84.5 (51-115)	0.114*
C4(mg/dL)	9.9 (7-12.7)	18 (8.2-29)	0.018*
AntiC1q	111(29-240)	53(19-97)	0.029*
Serum Creatinine (mg/dl)	1.76 (0.9-2.8)	0.8 (0.7-0.9)	0.0001*
eGFR(ml/min)	46.5 (22-93)	98 (84-120)	0.0001*
Protinuria (gm/24h)	2.1 (1.5-3.2)	0.105 (0.1-0.22)	0.0001*

y = year BP = Blood pressure m = month BMI = Body mass index

eGFR=estimated glomerular filtration rate by EPI equation. ANA=Antinuclear antibody AntiC1q =antiC1q antibody

Anti-ds DNA=Anti-double stranded deoxy ribonucleic acid C3, C4= complement 3, complement 4

* probability of Mann-Whitney U –test. Values of its variables are expressed as median (Interquartile range).

** probability of Fisher's exact test. Values of its variables are expressed as n (%) number (percentage.)

*** probability of independent sample T–test. Values of its variables are expressed as mean ± standard deviation.

Table 2: Comparison between lupus nephritis, lupus non nephritis and healthy control groups as regard renal resistive index (RRI).

Factor	Lupus nephritis (n=46)	Lupus non nephritis (n=24)	Healthy control (n=18)	P1	P2	P3
RRI	0.59± 0.059	0.55± 0.058	0.55± 0.063	0.007	0.877	0.026

Values are expressed as mean± standard deviation.

P1: probability of independent sample T–test between lupus nephritis and lupus non nephritis groups.

P2: probability of independent sample T–test between lupus non nephritis and healthy control groups.

P3: probability of independent sample T–test between lupus nephritis and healthy control groups.

P: probability of One Way "ANOVA" between the three groups.

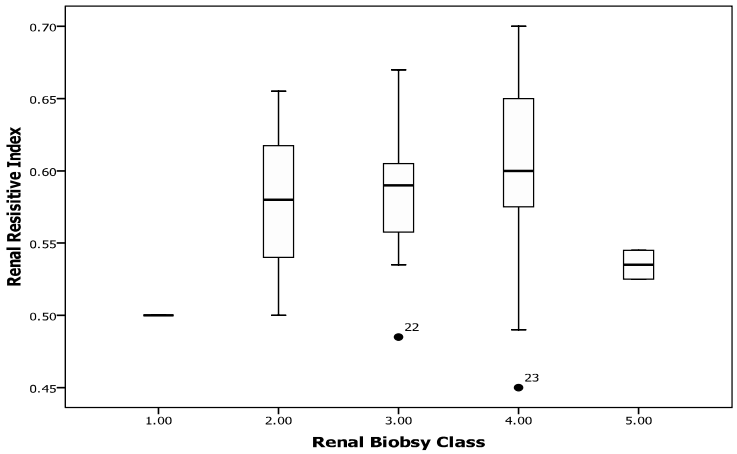

Fig1: Box plot; Renal resistive index (RRI) values in the different lupus nephritis classes. (median, quartiles and range, possible extreme values)

Table3. Demographic, clinical, and laboratory features of lupus nephritis patients grouped according to RRI values.

Factor	Lower50%percentile (n=23)	Uper50%percentile (n=23)	p
Age (y)	28 (22-35)	23 (19-30)	0.116*
Sex Males n (%) females n (%)	 3 (13%) 20 (87%)	 7 (30%) 16 (70%)	0.284**
Disease duration(m)	24 (4-36)	12 (3-54)	0.766*
BMI (kg/m$^{2)}$	27±6.4	26±4.8	0.354***
Mean BP (mmhg)	103 (96-118)	113 (103-123)	0.120*
Serum creatinine (mg/dl)	1.1 (0.8-2.5)	2.2 (1.1-6)	0.024*
eGFR (ml/min)	63 (27-105)	32 (10-76)	0.029*
Proteinuria (g/24h)	2.1 (1.6-3.3)	2.1 (1.4-3.2)	0.575*
Biopsy classes **Class I (n=1)** **Class II (n=3)** **Class III (n=11)** **Class IV (n=29)** **Class V (n=2)** **Class VI (n=0)**	 1(4%) 2(9%) 6(26%) 12(52%) 2 (9%) -	 0 (0%) 1 (4%) 5 (21%) 17 (75%) 0 (0%) -	-
AI	8.5 (5.7-11)	7.5 (4-12)	0.453*
CI	3 (2-4)	6 (4-9)	0.001*
TIF % <50% >50%	 19/20 (95%) 1/20 (5%)	 13/22 (59%) 9/22 (41%)	0.01**
Anti ds DNA	75 (40-102)	68 (40-80)	0.766*
ANA	3 (3-4)	3.3 (2.2-4)	0.243*
C3	60 (46-96)	65 (44-78)	0.800*
C4	9 (7-11)	11 (7-20)	0.230*
AntiC1q	90 (31-170)	142 (27-287)	0.717*
Renal echogenecity Normal n (%) Abnormal n (%)	 13 (57%) 10 (43%)	 7 (30%) 16 (70%)	0.074****

BP=Blood pressure m=month y=years BMI=Body mass index

eGFR=estimated glomerular filtration rate by EPI equation eGFR=estimated glomerular filtration rate by EPI equation. ANA=Antinuclear antibody AntidsDNA=Anti-double stranded deoxy ribonucleic acid antibody

C3,C4 = complement 3, complement 4 AntiC1q = Anti complement 1antibody

* probability of Mann-Whitney U –test . Values of its variables are expressed as median (Interquartile range).

** probability of Fisher's exact test. Values of its variables are expressed as n (%) number and (percentage)

*** probability of independent sample T–test. Values of its variables are expressed as mean ± standard deviation.

**** probability of chi square □2-test. Values of its variables are expressed as n (%) number and (percentage)

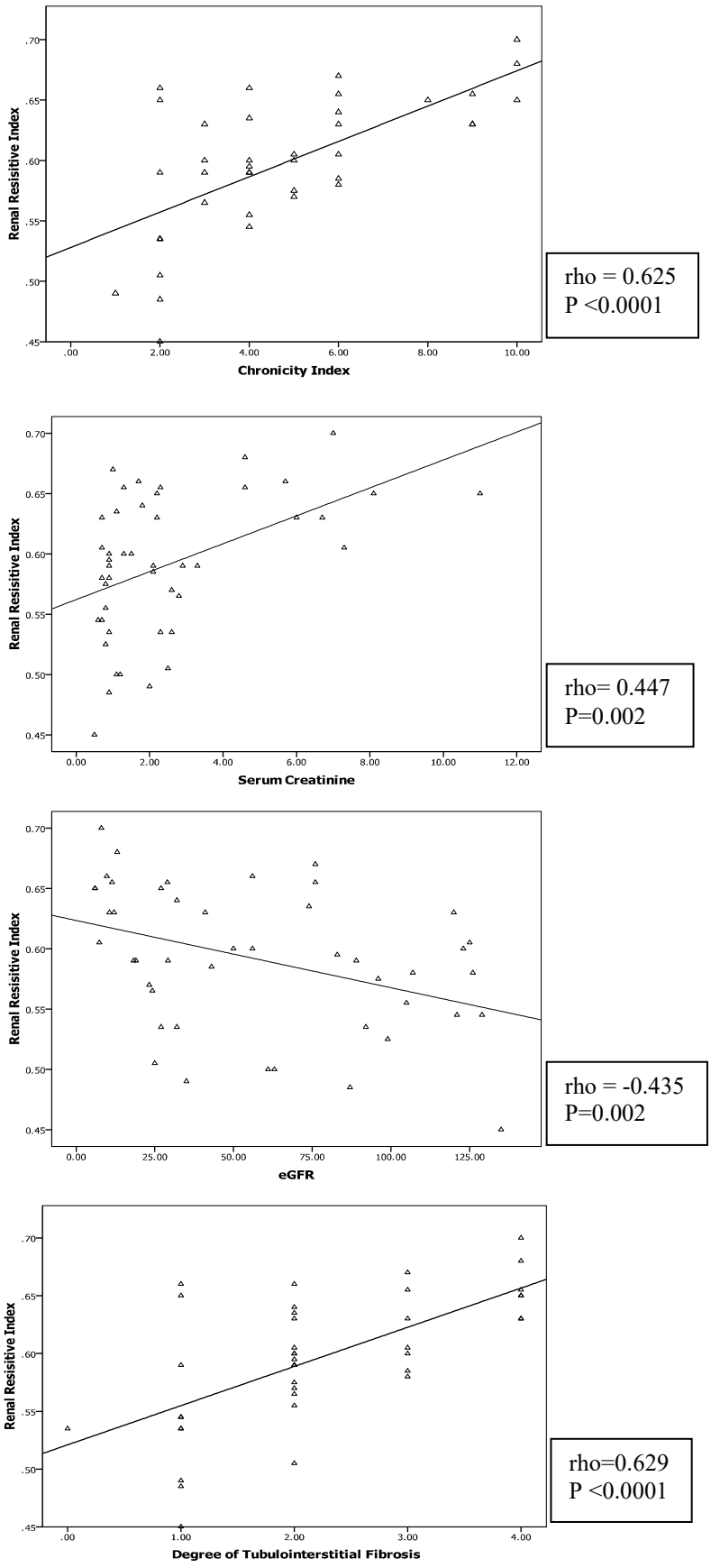

Fig2: Scatter/Dot; Correlation between renal resistive index (RRI) and serum creatinine, estimated GFR (eGFR), chronicity index, and degree of degree of tubule interstitial fibrosis (TIF;0<5%&1=5:19%&2=20:49%&3=50:74%&4=75:10% fibrosis).

Table 4: General Linear Model (GLM); Univarite regression analysis using chronicity index as dependant variable (after adjustment of age, sex, eGFR, and MABP).

variable	R square	F	P
Sex	**0.645**	**1.45**	**0.236**
Serum creatinine		**10.427**	**0.003**
eGFR		**2.366**	**0.133**
MABP		**0.920**	**0.344**
RRI		**16.091**	**<0.0001**

MABP= mean arterial blood pressure

eGFR= estimated glomerular filtration rate by EPI equation RRI=renal resistive index

Table 5: General Linear Model (GLM); Univarite regression analysis using tubulointerstitial fibrosis as dependant variable. (After adjustment of eGFR, and MABP).

variable	R square	F	P
Serum creatinine	**0.563**	**7.442**	**0.01**
eGFR		**0.563**	**0.458**
MABP		**0.271**	**0.606**
RRI		**13.32**	**0.001**

MABP=mean arterial blood pressure

eGFR=estimated glomerular filtration rate by EPI equation RRI=renal resistive index

Discussion:

Renal doppler ultrasound derived resistive index (RI) has been used before to assess the heamodynamic changes that might occure with a renal diseases . Its role was heilpful in the diagnosis of some renal disease like acute graft rejection,[22] acute urinary tract obstruction[23], and renal artery stenosis [24] .

Renal resistive index is derived from of both peak systolic velocity (PSV) and end diastolic velocity (EDV) through this equation [(PSV – end EDV) / PSV]. The PSV is a semi quantitative indicator of intrarenal blood flow, and markedly depends on the distension of the small arteries in the kidney. Thus, the PSV is associated with renal vascular compliance and vascular resistance. An elevated renal interstitial pressure would counteract the distention of arteries and arterioles during systole and diastole and hence alter RRI.[25, 26]

From the above, we hypothesized that renal resistive index might be affected by the factors that increase the interstitial pressure. These factors may be the interstitial infiltration (one of the activity parameters on renal biopsy) or fibrosis (one of the chronicity parameters by renal biopsy) which are associates to lupus induced glomerulopathy.

So, we raised these questions before starting the current study? Does renal Doppler derived resistive index can be used as an alternate noninvasive tool for the assessment of the kidneys of patients with lupus nephritis especially for those who have a contraindication of renal biopsy? Can it predict ISN/RPS renal biopsy classes? Can it predict an active nephritis? Could it be a promising noninvasive tool for the assessment of disease progression or remission for such patients especially that periodic sampling of renal biopsy is not clinically suitable?

