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Editorial & Advisory Board

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Provide a title. Include first name, middle initial, last name, and affiliation of each author. Indicate the name, address, and e-mail address of the author to whom correspondence and reprint requests should be addressed. Provide also a short running title (70 characters and spaces) including first author's name and initials, et al (e.g., SH Asaad-Khalil, et al; Autoantibodies and sibs of children with type 1 diabetes mellitus)

Abstract

Limit the abstract to 250 words. Use a structured format, including Aim, Subjects and Methods, Results, and Conclusions. Provide 3-6 key words for indexing at the end of the abstract Provide a list of abbreviations used throughout the manuscript, arranged alphabetically. at the bottom of the first page.

Text

Articles should be written in clear, concise English according to the Concise Oxford Dictionary Minimize use of abbreviations; any abbreviations used must be defined at first mention (except for units of measurement when used with numbers).Abbreviations may be used in tables and figures for space considerations but must be defined in the accompanying footnotes or legends. The AMA Manual of Style lists standard scientific abbreviations. In general, use generic names for drugs. To maintain anonymity, do not use patient names, initials, or any unnecessary identifying details (Individual cases should be labeled as "case 1," "case 2," and so forth.) The text should be structured as follows:

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Journal

1. Van den Berghe G, Wouters P, Weekers F, et al. Intensive insulin therapy in critically ill patients. N Engl J Med 2001; 345:1359-1367.

Book

2. Falk SA ed. Thyroid Disease: Endocrinology. Surgery Nuclear Medicine, and Radiotherapy 2nd, ed. Philadelphia: Lippincott-Raven, 1997,

Chapter in Book

3- Flier JS, Foster DW. Eating disorders: obesity, anorexia nervosa, and bulimia nervosa. In: WilsonJD, Foster DW, Kronerberg HM, Larsen PR, eds Williams Textbook of Endocrinology 9th ed.

Philadelphia: WB Saunders, 1998: 1001-1097.

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Conflict of Interest and Acknowledgments

Authors are required to disclose any potential conflict of interest Acknowledgments should list brief statements of assistance, financial support, and prior publication of the study in abstract form, if applicable.

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Contents

Original Articles

Letter from The Editor

Dear Colleague,

In this volume, the COVID-19 pandemic has thrown its shadow on the publication. We hope all the best for you and your families; keep safe.
Once again, we hope to meet your expectations, and until we meet in our next issue, deepest regards and best wishes.

The Editor

Prof. Samir Helmy Assaad Khalil

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Cover of the Journal: The gods and goddesses of the ancient Egyptian civilization including: Isis (goddess of prosperity and female fertility), Min (god of male fertility), ect… Designed by: S.H. Assaad Khalil

Original Article

Circulatory Level of Serum Osteoprotegerin (OPG) in Patients with Type 2 Diabetes Mellitus (T2DM) and Its Association with Peripheral Arterial Disease (PAD)

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Abstract

Background: Peripheral arterial disease (PAD) is a very common problem in diabetic population. It constitutes a major economic and social burden. Early diagnosis and prevention is very important to reduce this burden. Osteoprotegrin (OPG) is thought to be closely associated with the presence of PAD and its severity.

Objective: The aim of the current study was to test this hypothesis among Egyptian patients with T2DM.

Patients and Methods: The study included 180 subjects equally divided into 3 groups; Group I consisted of 60 type 2 diabetic patients with evidence of PAD, group II consisted of 60 type 2 diabetic patients without evidence of PAD, group III consisted of healthy adults who were age- and gender-matched to the studied patients. OPG in addition to lab investigations that included (lipid profile, HbA1c, HOMA IR) were compared in the three groups.

Results: The differences between all groups were significant as regards to HOMA IR level. The differences were also significant between the all three groups according to lipid profile except for triglycerides which were significant only when comparing three groups but not Group I and group II together. With respect to osteoprotegrin level, group I was significantly higher than group II which was significantly higher than group III. The mean level was 0.32 ± 0.23, 0.14 ± 0.05 and 0.05 ± 0.05 for group I, II and III respectively. ($p<0.001$) (Table 6) The results of group I as regards to Doppler were as follows; percentage of monophasic, biphasic and triphasic was 33.3%, 60.0% and 0.0% for right foot respectively while for left foot the percentage was 23.3%, 66.7% and 6.7% respectively.

Conclusion: OPG level is closely related to the presence of peripheral arterial disease and it may have the merit of being an early marker for the disease.OPG level may have a correlation with duration of diabetes, age and ankle brachial index (ABI).The relation between ABI and OPG level may reflect a role of the later as a marker of not only the presence but also the severity of PAD.