We found the renal resistive index values were significantly higher in LN group than LNN, and healthy control group, although no significant difference was detected between LNN and healthy control group. So, it seems that lupus induced renal affliction is the one incriminated in this differences, specially that

most of the known etiologies that might affect renal resistive index were excluded from the study See exclusion criteria. Conti, et al compared the renal resistive index in 42 patients with renal SLE, 10 with non-renal SLE and 14 healthy patients. Although he found no statistical difference between the three group, the highest values were recorded in the lupus nephritis group, he found 5/42 of LN patients had RI > 0.7 while not detected in LNN and healthy control group. [19]

Another similar results to conti , et al have been reported in the literature although no control groups were evaluated in comparison to LN patients, platt, et al found 10/34 LN patients had an increased resistive index >0.7[9] and Wang et al also found 21/44 of LN patients had increased RI >0.7[18]. On the contrary Ozebek et al., found no abnormality in renal Doppler parameters including resistive index (RI), but their study was carried on a smaller population (n=21) of SLE patients (only 11/21 had proteinuria >0.5 gm /24 hour and 3/21 had renal impairment) so not all patients included in this study were lupus nephritis, moreover it doesn't include any of control groups for a comparisons that might explore any additional findings.[15]

We didn't find any of the included subjects had a resistive index above 0.7, however the normal value of resistive index is debatable although most of the researchers have concluded that the cut off value of normal RI is 0.7.[27] Several studies have shown that a normal mean renal RI is approximately 0.60. One of the large series (58 patients) reported a mean (±SD) RI of 0.60 ± 0.01 for subjects without preexisting renal disease.[28-30] and generally, it was accepted that RI more than 0.7 was distinctly high[29] Exceptions to this are children < 4 years, adults >60 yrs. Moreover as cited before , a different value of RRI may be more appropriate for different renal diseases.[29] Prabahar and his colleague have shown that RI of 0.6 should be accepted as a cut off value in glomerular disease in Indian population. [31]

RRI was evaluated as regard the renal biopsy classes according to (ISN/RPS) classification. Although the highest values were recorded in class IV, there was no statistical difference between RRI in class III, and class IV lupus nephritis patients. The limited number of patients in class I, II, V made the comparison of RRI between all classes difficult and also the comparison of each of these classes to class III, or IV also was difficult. Moreover, no difference was seen between proliferative classes (III,IV) and non-proliferative classes (I,II,V) also, no difference was seen between diffuse proliferative LN (class IV) and non-diffuse proliferative LN (I,II,III,V). So, although the higher values of RRI in the current study were detected in class IV, it seems that RRI failed to differentiate between the renal biopsy classes.

To date the only study that compared RRI as regard the renal biopsy classes is that of Conti, and his co-workers, they have studied 42 patients of LN (7, 13, 18, 4 were class II, III, IV, V) LN respectively , they found 5/42 of LN patients had a RRI above 0.7 , and 4 of them were class IV LN , so they conclude that there was a significant correlation between pathologic RI and class IV nephritis. [19] We have two criticisms on the conti's study : first, the study didn't include patients with class I, or class VI LN making RRI not ideal for definite class identification ; second , four cases only (of class IV LN who had RRI > 0.7) is a very limited number to be involved in any of comparison and correlation studies and give a trusted results.

We held a comparison between LN patients grouped according to RRI values "lower50%percentile (n=23 , RRI < 0.6) and upper 50%percentile (n=23, RRI >=0.6)" as regard demographic, laboratory, radiological, and biopsy findings, we found a higher statistical difference of serum creatinine, eGFR, chronicity index and degree of tubulointerstitial fibrosis in patients with RRI>=0.6 than patients with RRI<0.6 and there was no significant difference between both group as regard disease activity markers and activity score in renal biopsy

So, we suggest a significant association of high RRI to more chronic rather than active disease. This suggestion is augmented by the significant correlation of RRI to serum creatinine (r=0.447, p=0.002) and eGFR (r= -0.435,p=0.002) in consistence t with Chen et al[9,17,19,26,30,32-36] tubulointerstial fibrosis (r=0.629. p<0.0001) in consistence with Chen et al[12,15,17,32,37] and with Chronicity index (r=0.625, p<0.0001) in consistence with Platt et al and Wang et al.[9,18]

In this trial, the activity index was correlated with anti-ds DNA, antiC1q,C3,C4 in consistence with,[38] while chronicity index and degree of tubulointerstitial fibrosis were correlated with MABP, RRI, serum creatinine, and eGFR. Wang and his colleague have demonstrated a positive correlation of CI to RRI.[18] In univariate regression analysis using a general linear model (GLM) we found serum creatinine and RRI to be the most significant predictor to both chronicity index and tubulointerstial fibrosis after adjustment of other covariates.

To conclude: Although Renal resistive index has a limited ability to differentiate between renal biopsy classes , we suggest its possible role as a marker of chronicity and interstitial fibrosis in patients with lupus nephritis and may be a useful noninvasive tool that help decision making as regard patient selection for aggressive immunosuppressant.

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Original Article

Study of Microalbuminuria in Patients with Systemic Sclerosis as an Indicator of Early Renal Damage and Increased Morbidity.

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Abstract:

Introduction: The kidney is one of the most clearly affected internal organs that show the complications of vascular insult in Systemic sclerosis. **Aim of work:** Microalbuminuria detection in SSc patients as an indicator of early renal involvement and its correlation with various SSc clinical, laboratory, radiological parameters and severity of organ systems' damage assessed by Scleroderma Assessment Questionnaire. **Patients and methods:** Thirty patients with systemic sclerosis (27 females and 3 males) with a mean age of 42.57years ± 8.77SD and mean disease duration of 13.83 ± 6.15 SD were included. Twenty-three (76.5%) had diffuse SSc (dSSc) seven (23.5%) had limited SSc (lSSc). Ten normal persons of matched age and sex as controls. **Results:** Eight (26.7%) had microalbuminuria and five (16.7%) patients had decreased creatinine clearance. Albumin/creatinine ratio was significantly higher among dSSc patients compared to those with lSSc (X^2 =7.061 ; p = 0.023). Albumin/creatinine ratio showed significant positive correlations with telangiectasia (Z = 1.975; p = 0.048) and modified Rodnan's skin score (r = -0.398; p = 0.029). Albumin/creatinine ratio correlated significantly and positively with IVS (index of vascular status) and IDS (index of disease status) of the SAQ (P= 0.018 and 0.009 and r= -0.429 and -0.467) respectively. No statistically significant correlations were found between creatinine clearance and the different demographic, clinical features, laboratory parameters and the indices of SAQ, except for; significant correlrtion with disease duration, where creatinine clearance decreases with increased disease duration. **Conclusion:** Microalbuminuria compared to creatinine clearance may be a more sensitive indicator of early renal affection and predictor of increased morbidity.

Keywords: Systemic sclerosis; Renal involvement; Scleroderma Assessment Questionnaire.

Introduction:

Systemic sclerosis (SSc) is a heterogeneous disease whose pathogenesis is characterized by three hallmarks: small vessel vasculopathy, production of autoantibodies, and fibroblast dysfunction leading to increased deposition of extracellular matrix.(1)

Almost all organ systems could be affected; however, the most predominantly affected organs are the cutaneous, musculoskeletal, pulmonary, cardiovascular, gastrointestinal and renal systems. Renal involvement and systemic vascular damage have been shown to significantly affect prognosis in SSc.(2)

In autopsy studies, the incidence of renal disease on a histopathological level was reported to be 70–90 %.(3)

An increased excretion of total urinary protein is a classical hallmark of renal disease. Assay of total urinary protein is non-specific and insensitive and the advent of sensitive and specific assay for albumin, the dominant urinary protein in patients with renal glomerular dysfunction, has enabled the detection of abnormalities at an earlier stage of the disease process.(4)

It is often difficult to assess damage to the renal and systemic vasculature in SSc patients. Using detailed urinary protein analysis is thought to detect scleroderma renal disease at an early stage and to assess systemic vasculopathy. Abnormal urinary protein excretion is associated with parameters of increased morbidity such as diffuse cutaneous disease and visceral involvement.[5]The term 'microalbuminuria' has been coined to describe increased excretion of a very small amount of proteins, primarily composed of albumin that could not be detected by routine methods, i.e., dip stick method.[6]According to the American Diabetes Association, microalbuminuria is defined as urinary albumin excretion (UAE) of 30–299 mg/ 24 h or albumin/creatinine ratio of 30–299 µg/mg creatinine.[7]

In SSc, the inflammatory process is very mild and organ damage develops often insidiously and slowly. Mostly used diagnostic procedures are not sensitive enough to detect organ damage in the early phase and to notice the minimal change in damage or function over time. Symptoms and subjective patient's complaints are very important in the evaluation of disease status in SSc. Scleroderma Assessment Questionnaire (SAQ) demonstrates the severity of damage of the different organ systems and the degree of change in the severity of affection over time.[8]

The aim of this study is microalbuminuria detection in SSc patients and its evaluation as an indicator of early renal damage and to find out its correlation with the various SSc clinical, laboratory, radiological disease parameters and severity of organ systems' damage assessed by SAQ.

Patients and Methods:

Thirty patients with systemic sclerosis (27 females and 3 males), who fulfilled the criteria for SSc described by 2013 ACR-EULAR systemic sclerosis diagnostic criteria, attending the Rheumatology and Rehabilitation Department and outpatient clinic,main University Hospital, faculty of Medicine, Alexandria University.

SSc patients were subdivided according to the classification subsets of SSc patients, which is based on the extent of skin affection, presence of Anti-Scl-70 and Anti centromere antibody.

Twenty-three (76.5%) had diffuse SSc, seven (23.5%) had limited SSc. Ten normal persons of matched age and sex as controls.

• **Exclusion criteria**:

Localized forms of scleroderma, various forms of pseudo-scleroderma, SSc patients with associated arterial hypertension, diabetes mellitus, cardiovascular diseases including pulmonary hypertension, heart failure, preexisting renal disease (e.g polycystic kidney disease, nephrotic syndrome), urinary tract infection and hematuria. Other conditions that may invalidate urine albumin excretion, e.g., exercise within 24 hours, acute febrile illness and pregnancy.

All patients included in the study were subjected to:

(1) Full history taking including Scleroderma Assessment Questionnaire (SAQ).[9]

• SAQ consists of twenty-three questions divided into four subgroups: four items related to vascular, six items to respiratory, five items to gastrointestinal and eight items to musculoskeletal dysfunctions. Some items are derived from the Systemic Sclerosis Questionnaire (SySQ), with permission of the authors. The range of all indices is from 0 to 3. Answering categories: (A) intensity of symptoms (no = 0, some = 1, moderate = 2, very intensive = 3), (B) frequency of symptoms (never = 0, sometimes = 1, frequently = 2, always = 3) and (C) ability to perform activities (without difficulty = 0, with some difficulty = 1, with much difficulty = 2, not able to do = 3). The Index of Vascular Status (IVS), Index of Respiratory Status (IRS), Index of Gastrointestinal Status (IGS) and Index of Musculoskeletal Status (IMSS) are calculated by dividing the total score for a particular group by the number of questions for that group. A higher index value indicates more severe damage of a particular organ system. By dividing the total score for the entire questionnaire by the number of questions, the Index of Disease Status (IDS) was obtained.

(2) Thorough general (including inter incisor and finger to palm (in flexion) distances in cm.[10] cardiopulmonary, abdominal, musculoskeletal and neurological system examinations.

(3) Skin examination including calcinosis, digital lesions and assessment of skin sclerosis severity by modified Rodnan's skin score (mRss).[11]

(4) Vascular system examination including Raynaud's phenomenon, digital pitting scar, digital gangrene and bone resorption.

(5) Laboratory investigations:

Ten ml venous blood were with drawn under complete aseptic technique, and divided into:

Two ml in lavender-top vacutainer tubes containing K2EDTA (dipotassium ethylene diamine tetra acetic acid) for CBC.

1.6 ml in black-top vacutainer tubes containing 3.2% sodium citrate for ESR.

Three ml in red-top plain vacutainer tubes for serum chemistry analysis.

Three ml in red-top plain vacutainer tubes for ANA analysis.

- Complete blood picture was done by differential automated cell counters XT1800 and Advia2120-1.
- ESR was done by Westergren technique.
- Liver enzymes (AST, n: 15–37 U/L and ALT, n: 30–65 U/L).
- Kidney functions: serum Creatinine (n: 0.6–1.00 mg/dl).
- Blood urea (n: 15-45 mg/dl).
- Muscle enzymes (CPK, n: upto 195 U/L and LDH, n: 100–190 U/L).
- All chemical analysis of blood were performed using Dade Behring Dimension RxL clinical chemistry auto-analyzer.
- Anti-nuclear Ab (ANAs): were identified by indirect immunofluorescence (IIF).
- Anti-topo-1/Scl-70, Anti centromere antibody were done.
- Complete urine analysis (physical appearance, chemical analysis and microscopic examination).
- 24 h urinary protein (n: up to 0.15 gm/24 h): was assayed by turbidimetry.
- Creatinine clearance (n: 80–120 ml/min): was calculated using the following equation C= UV/P, where C is creatinine clearance, P is the plasma concentration of the creatinine, U is the urinary concentration of creatinine and V is the urine flow rate. It is considered as a measure of glomerular filtration rate.
- Urinary albumin/creatinine ratio: was assayed by Nephelometryin random spot urine samples. Normal: up to 30 µg/mg creatinine, microalbuminuria: 30–300 µg/mg creatinine and macro albuminuria >300 µg/mg creatinine).

(6) Radiological investigations:

- Plain X-ray of the hands and chest.
- High resolution computerized tomography of the chest.
- Ultrasound abdomen for renal assessment (kidney size, coticomedullary differentiation, grade of medical nephropathy).

(7) Electrocardiography (ECG).

(8) Echocardiography.

Statistical analysis:

Data were fed to the computer and analyzed using IBM SPSS software package version 20.0. Qualitative data were described using number and percent. Quantitative data were described using rang (minimum and maximum), mean and standard deviation and median. Comparison between different groups regarding categorical variables was tested using Chi-square test. The distributions of quantitative variables were tested for normality, if it reveals normal data distribution, parametric tests was applied. If the data were abnormally distributed, non-parametric tests were used. For normally distributed data, comparison between two independent population were done using independent t-test. For abnormally distributed data, comparison between two independent population were done using Mann Whitney test. Significance of the obtained results was judged at the 5% level.[12,13]:

Results:

The clinical features and laboratory parameters of the study group are shown in Tables I and II.

Complete urine analysis revealed within normal results, none of patients had macroalbuminuria.

We found no significant difference between control group and the patient group after comparing demographic data, clinical features and laboratory parameters except for some laboratory parameters which showed significant difference in SSc patients, which are ESR, CRP, CPK, LDH, ANAs, albumin/creatinine ratio and creatinine clearance.

Eight (26.7%) patients had microalbuminuria; six (20%) had dSSc and two (6.7%) lSSc patients. Five(16.7%) patients had decreased creatinine clearance. ANAs positivity was detected in twenty six patients (86.7%).

No significant correlation was found between albumin/creatinine ratio and demographic (age, sex and disease duration) features. Albumin/creatinine ratio was significantly higher among dSSc patients compared to those with lSSc (Pearson's X^2 =7.061, p = 0.023)

Albumin/creatinine ratio showed significant positive correlations with telangiectasia (P=0.048, Z=1.975) and mRodnan's skin score (P=0.029, r=-0.398).

Albumin/creatinine ratio correlated significantly and positively with IVS (P=0.018,r=-0.429) and IDS indices of SAQ (p = 0.009, r=-0.467), as shown in Table III & IV.

We found no statistically significant correlation between creatinine clearance and the different demographic, clinical features and laboratory parameters of SSc patients, except for significant correlation with disease duration, where creatinine clearance decreases with increased disease duration.

The comparisons between creatinine clearance and the different laboratory parameters could not reveal any statistically significant correlation.

There was no significant correlation between creatinine clearance and the different indices of the SAQ.

Table I: Clinical features of the study (Patients) group.

Clinical features	**Number (n=30)**	**Percent**
Calcinosis	8	26.7
Telangiectasia	6	20.0
	Range	**Mean±SD**
Modified Rodnan's skin score	11-42	22.87±9.8
Interincisor distance in cm	2-5	4.29±0.65
Finger to palm distance in cm	0-8	1.10±1.86
	Number (n=30)	**Percent**
Musculoskeletal manifestations		
Myositis	6	20.0
Myopathy	1	3.3
Arthritis	12	40.0
Vascular manifestations		
Raynaud's phenomenon	28	93.3
Digital pitting scars	20	66.7
Digital gangrene	4	13.3
Bone resorption	6	20.0
Pulmonary manifestations		
Pleurisy	4	13.3
Pleural effusion	1	3.3
Interstitial pulmonary fibrosis (IPF)	6	20.0
GIT manifestations		
Esophageal dysmotility	20	66.7
Dyspepsia	14	46.7
Malabsorption	6	20.0
Neurological manifestations		
Carpal tunnel syndrome	2	6.7
Peripheral neuropathy	5	16.7
Cranial nerve involvement	1	3.3
Sicca manifestations	6	20.0

Table II: Laboratory parameters of the patients group.

Laboratory parameters	Range	Mean±SD
ESR in the 1st hour in mm	8-167	53.47±32.4
Hbgm/dl	7.6-13	10.96±1.32
TLC×10^3/ mm^3	3.22-16.4	7.90±2.52
AST U/L	15-97	41.57±19.89
ALT U/L	11-144	37.17±32.01
CPK U/L	16-1,860	295.33±418.59
LDH U/L	58-2,060	486.37±470.85
Creatinine mg/dl	0.3-1.0	0.66±0.20
Albumin / Creatinine Ratio µg/mg	5-140	36.97±42.75
Creatinine Clearance ml/min	52-155	99.37±24.32

ESR: erythrocyte sedimentation rate; TLC: total leucocytic count; AST: aspartate transaminase; ALT: alanine transaminase; CPK: creatinine phosphokinase; LDH: lactate dehydrogenase.

Table III: Different indices of the SAQ in the study (Patients) group.

Indices of SAQ	Range	Mean±SD
IMSS	0.00-2.20	0.90±0.78
IVS	0.00-2.80	1.34±0.92
IRS	0.0-2.0	0.28±0.50
IGS	0.0-2.80	1.47±0.77
IDS	0.0-20	1.05±0.54

SAQ: Scleroderma Assessment Questionnaire; IMSS: index of musculoskeletal status; IVS: index of vascular status; IRS: index of respiratory status; IGS: index of gastrointestinal status; IDS: index of disease status.

Table IV: The correlation between albumin/creatinine ratio and the different indices of SAQ.

Indices of SAQ	r_s	P value	Significance
IMSS	-0.031	0.871	Non-Significant
IVS	-0.429*	0.018	Significant
IRS	0.244	0.129	Non-significant
IGS	0.201	0.213	Non-significant
IDS	-0.467*	0.009	Significant

IMSS: index of musculoskeletal status; IVS: index of vascular status; IRS: index of respiratory status; IGS: index of gastrointestinal status; IDS: index of disease status.

Discussion:

The kidney is one of the most clearly affected internal organs that show the complications of vascular insult in SSc.[14]

Renal involvement and systemic vascular damage have been shown to significantly affect prognosis in SSc.[2]

Autopsy studies reveal histological evidence of renal involvement in 60–80% of patients with SSc.[15] In other autopsy study in SSc patients, the incidence of renal disease on a histopathological level was reported to be 70–90 %.[3]

Owing to the cumbersome collection of 24 hr urine volume and the inaccuracy of the result obtained by the dip stick method as a consequence of the variation of urinary protein concentration according to the urinary volume [16], estimation of albumin/creatinine ratio (µg/mg) on a urine sample is used to accurately measure urinary protein excretion.[17]

Moreover, microalbuminuria can detect early renal disease before progression of the condition to structural renal damage and frank macro albuminuria.[18]

Eight (26.7%) of our patients were positive for microalbuminuria. This coincides with the results of the studies done by Dawnay et al[19], Livi et al[15] and Seiberlich et al.[5] who detected microalbuminuria in 17.9%, 19.04% and 17.5% of the study population respectively after excluding those with concomitant hypertension, diabetes mellitus and other exclusion criteria described before.

In disagreement with Seiberlich et al.[5] who found that almost half (45.5%) of SSc patients who had albuminuria in their study were males, we found no significant correlation between the presence of microalbuminuria and gender.

In our study, we found no significant correlation between albumin/creatinine ratio and patient's age or the disease duration, this agrees with the results of Dawnay et al.[19] In disagreement with Seiberlich et al.[5] who reported that albuminuria was significantly more prevalent in SSc patients whose disease duration was more than four years, we didn't find significant correlation between albumin/creatinine ratio and the disease duration.

We found that albumin/creatinine ratio was significantly higher among patients with d SSc as compared to those with l SSc. This coincides with the results of the study done by Seiberlich et al.[5] who found that albuminuria was more prevalent among diffuse SSc patients.

Moreover, we found that albumin/creatinine ratio increases significantly with higher modified Rodnan's skin score. This coincides with the results of El Sayed et al.[20] who also revealed that patients with microalbuminuria had more extensive skin disease .

There was a significant positive correlation between albumin/creatinine ratio and telangiectasia. Moreover, There was a significant positive correlation between albumin/creatinine and IVS (index of vascular status)of the SAQ which can emphasize the association between renal affection and vasculopathy in SSc. This agrees with Deckert et al.[21] who reported that; an increased excretion of urinary albumin may reflect not only glomerular disease but also more generalized vascular dysfunction. This coincides with Dawnay et al.[19]as well who reported that; increased albumin excretion in patients with systemic sclerosis may reflect the underlying vascular pathology of the disorder and may also herald the onset of renal disease. Also, this is supported with Shanmugam et al.[22]who reported that proteinuria as an early preclinical marker of renal pathology might also serve as a surrogate marker for the severity of renal vascular pathology and for prognosis in SSc. Also, coincides with Seiberlich et al.[5] Who reported that; leakage of proteins into the urine is an indicator of vascular damage in SSc patients.

In disagreement with El Sayed et al.[20] Who detected a significant positive correlation between the degree of microalbuminuria and serum creatinine level, we could not find a significant correlation between albumin/ creatinine ratio and serum creatinine level. On the contrary this agrees with Reem et al.[23] who detected that serum Create nine is a poor marker of renal function in SSc patients.

There was a significant positive correlation between albumin/creatinine ratio and IDS (index of disease status) of SAQ. This agrees with Seiberlich et al.[5] Who reported that; leakage of proteins into the urine is an indicator of vascular damage in SSc patients which is highly relevant for mobidity in these patients. Also, this is supported with Shanmugam et al.[22]who reported that proteinuria as an early preclinical marker of renal pathology might also serve as a surrogate marker for the severity of renal vascular pathology and for prognosis in SSc. This agrees with Hillege et al.[24] who reported that microalbuminuria is commonly used as an indicator of early kidney damage and the detection of increased albumin excretion is associated with poorer prognosis. This supports the fact that microalbuminuria is a sensitive indicator of increased morbidity and disease severity rather than just a marker of renal affection.

Five patients (16.7%) had decreased creatinine clearance. This result coincides with the results of Clements et al[25] who reported low creatinine clearance in 16% of their SSc patients.

There was nosignifigant correlation between creatinine clearance and any of the demographic, clinical features and laboratory parameters of the SSc patients except for signifigant correlrtion with disease duration, where creatinine clearance decreases with increased disease duration. This agrees with the results of the studies of Desai et al.[26]and Gupta et al.[27] who found no correlation

between creatinine clearance and any of patient's age, interstitial lung disease, gastrointestinal tract involvement, modified Rodnan's skin score and muscle affection.

The significant correlation between creatinine clearance and disease duration coincides with El Sayed et al.[20] who detected that mean measured GFR in patients with disease duration more than forty eight months is lower than that of those with disease duration less than forty eight months. Also agrees with Gupta et al[27] who detected that patients with reduced estimated GFR had longer disease duration than those with normal GFR.

We could not find a relation between microalbuminuria and decreased creatinine clearance. This may indicate that microalbuminuria is a sensitive indicator of early renal affection even before impairment of renal function.

In contrast to albumin/creatinine ratio, we could not find a relation between creatinine clearance and any of the indices of the SAQ.

We can conclude that microalbuminuria is a more sensitive indicator of early renal affection when compared to creatinine clearance with the additional advantage of being a predictor of systemic vasculopathy and increased morbidity of the different organ systems. Subsequently, regular screening of SSc patients for the detection of microalbuminuria is recommended. Microalbuminuria patients should have more frequent follow up visits for early detection systemic organ affection

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Original Article

Death Receptor 4 (DR4) Polymorphism and Susceptibility to Hepatocellular Carcinoma in Egyptian Patients.