Keywords: Serum Osteoprotegerin (OPG), T2DM, PAD.

Introduction

Over the last years, our knowledge on the mechanisms involved in the pathogenesis of cardiovascular disease has been enriched by the discovery of new molecules emerging as novel risk factors. Osteoprotegerin (OPG) is a soluble glycoprotein, member of the tumor necrosis factor (TNF)-related superfamily, involved in bone resorption. It was first described as a key regulator of bone homeostasis and vascular calcification in mice.[(1)] Clinical studies have suggested that serum OPG is associated with vascular calcification in humans.[(2)] The role of OPG in the development of macroangiopathy in diabetes is not yet clear.[(3)] It is possible that the increased OPG levels in diabetes reflect a compensatory response to arterial injury and that it is not involved in the pathogenesis of atherosclerosis.[(4)] Whether harmful or not, determination of serum OPG levels has been suggested as a prognostic biomarker of cardiovascular disease. In addition, increased OPG levels have been reported in diabetic patients with microvascular complications.[(3)] The potential of

OPG administration for therapeutic reasons is challenging for future investigators.

The mechanism underlying the effect of OPG on the development of vascular disease in diabetes is subject to controversies. TNF, a proinflammatory cytokine, is closely related to the development of vascular calcification and atherosclerotic plaque. TNF-a, which has been found to be increased in the plasma of patients with both type 1[(5)] or type 2 diabetes, induces OPG production from the arterial wall of diabetic patients.[(6)] Peroxisome proliferator –activated receptor (PPAR), found predominantly in adipose tissue, is an important nuclear transcription factor with anti-inflammatory and anti-atherogenic effects.[(7)] Several reports have demonstrated that PPAR ligands inhibit proliferation and migration of VSMCs and activation of macrophages, both functions involved in the progression of atherosclerosis. [(8)] PPAR has recently been shown to down regulate OPG expression in human aortic smooth muscle cells. CRP level has intensively been studied as a marker of atherosclerosis, especially in diabetic patients. The positive association between OPG, CRP and CAD has been reported in many previous studies. Whether elevated OPG levels represent simply a new marker of inflammation or part of the atherosclerotic process is unclear. [(9)]

The aim of this study was to test the association of OPG with the presence and/or severity of peripheral arterial disease in Egyptian patients with type 2 diabetes.

Patients and Methods

The study included 180 subjects classified as: **Group I:** 60 type 2 diabetic patients with peripheral arterial disease recruited from the Diabetes Clinic in Alexandria Main University Hospital. **Group II:** 60 type 2 diabetic patients without peripheral arterial disease recruited from the Diabetes Clinic in Alexandria Main University Hospital. **Group III:** 60 non- diabetic healthy control group with matched age and sex with group **I** and group **II**. All participants gave a written informed consent after explaining the nature and the aim of the study. The healthy control group included those without any chronic cardiovascular or metabolic disease and not receiving any long-term medication for both conditions.

All patients with the following criteria were excluded from the study; patients who had arteriovenous grafts/shunts, with recent vasculitis, patients known to have chronic kidney disease, previous cerebral infarction, coronary artery disease, malignancies, osteoporosis or those receiving systemic glucocorticoids or immune-suppressants.

All participants were subjected to full history taking, complete physical examination including the average of the right and left brachial artery pressures. PAD is defined as an ABI of <0.9 in at least one leg. In addition, full routine Laboratory investigations Blood drawn for metabolic, biochemical and hematological parameters after a 10-12 hours overnight fasting and estimate the following:[(10-13)]Fasting serum glucose, Homeostasis Model Assessment 2 (HOMA2) calculator was used to estimate steady state beta cell function (%B) and insulin resistance (%S) (HOMA-IR) according to the updated computer based HOMA2 mode in subjects with normal or impaired glucose tolerance, Glycated haemoglobin (HbA1C), Serum uric acid, Serum triglycerides, Total serum cholesterol, Low density lipoprotein cholesterol (LDL- Cholesterol), High density lipoprotein cholesterol (HDL-Cholesterol), Serum Osteoprotegerin level by using ELISA. In addition to serum Osteoprotegerin level by using ELISA.

Statistical analysis of the data

Data were fed to the computer and analyzed using IBM SPSS software package version 20.0**.** **(**Armonk, NY: IBM Corp**)** [(14)] Qualitative data were described using number and percent. Quantitative data were described using range (minimum and maximum), mean, standard deviation and median. Significance of the obtained results was judged at the 5% level.