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Abstract:

Hepatocellular carcinoma (HCC) is one of the most common cancers in the world. Genetic polymorphisms have been reported to play a role in susceptibility to HCC. The present study aimed to study the possible association between DR4 gene polymorphisms and susceptibility to HCC in Egyptian patients. The study was carried out on 160 subjects divided into 3 groups: Group (A) included 78 HCC patients, group (B) included 42 chronic hepatitis C patients (HCV) and group (C) included 40 age and sex matched healthy control. All subjects were submitted to full history taking, liver function tests and DR4 gene polymorphisms by (PCR- RFLP). This study found a significant difference between HCC and each of HCV and control group, while there is no significant difference between HCV group and control group as regarding DR4 genotyping. The rs20575 polymorphism CC and CG genotypes occurred at increased frequencies in patients with HCC in relation to other groups and the risk of HCC was linked to carriage of rs20575 C allele (OR:1.66) when compared to HCV group and (OR:2.05) when compared to controls respectively.

The present study reported that carriage of DR4 rs20575 CC, CG genotypes have a role in susceptibility to HCC and we recommend performance of this work on a large scale to confirm these results.

Keywords: DR4 / HCC

Introduction:

Hepatocellular Carcinoma (HCC) is the third most common cause of cancer-related death world-wide and each year approximately 750.000 new cases are diagnosed (Jemal et al., 2011).

Chronic hepatitis B virus (HBV) and hepatitis C virus (HCV) infections, alfatoxin B_1, alcohol and non alcoholic steatohepatitis are regarded as the main carcinogenic factors (Kew., 2014).

However, not all individuals infected with HBV/HCV develop HCC indicating implication of other environmental and genetic risk factors in multistage process of this complex disease (Li et al., 2009).

Tumor development is normally prevented by the immune system, which eliminated transformed cells via induction of apoptosis by tumor necrosis factor related apoptosis inducing ligand (TRAIL) (Mellier et al., 2010).

TRAIL induces apoptosis by binding to death receptors (DR) and subsequent activation of the apoptotic cascade (Horak et al., 2005), and it induces apoptosis in a variety of transformed or tumor cells but not normal cells, making it an attractive agent for cancer therapy (Chen et al., 2009).

Death receptor 4 (DR4) was the first DR for TRAIL to be identified and it is located on chromosome 8 p21 (Hazra et al., 2003).

DR4 is type 1 membrane protein receptor which contains 486 amino acids which form two extra cellular cystein rich ligand binding pseudo repeats (50s and 90s loops), a single transmembrane helix and a cytoplasmic death domain (ectodomain) which provokes apoptosis upon TRAIL binding (Frank et al., 2005) .

Human death receptor gene consists of 10 exons and 9 introns in the coding regions. Exon sizes vary form 32 bp to 320 bp and intron sizes vary from 86 to 4700 bp and the principle elements of the DR4 ligand- binding domain are encoded by exons 3 and 4 (Fisher et al., 2001).

DR4 gene is highly polymorphic and DR4 mutation have been described in different human cancers (Tastemir-KorKmaz et al., 2013) However, the most studied polymorphisms are rs20575 with C to G substitution at position

626 (C626G) in the ectodomain of DR4 and rs20576 with A to C substitution at position 683 (A683C) in the extracellular cystein- rich domain of DR4 (Chen et al., 2014).

The present study aimed to study the possible association between DR4 gene polymorphisms namely rs20575 (C626G) with susceptibility to HCC patients in Egyptian patients.

Subjects and Methods:

This study was carried out on 160 subjects, 78 patients with HCC, 42 with chronic hepatitis C virus infection and 40 age and sex matched healthy controls, the patients were selected from National Liver Institute and Internal Medicine, Menofia University Hospitals in the period from Jan 2014 to July 2014.

The subjects were divided into 3 groups:

Group (A): It included 78 HCC patients (61 males and 17females). Their mean age was (44.53 $\pm$ 4.63 years). All patients were Hepatitis C antibody positive and Hepatitis B surface antigen negative, cancer was on top of cirrhosis. This group was diagnosed according to (Barcellona class), which depend on the combination of CT and ultrasound (US) results or on the combination of Alfa fetoprotein and either of CT or US results.

Group (B): It included 42 chronic hepatitis C virus patients HCV group (35 males and 7females). Their mean age was (43.66 $\pm$ 5.30 years).

Group (C): It included 40 age and sex matched healthy controls (32 males and 8 females). Their mean age was (42.40 $\pm$ 5.60 years). All controls are HCVAb and HBsAg negative.

Exclusion criteria included patients with autoimmune liver disease, suspected drug- induced cirrhosis or those with cryptogenic cirrhosis.

All Subjects were submitted to full history taking, clinical examination, ultrasonography, CT scan., laboratory investigation including liver function tests, complete blood picture, HBsAg, HCVAb, alpha fetoprotein (αFP) and assessment of DR4 rs20575 gene polymorphisms using PCR- RFLP technique.

Sample collection and assay:

10 ml of venous blood were divided into 2 parts, five ml were transferred into a plain tube, the clear supernatant serum was used for determination of liver function tests using fully automated auto analyzer SYNCHRON CX9ALX (Beckman Coulter Inc., CA, USA), viral markers HBsAg and HCVAb were determined by eletrochemiluminecence immunoassay using Cobase immunoassay analyzer (Roche Diagnostic, Germany).

The remaining 5 ml blood were transferred into EDTA containing tube and used for lymphocyte separation for further molecular analysis, then lymphocytes was mixed with phosphate buffer solution and stored in cryotubes at -80^{o}C till analysis (Bio test AG, Dreieich, Germany). DNA from lymphocytes samples was isolated using the QIAGEN extraction kit (Hilden, Germany) and eluted DNA was stored at-20^{o}C for PCR - RFLP.

PCR for the DR4 gene was carried out to a total volume of 25 ul, containing 10 ul genomic DNA, 0.25 ul of each primer (50μM)(Midland, Texas), 2.5 ul of 10x Taq polymerase buffer, 1.5 ul of 25 mM Mg Cl_2 (Qiagen Hiden, Germany), 0.25ul of ampil taq DNA polymerase (5 units / ul) (Qiagen Hiden, Germany), 0.5 ul of dNTPs (10mM) (Qiagen Hiden, Germany), 3ul of DMSo and 6.75 ul of H_2o.

The DR4 gene polymorphisms were analyzed using the following primers:

Polymorphism	Primers	RE	PCR products
rs20575 exon 4 [C626G]	Forward 5`-AAGGTCAAGGGAC ACGT CAGG-3` Reverse 5`-GCTTCTGT GG TTTCTTT GAGG-3`	DraIII	Allele C 164 bp, 56 bp Allele G: 220 bp

PCR amplification for these polymorphisms were performed in a programmable perkin elmer thermal cycler 2400 (USA), at 94°C for 5 min, followed by (35 cycles) at 94°C for 30s , 62°C for 30s (for A683C, annealing temperature is 58°C), 72°C for 30s and one final cycle of extension at 72°C for 7 minutes.

Then the amplification products were separated by electrophoresis through 3% agarose gel stained with ethidium bromide, one band was observed 220 bp for rs20575 (Tastemir-Korkmaz et al., 2013).

The DR4 genotyping using restriction fragment length polymorphism:

The PCR product of the rs20575 of DR4 gene was digested by DraIII restriction enzyme (RE) (provided by fermentas), the digestion products were resulted in 164 bp and 56 bp for CC genotype and 164 bp, 56 bp and 220 bp for CG genotype and 220 bp for GG genotype.

The reaction conditions were, 17ul nuclease- free water, 2ul 10x buffer, 10 ul DNA and 1 μl (2units) restriction enzyme. The mixture was incubated for 2 hours at 37°C then 10 ul of the products were loaded into 3% agarose gel containing ethidium bromide for electrophoresis.

Statistical Analysis:

Results were collected, tabulated, statistically analyzed by IBM personal computer and statistical package SPSS version 16 (SPSS inc. Chicago, Illinois, USA). All data were expressed as Mean ± standard deviation and number and percent. P-value of <0.05 was considered statistically significant.

Results:

Table (I): Shows no significant difference among the three studied groups regarding age and gender.

Table (II): Shows a significant difference among the three studied groups as regarding liver function tests, there is a significant increase of ALT, AST, ALP, TBil, while there is a significant decrease as regarding TP, ALB, PC% in each of HCC and HCV groups when compared to control group. There is a significant increase of TBil, αFP, ALP while there is a significant decrease as regarding PC% in HCC carcinoma group when compared to HCV group while there is no significant difference as regarding other parameters.

Table (III): Shows a significant difference between HCC group and HCV group as regarding DR4 genotyping and allele distribution. In the rs20575 polymorphism the frequency of CC and CG genotypes were associated with 3.12 and 3.01 times higher risk of HCC than GG genotype respectively and frequency of C allele was associated with 1.66 times higher risk of the HCC than G allele.

Table (IV): Shows a significant difference between HCC group and control group as regarding DR4 genotyping and allele distribution. In the rs20575 polymorphism the frequency of CC and CG genotypes were associated with 4.80 and 3.24 times higher risk of HCC than GG genotype respectively and frequency of C allele was associated with 2.05 times higher risk of the HCC than G allele.

Table (V): Shows no significant difference between HCV group and control group as regarding DR4 genotyping and allele distribution.

Table (I): Statistical comparison of demographic criteria between the three studied groups.

Criteria	Studied groups						P value
	Controls (n=40)		HCV patients (n=42)		HCC patients (n=78)		
Age (years):	42.40 ± 5.60		43.66 ± 5.30		44.53 ± 4.63		0.09
Sex:	**No.**	**%**	**No.**	**%**	**No.**	**%**	
Male	32	80.0	35	83.3	61	78.2	0.79
Female	8	20.0	7	16.7	17	21.8	

Table (II): Statistical comparison between the studied groups regards laboratory parameter rs.

Laboratory parameters	Studied groups			P value
	Controls (n=40)	HCV patients (n=42)	HCC patients (n=78)	
Prothrobin concentration (PC%)	99.80 ± 0.41	93.09±6.38	71.47±11.48	P1 0.001 P2 0.001 P3 0.001
Alanine aminotransferase (ALT U/L)	26.77±5.21	63.43±6.10	70.23±25.23	P1 0.001 P2 0.08 P3 0.001
Aspartate aminotransferase (AST U/L)	28.45±5.95	62.35±10.70	68.14±17.17	P1 0.001 P2 0.07 P3 0.001
Total bilirubin (T Bil mg/dl):	0.88±0.15	1.17±0.25	2.33±0.95	P1 0.001 P2 0.001 P3 0.05
Alfa fetoprotein (αFP ng/ml)	2.41±0.75	2.60±0.63	34.14±12.83	P1 0.001 P2 0.001 P3 0.52
Total protein (TP gm/dl)	6.68±0.46	5.66±0.58	5.38±0.92	P1 0.001 P2 0.12 P3 0.001
Seurm Albumin (ALB mg/dl)	3.77±0.38	3.04±0.22	2.91±0.58	P1 0.001 P2 0.15 P3 0.001
Alkaline phosphatase (ALP U/l)	54.65±10.15	59.85±7.32	91.19±12.86	P1 0.001 P2 0.001 P3 0.03

P 1 (HCC versus controls)
P2 (HCC versus HCV)
P3 (HCV versus controls)

Table (III): Distribution of DR4 genotypes and alleles between HCV and HCC patients.

Genotype	Studied groups				X^2 test	P value	OR (95% CI)
	HCV patients (n=42)		HCC patients (n=78)				
rs20575 (C626G):							
GG	13	30.9	10	12.8			
CG	19	45.3	44	56.4	5.08	0.02	3.01 (1.12–8.06)
CC	10	23.8	24	30.8			3.12 (1.03–9.43)
rs20575 (C626G):							
G allele	45	63.6	64	41.0	3.47	0.05	1.66 (0.97-2.83)
C allele	39	46.4	92	59.0			

Odds ratio (OR) at 95% confidence interval (CI)

Table (IV): Distribution of DR4 genotypes and alleles between HCC patients and controls.

Genotype	Studied groups				X^2 test	P value	OR (95% CI)
	Controls (n=40)		HCC patients (n=78)				
rs20575 (C626G):							
GG	14	35.0	10	12.8			
CG	19	47.5	44	56.4	8.6	0.014	3.24 (1.22–8.58)
CC	7	17.5	24	30.8			4.80 (1.49–15.46)
rs20575 (C626G):							
G allele	47	58.7	64	41.0		0.009	
C allele	33	41.3	92	59.0	6.67		2.05 (1.18-3.54)

Table (V): Distribution of DR4 genotypes and alleles between HCV patients and controls.