The used tests were: Chi-square test: For categorical variables, to compare between different groups, Fisher's Exact or Monte Carlo correction: Correction for chi-square when more than 20% of the cells have expected count less than 5 , F-test (ANOVA): For normally distributed quantitative variables, to compare between more than two groups, and Post Hoc test (Tukey) for pairwise comparisons , Mann Whitney test: For abnormally distributed quantitative variables, to compare between two studied groups , Kruskal Wallis test: For abnormally distributed quantitative variables, to compare between more than two studied groups, and Post Hoc (Dunn's multiple comparisons test) for pairwise comparisons and Regression: To detect the most independent/ affecting factor for

interpretation.

Results

The studied population included 46.7% males (n=84) and 53.3% females (n=96). The largest age category was people aged from 50 to 60 years which represented 38.9 The mean age for the whole study population was 52.28 ± 9.30.(TableI)

When comparing the three studied groups according to gender distribution, the percentage of males was 50.0%, 43.3% and 46.7% for group I, II, and III respectively.(p=0.764) with no significant statistical differences between the studied groups for gender distribution. The mean age for group I (59.37 ± 5.99) was significantly higher than group II (49.37 ± 7.41) and group III (48.10 ± 9.71) (p<0.001).

The percentage of hypertensive patients was 76.7% and 43.3% for group I and II respectively while the percentage of smokers was 33.3% and 16.7% respectively the p value was <0.001 for both and the differences were statistically significant. Nevertheless, no patient in group III was smoker or hypertensive.

In group I, 56.7% were on insulin alone and 43.3% were on oral medications alone while in group II 13.3% were on insulin and 83.3% were on oral medications. It is worth mentioning that no patients in group I was on both insulin and oral medication while in group II the percentage was 3.3%, patients in group I were more likely to be on insulin while patients in group II were more likely to be on oral medications with p value <0.001. With respect to statins, 56.7% were on this medication in group I while in group II the percentage was 50.0%, p=0.646 The mean duration of illness was 13.80 ± 4.94 and 6.90 ± 3.74 for group I and group II respectively (p<0.001), so duration of illness was significantly higher in group I.

There were significant statistical differences between the three studied groups as regards to BMI, Waist circumference, systolic and diastolic pressure. The mean BMI was 27.17 ± 4.65, 30.57 ± 4.15 and 23.23 ± 0.89 for group I, II and II respectively while the waist circumference was 96.07 ± 7.32, 98.73 ± 5.35 and 90.93 ± 7.24 respectively. The mean systolic BP was 138.0 ± 13.63, 126.7 ± 14.57 and 115.0 ± 5.67 for group I, II and II respectively while the diastolic BP was 89.67 ± 9.56, 81.0 ± 8.38 and 74.83 ± 5.29 respectively. (p<0.001) for all parameters.

While group I was significantly lower as regards to the monofilament score (6.65 ± 1.27) than group II (7.80 ± 1.34) and group III (9.0 ± 0.0), also it was significantly lower as regards to VPT score (22.08 ± 4.01) than group II(17.77 ± 4.) and group III(8410.45 ± 0.91). (Table II) There were also significant statistical differences between the two groups as regards to right, left and average ABI. The mean ABI was 0.63 ± 0.21, 1.12 ± 0.07 and 1.03 ± 0.03 for group I, II and III respectively. (Table III)

There were no significant statistical differences between group I and II with respect to FBG, HbA1c and Uric acid, however, when comparing the three groups together the differences were statistically significant. The differences between all groups were significant as regards to HOMA level. The differences were also significant between the all three groups according to lipid profile except for triglycerides which were significant only when comparing three groups but not Group I and group II together. The mean values are mentioned in details in tables IV and V.

With respect to osteoprotegrin level, group I was significantly higher than group II which was significantly higher than group III. The mean level was 0.32 ± 0.23, 0.14 ± 0.05 and 0.05 ± 0.05 for group I, II and III respectively. (p<0.001)(Table VI) The results of group I as regards to Doppler were as follows; percentage of monophasic, biphasic and triphasic was 33.3%, 60.0% and 0.0% for right foot respectively while for left foot the percentage was 23.3%, 66.7% and 6.7% respectively.

Nine parameters were significantly related to osteoprotegrin when univariate analysis was used namely, age, duration of illness, BMI, blood pressure, monofilament score, VPT score, average ABI, uric acid and LDL. However, only three parameter were significant when multivariate analysis was used which were age, duration of illness and average ABI. (Table VII)

ROC curve analysis of OPG to predict Diabetes with PAD showed that the area under the curve was 89% with 95% confidence intervals (0.835–0.945) the cutoff point was more than 0.1 IU/ml, the sensitivity was 100%, the specificity was 63.33%, the positive predictive value was 73.2%, and the negative predictive value was 100. (Figure 1)

Table (I): Distribution of the studied cases according to demographic data (n = 180)

	n	%
Sex		
Male	84	46.7
Female	96	53.3
Age (years)		
<40	14	7.8
40 – <50	48	26.7
50 – <60	70	38.9
≥60	48	26.7
Min. – Max.	35.0 – 72.0	
Mean ± SD.	52.28 ± 9.30	
Median	54.0	
HTN	72	40.0
Smoker	30	16.7

Table (II): Comparison between the three studied groups according to average of monofilament and VPT

Average	**Group I (n = 60)**	**Group II (n = 60)**	**Group III (n = 60)**	F	**P**
Monofilament					
Min. – Max.	4.0 – 9.0	5.0 – 9.0	9.0 – 9.0		
Mean ± SD.	6.65 ±1.27	7.80 ±1.34	9.0 ±0.0	72.867	<0.001*
Median	6.0	8.0	9.0		
Significance between groups	p1<0.001*,p2<0.001*,p3<0.001*				
VPT					
Min. □Ma□	□□.□□□□.□□	□□.□□□□.□	□.□□□□.□□		
Mean ± SD.	22.08 ±4.01	17.77 ±4.84	10.45 ±0.91	154.50*	<0.001*
Median	22.50	16.50	10.50		
Significance between groups	p_1<0.001*,p_2<0.001*,p_3<0.001*				

F: **F for ANOVA test**, Pairwise comparison bet. each 2 groups was done using **Post Hoc Test (Tukey)**

Group I: Diabetes with PAD Group II: Diabetes without PAD Group III: Healthy control

Table (III): Comparison between the three studied groups according to ankle brachial index (ABI)

ABI	Group I	Group II	Group III	H	P
Right	(n = 56)#	(n = 60)	(n = 60)	143.253*	<0.001*
Min. – Max.	0.20 – 0.84	1.01 – 1.30	1.01 – 1.12		
Mean ± SD.	0.61 ±0.22	1.11 ±0.07	1.02 ±0.03		
Median	0.73	1.12	1.01		
Significance between groups	p1<0.001*,p2<0.001*,p3<0.001*				
Left	(n = 58)#	(n = 60)	(n = 60)	147.874*	<0.001*
Min. – Max.	0.20 – 1.0	1.02 – 1.26	1.0 – 1.13		
Mean ± SD.	0.67 ±0.21	1.13 ±0.07	1.03 ±0.03		
Median	0.75	1.14	1.02		
Significance between groups	p1<0.001*,p2<0.001*,p3<0.001*				
Average				151.388*	<0.001*
Min. – Max.	0.20 – 0.86	1.02 – 1.28	1.01 – 1.13		
Mean ± SD.	0.63 ± 0.21	1.12 ± 0.07	1.03 ± 0.03		
Median	0.75	1.13	1.02		
Significance between groups	p1<0.001*,p2<0.001*,p3<0.001*				

H: H for **Kruskal Wallis test,** Pairwise comparison bet. each 2 groups was done using **Post Hoc Test (Dunn's for multiple comparisons test)**

Group I: Diabetes with PAD Group II: Diabetes without PAD Group III: Healthy control

Table (IV): Comparison between the three studied groups according to laboratory investigations

Laboratory investigations	Group I (n = 60)	Group II (n = 60)	Group III (n = 60)	Test of Sig.	p
FBS					
Min. – Max.	71.0 – 288.0	80.0 – 289.0	73.0 – 99.0		
Mean ± SD.	159.8 ±60.58	148.8 ±50.12	88.93 ±5.96	H= 99.189*	<0.001*
Median	145.5	141.0	89.0		
Significance between groups	p1=0.599,p2<0.001*,p3<0.001*				
HOMA2					
Min. – Max.	0.50 – 5.50	0.35 – 5.13	0.75 – 0.90		
Mean ± SD.	2.22 ±1.30	1.50 ±1.0	0.83 ±0.05	H= 77.762*	<0.001*
Median	1.93	1.20	0.80		
Significance between groups	p1=0.030*,p2<0.001*,p3<0.001*				
HbA1c					
Min. – Max.	6.10 – 7.90	6.30 – 8.40	1.90 – 5.80		
Mean ± SD.	11.28 ±12.87	11.10 ±13.79	5.03 ±0.66	H= 119.919*	<0.001*
Median	8.30	7.80	5.10		
Significance between groups	p1=0.519,p2<0.001*,p3<0.001				
Uric acid					
Min. – Max.	3.10 – 11.50	2.20 – 7.40	2.40 – 6.0		
Mean ± SD.	5.64 ±1.72	5.16 ±1.29	4.33 ±1.16	F= 13.273*	<0.001*
Median	5.75	5.45	4.35		
Significance between groups	p1=0.160,p2<0.001*,p3=0.004*				