Genotype	Studied groups				X^2 test	P value	OR (95% CI)
	Controls (n=40)		HCV patients (n=42)				
rs20575 (C626G):							
GG	14	35.0	13	30.9			
CG	19	47.5	19	45.3	0.518	0.077	0.93 (0.35–2.49)
CC	7	17.5	10	23.8			0.65 (0.19–2.21)
rs20575 (C626G):							
G allele	47	58.7	45	53.6	0.45	0.50	0.81 (0.44-1.50)
C allele	33	41.3	39	46.4			

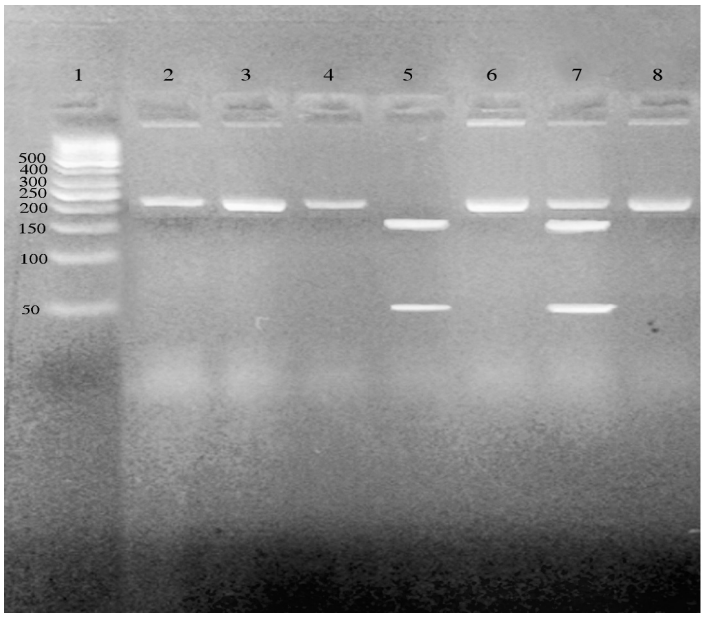

Figure (1): Shows the agarose gel electrophoresis for DR4 rs20575 polymorphism after digestion by Dra III

Lane 1 indicate ladder (50bp)
Lanes 5 indicate CC genotype (164bp & 56bp)
Lanes 7 indicate CG genotype (164bp, 56bp & 220bp)
Lanes 2,3,4,8 indicate GG genotype (220bp)

Discussion:

Death receptor-4 is a tumor suppressor gene and plays as an important mediator of apoptosis. Binding of these rec eptors to their cognate ligands lead to receptor aggregation and recruitment of adaptor proteins forming death inducing signaling complex which in turn activate Caspase 8 and 10 for the triggering of apoptosis (Shirley et al., 2011). Polymorphism in DR_4 gene may reduce apoptotic capacity and provoke proliferation of cell and cancer (Mittal et al., 2015).

Normal variations within the sequence of the apoptotic genes are suggested to lead suboptimal apoptotic capacity finally increasing cancer risk (Geoge et al., 2012).

This study found that, there is a significant increase of AST, ALT, total bilirubin, while there is a significant decrease as regarding serum albumin, total protein and prothrombin concentration in HCC and HCV groups when either of them is compared to the control group.

These results are matched with (Awadellah et al., 2011) who reported a significant deterioration of liver function in HCC and HCV patients when compared to controls.

The present study found a significant increase of α FP in HCC group when compared to HCV group and controls. These results are in accordance with (Spadaro et al., 2005).

This study detected a significant difference among the three studied groups as regarding DR4 genotyping, there is a significant difference between HCC group when compared to either HCV group or control group, while there is no significant difference between HCV group and the controls. On comparing HCC & HCV groups, as regarding rs20575 polymorphism, the frequency of CC and CG genotypes are associated with 3.12 and 3.01 times higher risk of HCC than GG genotype and C allele was associated with 1.66 higher risk of HCC than G allele While on comparing HCC to controls as regarding rs20575 polymorphism, the frequency of CC and CG genotypes are associated with 4.80 and 3.24 times higher risk of HCC than GG genotype and C allele was associated with 2.05 higher risk of HCC than G allele,

These results are in agreement with (Komer et al., 2012) who found that TRAIL receptor genetic variants are associated with an increased risk of HCC in patients with chronic hepatitis C. Lan et al., 2008, found that the increased HCC risk in HCV infected patients carrying the C626G risk variants of TRATL receptor 1 reflects less efficient immune control over HCV infection via TRAIL mediated mechanisms, the genetic variants in DR4 alter its affinity for TRAIL so, TRAIL- DR4 signaling is less efficient in these patients and reduced susceptibility of hepatocytes towards TRAIL –induced apoptosis facilitating HCC development. (Komer et al., 2012).

However previous studies investigating the role of DR4 gene polymorphism in Cancer, Golvez et al, 2014 suggested a possible role of TRAIL receptor, polymorphism in B cell lymphoma genesis.

Rai et al., 2014 concluded that DR4 haplotypes especially rs20575C and rs20576A significantly increased gallbladder cancer risk and this haplotype may change the apoptotic signals and modulate cancer susceptibility by promoting tumor cells survival and tumor growth rather than initiating tumor formation.

Harza et al., 2003 showed an increased risk of C to G transition in 626 position of exon 4 of DR4 gene in bladder cancer and meta

analysis by (Chen et al., 2014) indicated that C626G polymorphism in DR4 gene is associated with cancer susceptibility. On the other hand, (Martinez- Ferrandia et al., 2007) found no association between DR_4 polymorphism and breast cancer in spanish women and also in Turkish population with lung cancer (Tastemir- Korkmaz et al., 2013).

Conclusion:

The present study reported that carriage of rs20575CC, CG genotypes have a role in susceptibility to HCC and we recommend performance of this work on a large scale to confirm these results.

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Original Article

The Study of the Relationship between Insulin Resistance Index and Serum Apelin Level in Patients with Chronic Hepatitis C Virus.

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Abstract:

Objective: Highlighting the apelin system would present a new therapeutic target for liver disease. Apelin; endogenous ligand for the orphan receptor APJ, was recently suggested to be associated with fibrosis progression and cirrhosis in addition to insulin resistance (IR) and inflammation. The present study was conducted to detect the relationships between apelin serum levels and insulin resistance in patient with HCV in comparison to patients without HCV and calculating the apelin serum level and HOMA 2- IR among 60 egyptian patients with HCV and 20 non HCV. Serum apelin levels were significantly lower in HCV patients with median value (167.69±221.70) when compared with control group (867.0±158.95), at P <0.001 with significant apelin variations among asymptomatic carriers (ASC), fibrosis, and cirrhosis patients .Multiple Linear Regression models depicated that HbA1c, S.insulin and HCV were found to be the best predictors affecting apelin levels, and FBS, S.insulin were found to be the best predictors affecting HOMA IR with a decreasing and increasing effect respectively and there were negative significant correlation between S.apelin with BMI, FBS and Hb while there were positive significant correlation between HOMA IR with S.insulin (P < 0.001). **Conclusion:** Apelin level varies in HCV patients, which may contribute to fibrosis progression. In addition, IR could act as comorbid factors affecting apelin level in patients with HCV.

Keywords: Insulin Resistance Index, Serum Apelin, Chronic Hepatitis C Virus

Introduction:

Hepatitis C virus (HCV) has been recognized as a major cause of chronic liver disease(s) (CLD) in Egypt[(1)]. The emerging role of apelin in CLD is complex, as described in a recent report linking apelin to the initiation and maintenance of the inflammatory and fibrogenic processes occurring in the fibrotic liver[(2)], as well as to the vascular and haemodynamic abnormalities in cirrhosis and its complications[(3,4)]. However, clinical data demonstrating the role of apelin in CLD is limited, as depicted by Bertolani and Marra.[(5)]

Hepatic insulin signaling is markedly impaired in HCV patients; in addition, down regulation of hepatic insulin mediators is associated with enhanced hepatocyte apoptosis and fibrogenesis[(6)]. Currently some evidence supports a relationship between insulin resistance (IR) and hepatitis C on one hand, playing role in progression of liver disease[(7-10)], and between IR and apelin level.

The receptor APJ remained orphan until 1998, when Tatemoto and his coworkers isolated its endogenous ligand from bovine stomach extract. They isolated a 36-amino-acid peptide which was named apelin (from APJ endogenous ligand)[(11)] .Apelin exists in at least three forms, consisting of 13, 17, or 36 amino acids, all originating from a common 77-amino-acid precursor[(12)]. Apelin has been shown to be involved in vessel formation, where it exerts a pro-angiogenic role [(13,14)], and

in the regulation of cardiovascular function, by reducing arterial blood pressure, via stimulation of nitric oxide-mediated vasorelaxation[15,16]. Moreover, apelin has recently been added to the family of adipokines[17,18], which are adipocytokines mainly derived from adipose tissue as well as endothelial cells (ECs) in various parts of the body [12]

Subjects and Methods:

Subjects:

Eighty age matched subjects selected from outpatient clinic of the diabetes and metabolism unit, Alexandria Main University Hospital were enrolled in this study divided into 60 patients with chronic hepatitis c virus (Child-Paugh A,B,C) and 20 patients without HCV as a control group. After protocol approval, the study was conducted in the period from September 2013 to April 2015. All subjects gave written informed consent prior to participation .The following exclusion criteria were used for all subjects: Other causes of chronic liver disease as alcoholic and autoimmune, treatment with interferon beta, pregnancy and lactation, conditions reported to be associated with altered serum apelin level as under-weight (B.M.I < 18.5 Kg/m2), morbid obesity (B.M.I > 40 Kg/m2), unstable body weight within last three months, patients receiving corticosteroids for any reason, diabetes mellitus, known concurrent malignancy, severe chronic renal disease and recent major surgery.

Methods:

Data collection:

A detailed drug treatment(s) history was collected, and physical examination of the patients was carried out with special emphasis on previous surgical procedures and prior parenteral therapy. Complete clinical examination was done, on the day of sample withdrawal which included the manifestations of hepatitis such as hepatomegaly, tenderness in the right hypochondrium, splenomegaly, and lower limb edema, or liver cell failure such as jaundice, hepatic encephalopathy, bleeding varices, and ascites. abdominal ultrasonography were also done side by side with routine laboratory investigations including complete blood picture, liver, kidney function tests, fasting blood sugar, serum insulin ,HbA1c, lipid profile and HOMA-IR. HCV was diagnosed by anti-HCV antibodies, and HCV RNA by real time PCR. Medical records indicated that 60 patients were of HCV .

Body mass index (BMI) was calculated as an index of the weight (in kilograms) divided by the square of the height (in meters) measured on the same day of sample withdrawal. Centers for Disease Control and Prevention (CDC) classify the normal range of BMI to be between 18.5–24.9kg/m^2, overweight BMI between 25–29.9kg/m^2, and the obese BMI > 30 kg/m^2[19] . Since BMI could be influenced by the presence of ascites in patients with cirrhosis, earlier weights taken prior to the diagnosis of ascites were used for the calculation of BMI.[20]

Sample Preparation, Collection, and Storage:

All subjects were advised to take no medication on the morning before blood sample collection. Fasting blood (5mL) was obtained from the antecubital vein, after an overnight fasting period (10–12 hours). Samples were divided into two parts; one containing trisodium citrate (final concentration 1mg/mL) for prothrombin time (PT) determination. The other part was taken into vacutainer clotted tubes, where sera were separated by centrifugation at 3000rpm for 10min for other lab measurements. Other sera aliquots were kept frozen at −70°C for measurement of serum apelin (with no need for aprotinin pretreatment step applied to plasma samples [21]) TNF-α, and insulin.

Laboratory Assessments:

Sera were obtained, aliquoted for the measurement of LFTs: aspartate transaminase (AST), ALT, bilirubin, albumin, fasting blood glucose (FBG), and lipids (total cholesterol (TC) and triacylglycerol (TAG)) by using standard enzymatic techniques using appropriate kits and

semiautomated photometer 5010. Determination of serum apelin level using DRG® Apelin C-Terminus Elisa (Human/Mouse/Rat) (EIA-5178)., procedures were carried out according to the manufacturers' instructions.

Insulin resistance was determined by the homeostasis model of assessment (HOMA) [22] using the formula: fasting insulin (μIU/mL) × fasting blood glucose (mg/dL)/405.