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Table (V): Comparison between the three studied groups according to lipid profile

Lipid profile	Group I (n = 60)	Group II (n = 60)	Group III (n = 60)	Test of Sig.	p
Cholesterol					
Min. – Max.	81.0 – 286.0	126.0 – 250.0	109.0 – 198.0		
Mean ± SD.	172.6 ±50.64	192.3 ±32.85	171.1 ±20.99	F= 6.208*	0.002*
Median	181.5	200.0	175.0		
Significance between groups	p1=0.011,p2=0.973,p3=0.005*				
Triglycerides					
Min. – Max.	73.0 – 314.0	74.0 – 422.0	49.0 – 161.0		
Mean ± SD.	144.7 ±65.59	145.1 ±75.25	100.5 ±26.19	H= 18.802*	<0.001*
Median	130.5	111.5	98.50		
Significance between groups	p1=0.905,p2=0.973,p3=0.005*				
LDL					
Min. – Max.	24.0 – 174.0	50.0 – 184.0	56.0 – 132.0		
Mean ± SD.	97.73 ±40.10	120.1 ±30.81	106.6 ±20.37	H= 11.316*	0.003*
Median	104.5	124.0	108.5		
Significance between groups	p1=0.002*,p2=0.544,p3=0.010*				
HDL					
Min. – Max.	12.0 – 187.0	28.0 – 66.0	39.0 – 52.0		
Mean ± SD.	45.67 ±29.16	43.20 ±8.35	44.30 ±3.56	H= 4.990	0.082
Median	42.0	41.50	44.50		

Group I: Diabetes with PAD Group II: Diabetes without PAD Group III: Healthy control

Table (VI): Comparison between the three studied groups according to osteoprotegerin

	Group I (n = 60)	Group II (n = 60)	Group III (n = 60)	H	p
Osteoprotegerin **Min. – Max.** **Mean ± SD.** **Median**	 0.20 – 1.30 0.32 ±0.23 0.20	 0.10 – 0.20 0.14 ±0.05 0.10	 0.0 – 0.10 0.05 ±0.05 0.05	 131.693*	 <0.001*
Significance between groups	p1<0.001*,p2<0.001*,p3<0.001*				

H: H for **Kruskal Wallis test,** Pairwise comparison bet. each 2 groups was done using **Post Hoc Test (Dunn's for multiple comparisons test)**

p: p value for comparing between the three groups p1: p value for comparing between group I and group II p2: p value for comparing between group I and group III p3: p value for comparing between group II and group III

Group I: Diabetes with PAD Gro□□II□Diabe□es □i□ho□□PAD Gro□□III□Heal□hy □on□rol

Table (VII): Univariate and multivariate linear regression analysis for the parameters affecting osteoprotegerin for total cases (n = 120)

Osteoprotegerin	Univariate		#Multivariate	
	B(95%C.I)	p	B (95%C.I)	p
Age (years)	0.007*(0.003 – 0.011)	<0.001*	-0.005*(-0.009 - -0.001)	0.013*
Duration	0.021*(0.016 – 0.026)	<0.001*	0.017*(0.007 – 0.027)	0.001*
BMI (kg/m2)	-0.011*(-0.018 – -0.004)	0.002*	-0.001 (-0.008 – 0.005)	0.649
Waist circumference	-0.001 (-0.006 – 0.004)	0.724		
Systolic	0.003*(0.001 – 0.005)	0.004*	0.003 (-0.002 – 0.007)	0.233
Diastolic	0.005*(0.001 – 0.008)	0.008*	-0.004 (-0.011 – 0.002)	0.181
Monofilament	-0.054 (-0.076 - -0.032)	<0.001*	0.008 (-0.024 – 0.040)	0.617
VPT	0.015 (0.009 – 0.021)	<0.001*	-0.003 (-0.012 – 0.007)	0.591
ABI (Average)	-0.421 (-0.510 - -0.332)	<0.001*	-0.319*(-0.473 - -0.165)	<0.001*
FBS	0.000(0.00 – 0.001)	0.476		
HOMA2	0.002 (-0.027 – 0.030)	0.902		
HbA1c	0.00 (-0.002 – 0.003)	0.757		
Uric acid	0.034*(0.013 – 0.056)	0.002*	0.015 (-0.002 – 0.031)	0.080
Cholesterol	-0.001 (-0.002 – 0.00)	0.056		
Triglycerides	0.000 (0.00 – 0.001)	0.352		
LDL	-0.001*(-0.002 – 0.000)	0.015*	0.00 (0.00 – 0.0001)	0.405
HDL	0.000 (-0.002 – 0.001)	0.837		

Beta: Standardized Coefficients C.I: Confidence interval #: All variables with p<0.05 was included in the multivariate

*: Statistically significant at p ≤ 0.05 Both Group I& II Group I: Diabetes with PAD Group II: Diabetes without PAD

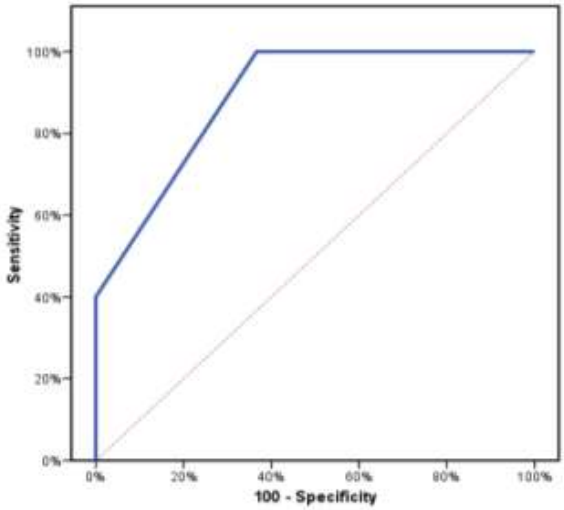

Figure (1): ROC curve for Osteoprotegerin to predict Diabetes with PAD cases vs without PAD (group I vs II)

Discussion

The main finding of the current study is that OPG is significantly higher in diabetic patients with PAD than in patients with DM without PAD.

The first evidence of an involvement of OPG in vascular calcification was derived from genetically altered laboratory animals, which in addition to osteoporosis displayed calcification of the large artery system, resembling vascular lesions of patients, suffering from atherosclerosis. [15, 16] The development of calcification of vessels was completely prevented by restoration of the OPG gene in these animals [15]. The hypothesis of a link between osteoporosis and vascular calcification was suggested also by the fact that in humans low bone mineral density often coincidences with vascular calcification. [17] Subcutaneous injection of OPG was shown to inhibit osteoclastic bone resorption in postmenopausal women. [18] Furthermore, in an animal model of artery calcification induced by Vitamin D intoxication, administration of OPG was able to prevent vascular lesions. [19] A recent clinical study on humans demonstrated elevated serum OPG levels to be an independent risk factor for progressive atherosclerosis and cardiovascular disease. [20] Schoppet et al.[21] and Jono et al.[22] observed a positive correlation of OPG serum levels with the severity of CAD.

Our results are in complete accordance to the study of Ziegler et al [23] which aimed to study the significance of OPG as a marker of PAD. They conducted their study on 67 patients who were undergoing percutaneous transluminal angioplasty (PTA) because of advanced symptomatic PAD of the lower extremities and same number of healthy controls. They found that OPG was significantly higher in patients with PAD than the age matched control. Their study has been different from ours in that they correlated between the severity of PAD and the level of OPG according to PTA unlike the current study which correlated between OPG level and severity of PAD according to ABI and Doppler pattern whether monophasic or biphasic. They found that in patients with PAD, plasma OPG concentrations were significantly higher in subjects with ischemic ulcerations than in those without and were positively correlated with higher severity grade of disease. Therefore, Ziegler et al [23] hypothesized that alterations of the OPG cytokine system may be related also to severe PAD, which represents a further manifestation of generalized artery occlusive disease. However, the origin and the molecular mechanisms by which OPG concentrations are elevated in patients with severe PAD were elusive.

To date, very few studies commented on a potential interaction between both Coronary artery disease (CAD) and PAD on serum OPG. Avignon et al published a study primarily designed to evaluate a number of biomarkers for silent myocardial ischemia (SMI) in asymptomatic patients with diabetes, Out of 6 serum markers of vascular inflammation measured, only OPG was independently predictive of SMI, but this predictive power was lost when the presence of PAD was controlled. [24] This suggests that the co-existence of both conditions could affect OPG levels. Given the established findings of high levels of OPG in patients with diabetes,[23,24] Avignon and his colleagues hypothesized that the combination of diabetes and PAD would be associated with significantly higher serum levels of OPG than either condition alone.[24]

One of the most recent studies that aimed to examine the association between serum OPG levels and both the presence as well as the severity of lower extremity arterial disease in patients with type 2 diabetes is Demková et al. [25] The study was conducted in 2018 and included 165 consecutive patients with T2DM. They differed from our study in that PAD was diagnosed by measurement of the toe-brachial index (TBI) not ABI. Their conclusion was similar to our conclusion which was that serum OPG level is significantly associated with both the presence and severity of PAD in patients with T2D and it might be a biomarker for the presence of atherosclerotic disease in patients with T2DM.