Statistical Analysis:

SPSS statistics (V. 19.0, IBM Corp., USA, 2010) was used for data analysis. Data was expressed as mean ± S.D for quantitative parametric measures, in addition to median and IQR for nonparametric data and percentiles for categorical data. Student's t test was used for comparison of two independent groups for parametric data and Wilcoxon Rank Sum for nonparametric data. However, for comparison between more than 2 patient groups for parametric data, we used analysis of variance (ANOVA). Multiple comparisons (Post hoc test: LSD (least significant difference)) were also followed to investigate the possible statistical significance between each 2 groups. Moreover, comparison between more than 2 patients' groups for nonparametric data Kruskall Wallis test was used. Finally, Spearman's ranked correlation test, to study the possible association between each two variables among each group for nonparametric data, using the probability of error at 0.05 was considered significant, while at 0.01 and 0.001 were highly significant. Multiple linear regression analysis was done on all measured parameters to allow for adjustment of apelin level.

Results:

No significant differences with regards to age or gender distribution existed among groups. The clinical and demographic characteristics as well as studied parameters of all participants (control and HCV patients) are shown in Table 1. Significant differences in median values of apelin and HOMA-IR levels existed between control and HCV patients as shown in Table I.

Comparison between the two studied groups according to HOMA IR were presented in Table 2, it showed that, HOMA IR ranged between 0.94 – 22.50 and 0.35-2.98 with the mean of 3.82±3.60 and 1.67±0.69 for HCV patients and control groups respectively, HCV group have values statistically higher than control group with statistically significanct difference. (P=<0.001) as shown in Table II.

Comparison between the two studied groups according to S.apelin were presented in Table (3), it showed that, S.apelin ranged between 7.0-860.0 and 400.0-1000.0 with the mean of 167.69±221.70 and 867.0±158.95 for HCV patients and control groups respectively, Control group have values statistically higher than HCV patients group with statistically significant differences. (P=0.001) as shown in Table III.

The correlation between HOMA IR and S.apelin with different studied parameters in HCV patients group was shown in Table 4 demonstrated that there were negative significant correlation between S.apelin with S.insulin (P= 0.001), while there were positive significant correlation between HOMA IR with FBS, S.insulin and Hb (P=0.003, 0.001 and 0.022 respectively), as shown in Table IV .

Correlation between HOMA IR and S.apelin with different studied parameters in control group demonstrated that, there were negative significant correlation between S.apelin with BMI , FBS and Hb while there were positive significant correlation between HOMA IR with S.insulin (P < 0.001) as shown in Table V.

Determining the best predictors which affect apelin and HOMA IR measurements was also evaluated by multiple linear regression analyses. All possible factors, which were defined in correlation analyses, were evaluated together in Multiple Linear Regression models. HbA1c, S.insulin and HCV were found to be the best predictors affecting apelin levels, and FBS, S.insulin were found to be the best predictors affecting HOMA IR with a decreasing and increasing effect respectively, Tables (VI,VII) shows the all possible factors that may affect apelin levels and HOMA IR with the Multiple Linear Regression model.

Table (I): Comparison between the two studied groups according to different parameters

	HCV Patients (n = 60)	Control (n = 20)	p
Sex			
Male	60 (100%)	20 (100.0%)	-
Female	0 (0.0%)	0 (0.0%)	
Age	37.67 ± 11.53	38.90 ± 6.22	0.547
Weight	82.03 ± 13.19	86.30 ± 5.96	0.052
Height	171.30 ± 6.86	174.25 ± 3.01	0.010*
BMI	27.97 ± 4.52	28.41 ± 1.74	0.527
SGOT	72.95 ± 25.71	21.25 ± 6.13	<0.001*
SGPT	92.82 ± 21.22	25.15 ± 6.45	<0.001*
S. albumin	2.93 ± 0.25	3.83 ± 0.17	<0.001*
Total Bilirubin	2.08 ± 0.45	1.10 ± 0.12	<0.001*
Direct bilirubin	1.34 ± 0.42	0.75 ± 0.09	<0.001*
PT	14.38 ± 0.64	14.0 ± 0.0	<0.001*
Hb	11.10 ± 1.45	14.17 ± 1.02	<0.001*
Creatinine	0.93 ± 0.22	0.85 ± 0.11	0.023*
Cholesterol	192.98 ± 43.42	173.85 ± 37.90	0.083
Triglyceride	163.30 ± 44.35	146.25 ± 46.41	0.145
FBS	92.10 ± 9.06	91.30 ± 10.71	0.745
S.insulin	9.55 (2.30 – 51.40)	7.55 (1.50 – 12.80)	0.090
HbA1c	6.07 ± 0.23	4.87 ± 0.52	<0.001*
HOMA IR	2.78 (0.94 – 22.50)	1.72 (0.35 – 2.98)	<0.001*
S. apelin	90.0 (7.0 – 860.0)	925.0 (400.0 – 1000.0)	<0.001*

Normally quantitative data was expressed in (Mean. ± SD) and was compared using t-student test while for abnormally quantitative data expressed in Median (Min. – Max.) and was compared using Mann Whitney test.

*: Statistically significant at p ≤ 0.05

Table (II): Comparison between the two studied groups according to HOMA IR

	HCV Patients (n = 60)	Control (n = 20)	Z	p
HOMA IR				
Min. – Max.	0.94 – 22.50	0.35 – 2.98		
Mean ± SD.	3.82 ± 3.60	1.67 ± 0.69	3.711*	<0.001*
Median	2.78	1.72		

Z: Z for Mann Whitney test

*: Statistically significant at p ≤ 0.05

Table (III): Comparison between the two studied groups according to S.apelin

	HCV Patients (n = 60)	Control (n = 20)	Z	p
S. apelin				
Min. – Max.	7.0 – 860.0	400.0 – 1000.0		
Mean ± SD.	167.69 ± 221.70	867.0 ± 158.95	6.394*	<0.001*
Median	90.0	925.0		

Table (IV): Correlation between HOMA IR and S.apelin with different studied parameters in HCV patients group.

	S. apelin		HOMA IR	
	r_s	p	r_s	p
S. apelin	-	-	-0.333*	0.009
Age	-0.039	0.768	-0.156	0.234
Weight	0.100	0.446	0.569*	<0.001
Height	0.029	0.826	0.099	0.452
BMI	-0.192	0.141	0.393*	0.002
PCR	-0.148	0.258	0.134	0.309
FBS	0.026	0.843	0.410*	0.001
HbA1c	-0.236	0.070	0.020	0.878
S.insulin	-0.402*	0.001	0.925*	<0.001
SGOT	-0.112	0.392	0.091	0.490
SGPT	-0.004	0.973	0.025	0.847
S.albumin	-0.194	0.138	-0.054	0.684
T.Bilirubin	0.053	0.689	-0.054	0.680
D.bilirubin	0.035	0.791	-0.082	0.533
PT	0.112	0.393	0.003	0.981
Hb	-0.069	0.599	0.316*	0.014
Creatinine	-0.136	0.302	0.139	0.291
Cholesterol	-0.025	0.847	0.122	0.353
Triglyceride	0.136	0.300	0.189	0.148

r_s: Spearman coefficient *: Statistically significant at $p \leq 0.05$

Table (V): Correlation between HOMA IR and S.apelin with different studied parameters in control group

	S. apelin		HOMA IR	
	r_s	p	r_s	p
S. apelin	-	-	0.488*	0.029
Age	0.039	0.870	0.263	0.262
Weight	0.044	0.854	0.100	0.673
Height	-0.005	0.982	0.242	0.305
BMI	-0.127	0.593	0.006	0.980
FBS	-0.134	0.573	0.243	0.302
HbA1c	-0.285	0.223	-0.398	0.082
S.insulin	0.545*	0.013	0.915*	<0.001
SGOT	0.308	0.186	0.166	0.485
SGPT	-0.266	0.257	0.184	0.438
S.albumin	-0.076	0.749	-0.261	0.265
T.Bilirubin	-0.450*	0.047	-0.157	0.508
D.bilirubin	-0.106	0.657	-0.160	0.502
Hb	-0.079	0.742	0.153	0.520
Creatinine	-0.343	0.139	-0.201	0.396
Cholesterol	0.281	0.229	0.037	0.879
Triglyceride	0.405	0.076	0.138	0.561

r_s: Spearman coefficient *: Statistically significant at $p \leq 0.05$

Table (VI): Linear regression for factors affecting S. apelin

	B	SE	t	p	95% CI	
					LL	UL
HbA1c	-143.998	69.375	2.076*	0.041*	-282.170	-5.826
S.insulin	-5.674	2.358	2.406*	0.019*	-10.372	-0.977
HCV	-494.107	99.967	4.943*	<0.001*	-693.209	-295.005

Table (VII): Linear regression for factors affecting HOMA IR

	B	SE	t	p	95% CI	
					LL	UL
FBS	0.077	0.026	2.914*	0.005*	0.024	0.129
S.insulin	0.206	0.026	7.839*	<0.001*	0.154	0.259
Hb	0.296	0.185	1.604	0.113	-0.072	0.664
HCV	1.335	0.828	1.612	0.111	-0.314	2.985

Discussion:

Recent emerging studies pointed to the possible multiple effects of the apelinergic system in the liver and related it to oxidative stress, inflammation[18], fibrosis[2], angiogenesis[23], as well as haemodynamic and vascular disturbances [3,4]

Hepatic insulin signaling is markedly impaired in HCV patients; in addition, down regulation of hepatic insulin mediators is associated with enhanced hepatocyte apoptosis and fibrogenesis[24]. Currently some evidence supports a relationship between insulin resistance (IR) and hepatitis C on one hand, playing role in progression of liver disease [1-5], and between IR and apelin level on the other hand in cases of obesity[25].

This study aims to detect the relationships between apelin serum levels and insulin resistance in patient with HCV in comparison to patients without HCV. 80 patients were selected from the out patient clinic of Main University Hospital, Alex, Egypt. Subjects will be divided into 2 main groups: Group A: 60 patients with chronic hepatitis c virus (Child-Paugh A,B,C). Group B: 20 patients without HCV as a control group.

Hala, et al., (2011) showed that, apelin was evaluated in all groups both before and after adjustment for BMI, TAG, and TC that acted as potential covariants. Studies assessing apelin-36 and apelin-12 levels in patients with nonalcoholic fatty liver disease (NAFLD) indicated that the elevation of this peptide did not persist after adjustment for potential confounders and rather attributed apelin elevation in these cases to obesity and IR that are closely associated with NAFLD [10,26].

In contrast to NAFLD models, the present study shows that serum apelin level is changed in patients with either fibrosis or cirrhosis due to HCV, with significant differences among the two groups being lower in the latter, even after apelin adjustment. This is in line with previous investigations demonstrating that patients with HCV showed significant increase in apelin circulating levels [3,27].

Moreover, recent emerging studies speculated that activated hepatic stellate cells (HSCs) represent a potential source for apelin in liver [3] and that apelin could be an important mediator of the profibrogenic gene induction that markedly stimulates collagen-I synthesis [2]. In addition, apelin contributes to platelet-derived growth factor-induced proliferation of HSC's in vitro [4], all of which are known to contribute

largely to fibrosis progression and extracellular matrix deposition [28,29]. In this setting, we also found apelin serum level to be significantly elevated in patients with HCV. Thus, apelin emerges as a major contributor to the fibrogenic process(es) occurring in liver disease[2] as well as playing role in disease progression.

On the other hand, this elevation in case of ASC disappeared after apelin adjustment to cofounders, which points to the interference of these cofactors in elevating apelin levels and may result in the early upset of the system in CHC patients.

Moreover, the current study demonstrated that this peptide is closely associated to ascites and portal hypertension complications. Tiani and his coworkers suggested that the expression of endogenous apelin/APJ signaling is associated with development of portal hypertension and contributes to the formation of portosystemic collateral blood vessels and splanchnic neovascularization in portal hypertensive rats [23].

In the last few years, several data have accumulated suggesting that obesity also plays role in development and progression of liver disease of well-defined etiology [30]. Recently, expression of both apelin and APJ has been described in adipocytes [17] and is suggested to stimulate blood vessel growth, due to its proangiogenic activity, thus leading to increased growth of adipose tissue[31]. Coinciding with that line, our study also demonstrated that obese subjects with CHC had significant increased circulating apelin levels than lean patients, regardless of the stage or grade of liver disease.