On the other hand, the results of Pennisi et al [26] are not in accordance with our findings. They found no difference in OPG concentrations in 36 patients with ultrasound-defined atherosclerosis of the carotid or femoral artery compared to a group of 30 age and gender matched controls. The reason for this apparently contradictory finding may be that the definition of atherosclerotic disease in this

study was on the basis of ultrasound defined disease in the carotid and femoral arteries; no patient in this study had ABI measurement performed. It is possible that atherosclerotic disease of different vascular beds may not have similar effects on serum OPG.

Elevated OPG serum levels in osteoporosis have been interpreted to result as a counter-regulatory mechanism to protect against bone loss. [27] Similarly, the paradox of increased OPG concentrations in vascular disease could also be interpreted as a compensatory self-defensive mechanism against other factors that promote vascular damage. Therefore, it could be assumed that raised OPG in human atherosclerosis is a response to, rather than a cause, of atherosclerosis, obviously in an attempt to prevent further vascular lesions. On the other hand, cell culture experiments suggested that the protective effect of OPG on the vascular phenotype starts already during the prenatal period, [28] suggesting a later role as a defense against progression of atherosclerosis.[29] Vascular diseases are known to be promoted by immune mediated mechanisms [30] OPG also was shown to be involved in immune responses, i.e. in B-cell maturation or the generation of efficient antibody responses. [31] A further important aspect of the immunological function of OPG is to act as a survival factor for endothelial cells due to its ability to inhibit TNF-related apoptosis inducing ligand (TRAIL)-induced apoptosis of cells.[32] Endothelial cells have an essential barrier function and altered endothelial cell survival may contribute to endothelial dysfunction, which precedes atherosclerosis.[33] It can be hypothesized that elevated OPG concentrations in patients with PAD may well represent an increased production of this molecule by endothelial cells and smooth muscle cells in order to repair advanced plaque calcification.

Hyperglycemia observed in DM modulates OPG release by endothelial cells via a tumor necrosis factor alpha-dependent pathway. [34,35] However, increased OPG concentrations in patients with diabetes are not accompanied by a concomitant increase in its ligand (receptor activator of nuclear factor kappa-B ligand [RANKL]).[36] An imbalance between OPG /RANKL, resulting from an isolated increase in OPG, but not its ligand, might explain why, despite increased concentrations in diabetes, additional vasculo-protective benefits are not seen. In fact, the United Kingdom Prospective Diabetes Study (UKPDS) indicated that tight glycaemic control does not reduce the risk of amputations due to diabetic foot. [37]

Another finding in our study is that OPG was significantly related to age, duration of DM when measured in Patients with DM with or without PAD (120 patients in our study)

As regards the correlation between OPG level and age, our results are in accordance with Ziegler et al [23] who found level of OPG was significantly correlated with the age of the diabetic patients. Szulc et al [38] also agreed with our results and they also suggested that OPG may be an important paracrine mediator of bone metabolism in elderly men. Another study that agreed with the current results i.e. the correlation between age and OPG was that of Pammer et al [39]who found that high OPG not only correlates with old age but also may be a predictor of pathological fracture in elderly. Nevertheless, O'Sullivan et al [40] found that OPG was independent of age in all patients.

With respect to correlation between OPG level and ABI, O'Sullivan et al [40] agreed with the current results and found that OPG correlated negatively with ABI, independent of age, gender, glycaemic status, hs-CRP and IL-6. Esteghamati et al[41] also found that OPG was a significant predictor of disease severity, determined by ABI and percentage of vessel occlusion in univariable and multivariable models. Alkac et al[42] found a weak and inverse correlation between OPG and atherosclerosis as measured by ABI in type 1 diabetics only, but not in type 2 diabetics.

We found that OPG levels correlated with the duration of DM when computing only diabetic subjects, and correlated with FBS. This was consistent with the study conducted by Secchiero and his colleagues[34] as they found a significant correlation between OPG and both FBS and HbA1c. Alshock et al[43] also agreed with our results and found that OPG significantly correlates with duration of DM and FBS. However, Alkac et al [42] found that OPG did not correlate with DM

duration or FBS. Atile et al[44] not only denied a correlation between OPG and FBS or DM duration but also suggested that OPG has no role in the pathogenesis of PAD in diabetic patients.