On the other hand, studies also demonstrated that apelin expression was higher in animal models of obesity associated with hyperinsulinemia [17], in addition to its role in adipogenesis[31] and steatosis[18]; all of which contribute largely to fibrosis progression, as well as higher degree of inflammation[30,32]. Besides, virus C infection may induce IR by blocking intracellular signaling [33]. Further insight in our study revealed that IR was significantly higher among all groups of CHC in comparison to the control group. Moreover, significant difference was also found between ASC and cirrhotic groups, being higher in the latter. Moreover, when comparing apelin levels in IR and non-IR groups, it was significantly higher in IR group. Additionally, there was significant positive correlation between IR and adjusted-apelin in patients with CHC. This is in agreement with a previous study conducted by Aktas et al., who depicted that apelin, the novel adipokine, was associated with the components of the metabolic syndrome (hyperlipidemia, obesity, and IR) in NAFLD patients[34]. However, our correlation was quite significant in ASC but was unexpectedly nonsignificant in the case of progressive stages of fibrosis and cirrhosis, suggesting that the upset of the apelin system in these stages may follow a unique different pattern irrelevant to IR.

Moreover, previous investigations had pointed to s.insulin as an inductor of apelin synthesis in adipocytes[35]. Hence, we sought to investigate such correlation in our CHC population. TNF-α was mostly elevated in cases of ASC, this was in agreement with Zekri and his coworkers[36] but was opposite to what was reported by Goyal et al.[37] and Toyoda et al.[38]. This could be attributed to the difference in the epitopes of the ELISA system used by the different investigating groups or to the difference in genotypes where all our patients were HCV genotype 4. Moreover, the striking elevation of this proinflammatory cytokine in carriers may reflect both insufficiencies of HCV elimination and/or a failure to control the cytokine cascade [36].

These findings point to the possible role of apelin in CLD progression. Moreover, this provides a rationale to investigate new drugs targeting the apelin/APJ signaling pathway to reduce fibrosis and to improve hemodynamics in those patients.

The strong correlation of apelin- with LDL and HDL-cholesterol is an interesting finding. Although apelin is secreted also from adipose tissue, this is not sufficient to explain the relationship. Malyszko et al.[39] found negative correlation between apelin and total cholesterol, LDL-cholesterol and triglyceride levels in HD patients. This difference from our study may be related with metabolic abnormalities like hyperglycemia, dyslipidemia and obesity that are more common in PD patients due to the glucose content of PD solutions. Moreover, Tasci et al.[40] found in their studies that apelin levels were lower in patients with high LDL-cholesterol in non-uremic population; and lowering LDL-cholesterol with life style changes and/or statins resulted in an increase in apelin levels. These different results between uremic and nonuremic population may be regarded as a clue for different lipid profile of uremic patients.

Conclusion:

From our results we can conclude the following:

HOMA IR in HCV patients group has values statistically higher than control group with statistically significanct difference, S.apelin level in control group has values statistically higher than HCV patients group with statistically significant differences, there were negative significant correlation between S.apelin with S.insulin, while there were positive significant correlation between HOMA IR with FBS, S.insulin and Hb and finaly Multiple Linear Regression models depicated that HbA1c, S.insulin and HCV were found to be the best predictors affecting apelin levels, and FBS, S.insulin were found to be the best predictors affecting HOMA IR with a decreasing and increasing effect respectively.

Conflict of Interests:

The authors declare that there is no Conflict of Interest.

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Original Article

Predictors of Hepatic Fibrosis in Chronic Hepatitis C Virus Patients in Comparison to Liver Biopsy.

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Abstract:

Background/Aim: Liver fibrosis is the main predictor of the progression of chronic hepatitis C, and its assessment by liver biopsy can help the plan of therapy. However, biopsy is an invasive procedure with occasional complications and poor patient acceptance. The aim of this work was to compare non - invasive and invasive methods for evaluation of fibrosis in patients with chronic hepatitis C. **Methods:** This cross-sectional study was carried out at the Liver Unit of Mansoura University Hospital and Mansoura Health Insurance Hospital. The study was carried out on 100 patients with chronic active hepatitis, biochemical and virological studies were performed in addition to abdominal ultrasonography and liver biopsy in all patients, and also Serum fibronectin, APRI and AST/ALT ratio were performed. **Results:** We found that fibronectin has the highest sensitivity and specificity , the independent variables related to fibrosis were fibronectin, APRI, and AST/ALT ratio. **Conclusion:** The biochemical tests including APRI, AST/ALT ratio and particularly fibronectin could be valuable non – invasive predictor for assessment of liver fibrosis in chronic hepatitis C infected patients.

Keywords: H.C.V, fibronectin, Liver biopsy.

Introduction:

Hepatitis C Virus (HCV) infection is a major health problem in Egypt, where the seroprevalence is (10–20) fold higher than that in the USA, Egypt has the highest prevalence of HCV worldwide, ranging from 6% to more than 40% across regions and demographic groups. At present, liver biopsy is considered the 'gold standard' to evaluate the grade of liver fibrosis.(1) Liver fibrosis is the main predictor of the progression of chronic hepatitis C, and its assessment by liver biopsy (LB) can help the plane of therapy.(2) However, biopsy is an invasive procedure with occasional complications and poor patient acceptance.(3) Diagnosis of HCV infection is based on the presence of both anti- HCV antibodies, detected by enzyme immunoassays, and HCV RNA, detected by molecular assays. HCV RNA testing is essential for the management of HCV therapy.(4) Advanced liver fibrosis results in cirrhosis that can in turn lead to liver failure, portal hypertension and hepatocellular carcinoma. Fibrosis develops with different spatial patterns and is a consequence of various prevalent mechanisms according to the diverse causes of parenchymal damage. Early detection of fibrosis would allow for initiation of anti-fibrotic therapies capable of halting and even reversing this process.(5)

Pain is the most common complication of percutaneous liver biopsy, occurring in up to 84% of patients, including those with relatively mild discomfort.(6) The most important complication of liver biopsy is bleeding, which when severe occurs intraperitoneally.(7) A number of other complications have been reported after liver biopsy, these include pneumothorax, haemothorax, perforation of any of several viscous organs, bile peritonitis, infection, haemobilia and neuralgia.(8) Bellest et al., 2007 found that there is some contraindications in using biopsy; Absolute contraindications include: Severe coagulopathy, infection of the hepatic bed, extrahepatic biliary obstruction and Relative contraindications: Ascites, morbid obesity, possible vascular lesions, amyloidosis, hydatid disease, of similar importance to adequate specimen size is the necessity that a pathologist experienced in liver disease interpret the biopsy, ideally in partnership with the clinician

who performed the biopsy and /or whom is caring for the patient. In the absence of this interaction, diagnostic errors by non-specialist pathologists have been reported in more than 25% of patients evaluated at an academic centre.[9] Complex scoring systems, such as the Knodell scoring system and its revised form, the Ishak scoring system, have been devised for grading and staging of chronic viral hepatitis, as shown in the following table;[10]

IASL	Metavir	Batts-Ludwig
Grade(Activity, Inflammation) Minimal chronic hepatitis	A1	Grade1
Mild chronic hepatitis	A1	Grade2
Moderate chronic hepatitis	A2	Grade3
Severe chronic hepatitis	A3	Grade4
Stage (Fibrosis) Mild—Portal fibrosis	F1	Stage 1
Moderate—Periportal fibrosis or portal-portal septa	F1	Stage 2
Severe—Bridging fibrosis (few)	F2	Stage 3
Severe—Bridging fibrosis (many)	F3	Stage 3
Cirrhosis	F4	Stage 4

Several non-invasive tests have become available for clinicians to assess liver fibrosis and determine the best course of management for their patients, especially those with chronic hepatitis C.[11] Serum markers of liver fibrosis offer an attractive, cost effective alternative to liver biopsy for both patients and clinicians. In addition to being substantially less invasive, there are practically no complications, little or no sampling errors and small observer related variability. Moreover, measurements may be performed repeatedly, thus, allowing for a dynamic monitoring of fibrosis.[12] Fibronectin (FN) is a glycoprotein, produced from hepatocytes, Kupffer cells and endothelial cells ,Circulating fibronectin represents a viable marker for the presence of significant and advanced liver fibrosis in chronic hepatitis C patients (CHC) and fibronectin was identified at 90 kDa and quantified in sera of individuals with CHC using ELISA,[13] Fibronectin is one of the molecules produced by hepatic stellate cells, It is also part of the extracellular matrix, so it is important for the assembly of a collagen matrix in vitro , Its continuous presence also supports matrix integrity, both in vitro and in vivo ,It further regulates cell proliferation and cell cycle progression.[14] Fibronectin has been related to liver fibrosis and subsequent , development of portal hypertension in chronic liver disease.[15] Interpretation of serum aminotransferase levels, coagulation parameters, and platelet counts have been used in clinical practice to determine whether cirrhosis compensated or decompensated. Several studies have also evaluated the accuracy of combinations (or ratios) of these measures.[16]

Patients and methods

This study was carried out at the Liver Unit of Mansoura University Hospital and Mansoura Health Insurance Hospital. In the period between April 2012 and May 2014. The study was carried out on 100 Adult patients with chronic active hepatitis, of them 53 were males (53%) and 47were females (47%), informed written consent to participation into the study was obtained from each patient, inclusion criteria: age from 18 to 60 years, positive anti- HCV and HCV- RNA, patients with liver biopsy proven chronic hepatitis., HBs Ag negative and exclusion criteria: Age <18 and >60 years, co-infection with HBV, active alcohol consumption or features of alcoholic disease in the liver biopsy, HBs Ag

positive, preexisting psychiatric condition, pregnancy or breast feeding and co-morbidities.

All patients have been subjected to thorough history taking and complete clinical examination. Laboratory investigations included:- CBC (WBCs, hemoglobin & platelets). Bleeding time (BT), Alanine amino transferase (ALT) (U/L), Aspartate amino transferase (AST) (U/L), Serum bilirubin (mg/dl), Serum albumin (g /dl), Alkaline phosphatase (U/L), International normalized ratio (INR).

Viral markers were done including; HCV- antibody, HCV- RNA (IU/ m1) and Hepatitis B surface antigen, AST/ALT ratio (AAR), APRI (AST-to-Platelet ratio Index), ANA titre, SMA , AMA and LKM., TSH level, α feto protein (ng/dL), pregnancy test for females in child bearing period, Serum fibronectin (ng/ml).

Samples and standards were added and incubated in plates at 37 º C for 90 min. no wash ,Biotinylated antibodies were added and incubated in plates at 37 º C for 60 min. and washing 3 times with 0.01M TBS,ABC working solution was added and incubated in plates at 37 º C for 30 min. with washing 5 times with 0.01M TBS,TMB color developing agent were added and incubated in plates at 37 º C in dark for 20-25 min ,TMB stop solution were added and readed (BOSTER BIOLOGICAL TECHNOLOGY Co) and abdominal ultrasound.

Liver biopsy by highly qualified specialist in a well-equipped place under complete aseptic conditions, examined by a pathologist unaware of the laboratory results, needle liver biopsy specimens (n = 100) were taken from all patients. Biopsies were processed for diagnostic purposes, fixed in 10% neutral, buffered formalin, embedded in paraffin, cut into 4 µm thick, routinely stained with haematoxyline and eosin, the biopsies were pathologically classified according to Metavir staging system into different stages of fibrosis and cirrhosis from (F0-F4)

Statistical analysis:

Descriptive statistics were calculated for the anthropometric measurements and laboratory data in the form of: Mean ±Standard deviation (SD), Median and IQR, Frequency (No-%), using one of the following tests: - Student's *t*-test ANOVA Mann-Whitney *U*- test. The sensitivity and specificity of Fibronectin, APRI, AST/ALT ratio and serum albumin to diagnose fibrosis were examined at different cutoff points using ROC curve analysis to determine the best cut off point as well as the diagnostic power of each test. A *P* value <0.05 was considered statistically significant (S). And a *P* value <0.0001 was considered highly significant (HS) in all analyses.

Results:

Table (I) stages of fibrosis in studied cases according to Metavir:

Metavir	NO (n=100)	%
F0	12	12
F1	25	25
F2	28	28
F3	29	29
F4	6	6

Table (II) Serum albumin in different stages of fibrosis:

	Mean	SD	Median	IQR	P [a]
F0 (n=12)	4.29	0.26	4.40	4.03-4.50	**0.85**
F1 (n=25)	4.34	0.45	4.40	3.95-4.60	
F2 (n=28)	4.26	0.53	4.30	3.90-4.60	
F3 (n=29)	4.24	0.52	4.20	3.80-4.60	
F4 (n=6)	4.15	0.26	4.10	3.90-4.43	

***SD**: Standard deviation **IQR** (interquatile range)*

***P**: Probability a: ANOVA **p** value**<0.05** is significant.*

There is no significant difference in serum albumin level in the different stages of liver fibrosis.

Table (III) Serum Bilirubin in different stages of fibrosis:

	Mean	SD	Median	IQR	P value[a]
F0 (n=12)	0.96	0.34	0.85	0.63-1.28	<0.001
F1 (n=25)	1.01	0.44	0.90	0.70-1.20	
F2 (n=28)	1.14	0.29	1.20	0.88-1.38	
F3 (n=29)	1.27	0.39	1.30	0.90-1.60	
F4 (n=6)	1.88	0.26	1.90	1.65-2.13	

Serum bilirubin level is significantly increased with the progress of the degree of hepatic fibrosis.

Table (IV) Platelets counts in different stages of fibrosis:

	Mean	SD	Median	IQR	P value
F0 (n=12)	2.21E+11	3.11E+10	2.22E+11	1.92E+11-2.50E+11	0.01
F1 (n=25)	2.11E+11	4.88E+10	2.10E+11	1.77E+11-2.31E+11	
F2 (n=28)	2.09E+11	7.04E+10	1.84E+11	1.62E+11-2.40E+11	
F3 (n=29)	1.91E+11	5.57E+10	1.78E+11	1.53E+11-2.32E+11	
F4 (n=6)	1.54E+11	2.14E+10	1.57E+11	1.41E+11-1.68E+11	

E+10 *means* 10^{10}, **E+11** means 10^{11}

The increase in the degree of fibrosis is significantiy associated with decrease in platelet count.

Table (V) AST level in different stages of fibrosis:

	Mean	SD	Median	IQR	P
F0 (n=12)	19.75	15.06	15.50	6.25-35.00	<0.001[a]
F1 (n=25)	19.68	18.22	10.00	9.00-27.50	
F2 (n=28)	25.89	13.92	23.50	12.00-36.00	
F3 (n=29)	34.03	17.41	33.00	21.50-40.00	
F4 (n=6)	50.67	25.34	42.00	34.00-66.00	

AST is increased significantly with the increase in the stages of fibrosis.

Table (VI) ALT level in different stages of fibrosis:

	Mean	SD	Median	IQR	P value
F0 (n=12)	20.33	15.75	19.00	6.00-36.00	0.9
F1 (n=25)	21.19	19.95	12.00	6.00-38.00	
F2 (n=28)	20.82	16.96	14.00	7.50-36.00	
F3 (n=29)	21.31	16.92	15.00	8.50-36.00	
F4 (n=6)	25.00	24.45	14.50	4.75-56.00	

There are no significant changes in ALT level with the progress of the degree of liver fibrosis.

Table (VII) AST/ALT ratio in different stages of fibrosis:

	Mean	SD	Median	IQR	P value
F0 (n=12)	1.04	0.33	1.04	0.92-1.29	0.003
F1 (n=25)	1.13	0.62	0.96	0.76-1.18	
F2 (n=28)	1.89	1.70	1.12	0.85-2.59	
F3 (n=29)	2.65	3.13	1.65	0.97-3.42	
F4 (n=6)	4.07	3.18	2.91	1.54-7.59	

AST/ALT ratio is significantly increased with the increase in degree of fibrosis from (F0 – F4).

Table (VIII) APRI in different stages of fibrosis:

	Mean	SD	Median	IQR	P[a]
F0 (n=12)	0.14	0.13	0.09	0.05-0.19	<0.001
F1 (n=25)	0.20	0.16	0.15	0.10-0.24	
F2 (n=28)	0.30	0.17	0.26	0.15-0.40	
F3 (n=29)	0.36	0.22	0.30	0.16-0.54	
F4 (n=6)	0.62	0.55	0.40	0.28-1.00	

The progress of the stages of fibrosis from (F0 - F4) is associated with significant increase in APRI.

Table (IX) Serum fibronectin in different stages of fibrosis:

	Mean	SD	Median	IQR	P[a]
F0 (n=12)	18.83	14.33	15.00	8.25-31.50	**<0.001**
F1 (n=25)	36.64	25.77	26.00	15.00-53.50	
F2 (n=28)	58.46	37.38	52.50	22.50-86.75	
F3 (n=29)	93.00	83.51	70.00	25.00-115.00	
F4 (n=6)	152.50	94.11	135.00	71.25-232.50	

There is significant increase in serum level of fibronectin with the increase in stages of liver fibrosis from F0 to F4.

Table (X) sensitivity and specificity of different laboratory parameters in detection of fibrosis:

	Fibronectin	APRI	AST/ALT ratio	Serum Albumin
Area under the curve (AUC)	83.3 %	79.6%	61.7%	51.1%
Cutoff value	16	0.13	1.087	<4.25
Sensitivity	80.7	80.7	55.7	47.7
Specificity	77	77	42	58.3
PPV	94.6	94.6	90.7	89.4
NPV	32	32	15.2	13.2

PPV: Positive predictive value.
NPV: negative predictive value.

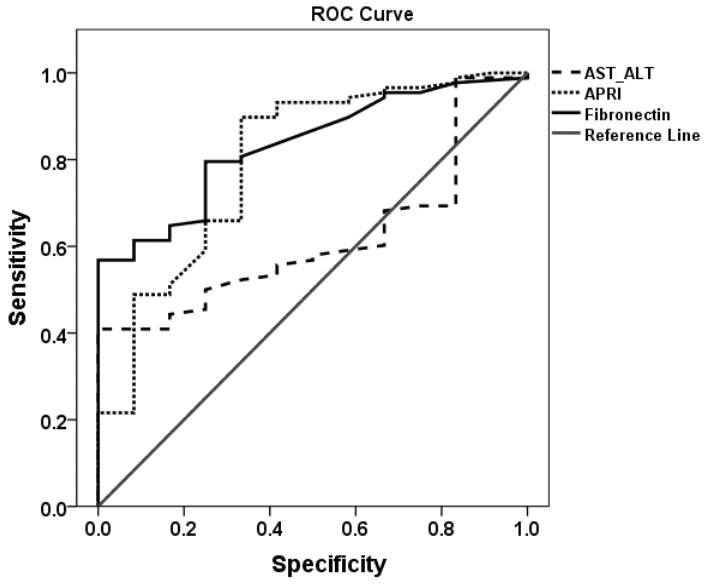

Figure (1): ROC Curve for Sensitivity & Specificity

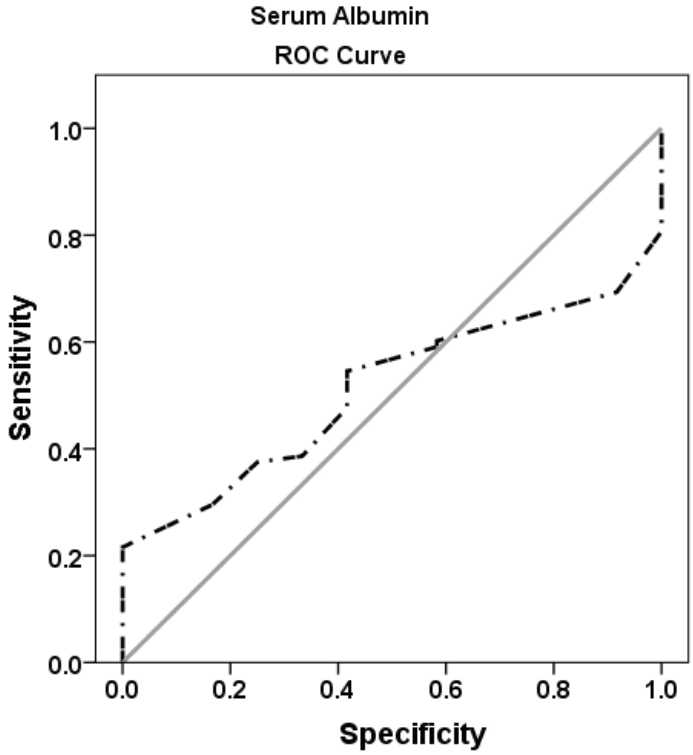

Figure (2): ROC Curve for Sensitivity & Albumin

As shown in **table X** and **figure (1**and **2)** above**,**

- ROC (receiver-operating characteristic) Curves of biomarkers for discriminating CHC patients **(F0)** from those with **(F1 - F4).**
- Using cutoff value (16, 0.13 ,1.087 and **<**4.25) the area under the curve (AUC) were 83.3, 79.6, 61.7 and 51.1 for fibronectin, APRI, AST/ALT ratio and albumin respectively.
- Increase sensitivity and specificity of fibronectin and APRI (80.7%and 77%) compared with AST/ALT ratio and albumin (55.7, 47.7, 42 and 58.3) respectively.
- PPV of fibronectin and APRI (94.6%) >AAR (90.7%) > albumin (89.4%).
- NPV of fibronectin and APRI (32%)> AAR (15.2%) > albumin (13.2%).

Discussion:

The gold standard for detecting liver fibrosis remains percutaneous liver biopsy, However, the procedure is limited by its invasive nature, expense, morbidity, intra- and inter-observer variability, and sampling errors,[17] Fibrosis prediction is an essential part of the assessment and management of patients with chronic liver disease. Blood-based biomarkers offer a number of advantages over the traditional standard of fibrosis assessment of liver biopsy, including safety, cost-savings and wide spread accessibility.[18] According to Metavir scoring system AST was increased (P value <0.001[a]) as shown in table (5). These results were in agreement with Ahmed et al,[19] who reported that AST was significantly increased with the progress of fibrosis stages. According to the progress in stages of fibrosis platelets count were decreased (P value =0.01[a]) as shown in table (4). Snyder et al. reported that low platelet count is caused by a variety of diseases as HCV.[20] In the current study the progress in the degree of fibrosis is associated with the increase in AAR (AST/ALT ratio) as

shown in table (7). Yu Hsieh et al. reported that the AAR scores increased significantly as the fibrosis advanced.[21] The level of the APRI was increased significantly with the progression of liver fibrosis stages as shown in table (8) ($P<0.001^a$).

Similar results were obtained by Ahmed et al. who reported that APRI was significantly increased with progression of fibrosis stages.[19] Similar results were obtained by Yilmaz et al., who reported that APRI was significantly associated with fibrosis scores in patients with CHC.[5]

In the current study Serum level of fibronectin was increased with the progress of the degree of fibrosis as shown in table (9) ($P<0.001^a$), similar results were obtained by Mosa et al.[15] In the current study the level of serum bilirubin was significantly increased within the progress of fibrosis stages ($P < 0.001$) as shown in table (3) ,This result was in agreement with Ahmed et al,[19] who reported that serum bilirubin was significantly increased with the progress in stages of fibrosis.

According to the stages of fibrosis (Metavir scoring system) the level of serum albumin was not significantly affected with the change in degrees of fibrosis ($P = 0.85$) as shown in table (2). Using ROC (receiver operating characteristic) curves to assess and compare the diagnostic accuracy of blood markers as fibronectin, APRI, AST/ALT ratio and serum albumin in patients with liver fibrosis ,In the current study as shown in table (10), and figure (1, 2), ROC curves of biomarkers for discriminating CHC patients with no liver fibrosis (F0) from those with liver fibrosis (F1 –F4). Using cutoff value (16, 0.13, 1.087 and <4.25) and the area under the curve (AUC) were 83.3, 79.6 , 61.7 and 51.1 for fibronectin, APRI, AST/ALT ratio and albumin respectively ,Sensitivity of fibronectin, APRI, AAR and albumin were(80.7%, 80.7%, 55.7% and 47.7%) respectively, Specificity of fibronectin, APRI, AAR and albumin were (77, 77, 42, and 58.3) respectively, PPV was (94.6, 94.6, 90.7 and 89.4) respectively for serum fibronectin, APRI, AAR and albumin, NPV was (32, 32, 15.2 and 13.2) for fibronectin, APRI, AAR and serum albumin respectively.

Conclusion:

Fibronectin has a better accuracy than APRI, AST/ALT ratio and albumin in prediction ability for fibrosis.

Recommendations:

In HCV patients because of its safety and cost effective. So, further studies aimed at reducing the need for liver biopsy.

Acknowledgment: Much gratitude is paid to Dr. Ibrahim Mohammed El Shawaf, professor of pathology in Faculty of Medicine, Mansoura University for his efforts.

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