The explanation of these different results may be that the other studies enrolled a different population with different inclusion criteria than our study like Ziegler et al [23] who enrolled patients with severe symptomatic PAD and Avignon et al[24] whose study material was subjects with both CAD and PAD . They also had different definitions of atherosclerosis diagnosis by ABI. Moreover, the difference in the sample size might have its impact in the statistical significance of the results.

Our ROC curve analysis of OPG to predict Diabetes with PAD showed that the area under the curve was 89% with 95% confidence intervals (0.835–0.945) the cutoff point was more than 100 pg/mL, the sensitivity was 100%, the specificity was 63.33%, the positive predictive value was 73.2%, and the negative predictive value was 100.

Another study evaluated the relationships between vascular risk biomarkers (including classic risk factors and OPG) and coronary artery calcification (CAC) extent in chronic kidney disease (CKD) patients and to establish within the markers the appropriate cut-off value to predict coronary artery calcifications. After adjustment for age, diabetes, smoking and gender, among biological markers, ROC curve analysis showed that the OPG best cut-off value predicting CAC was 757.7 pg/mL. [45]

Another study investigated whether plasma OPG levels were associated with the presence and severity of cerebral atherosclerosis. In multivariate ordinal logistic regression using the number of arteries with cerebral atherosclerosis as dependent variable, plasma OPG > 229.9 pg/mL was a significant predictor for the severity of cerebral vessel atherosclerosis (adjusted OR [95%CI] was 3.20 [1.26–8.12], p = 0.014). [46] Others conducted study aiming to determine whether plasma markers of bone remodeling osteoprotegerin and osteopontin (OPG, OPN) are in relation to endothelial dysfunction, which results in hypertension. They determined optimal cut-off for osteopontin OPN and osteoprotegerin OPG (19.7 ng/mL, 2.7 pmol/L, respectively) for differentiating between the hypertensive and asymptomatic subjects, but the sensitivity and specificity of these tests are not sufficient to use OPG and OPN as sole makers for recognizing endothelial dysfunction. [47]

To the best of our knowledge, the present study is the first to discuss the correlation between the OPG level and severity of PAD among the Egyptian population.

Strengths and Limitations

Our study has a main limitation, which is the relatively small number of studied patients as this may constrain the significance of multivariate adjustment analysis and decrease the strength of the evidence. Other limitation in the study is that the assay used in the present study, unlike some of the previously reviewed studies, [35,40] measures the total amount of OPG and therefore cannot discriminate between the free fraction of OPG and that complexed to RANKL. Ziegler et al [23] used angiography to define patients with PAD which is considered the gold standard investigation, contrary to the current study, which used Doppler that considered a safer procedure for diabetic patients.

The main strength points in the current study is the prospective design and the homogeneity of demographic data of the studied cohort that prevented any bias in the results.

Conclusion:

The current study found that osteoprotegrin (OPG) level was significantly higher in diabetic patients with peripheral arterial disease than both diabetic patients without peripheral arterial disease and healthy adults, so the study had the following conclusions:

1- OPG level is closely related to the presence of peripheral arterial disease and it may have the merit of being an early marker for the disease.

2- OPG level have a correlation with duration of diabetes, age and ankle brachial index ABI

3- The relation between ABI and OPG level may reflect a role of the later as a marker of not only the presence but also the severity of PAD.

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Original Article

STUDY OF DIABETIC FOOT DISEASE IN TYPE 2 DIABETIC PATIENTS WITH CHRONIC KIDNEY DISEASE.

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Abstract:

Background: Diabetes mellitus is probably one of the earliest diseases known to man , type 2 DM is the most common which is more vulnerable to microvascular complications such as diabetic neuropathy and diabetic nephropathy causing CKD which is an added risk factor for diabetic foot disease. **Subjects and Methods:** This cross-sectional study included 300 type 2 diabetic patients (aged 30-70 years) with chronic kidney disease classified into 5 groups according to eGFR. Clinical examination and full diabetic foot examination was done to all study subjects. HbA1c, LDL-C, serum creatinine, and urinary albumin creatinine ratio (ACR) were measured for all study subjects. Estimated glomerular filtration rate (eGFR) was calculated using CKD-EPI equation. **Results:** There was statistically significant difference between the five groups as regard DN score ($p<0.001$). The DNS was significantly higher in cases in group 4,5A and 5B CKD as compared with group1 and group2. Regarding ABI, it was significantly low in groups 4,5A and 5B as compared with groups 1 and 2. The mean serum cholesterol, mean TGs level, mean LDL level, mean creatinine level and UACR increased significantly with increasing the stage of CKD while the mean HBA1C and HDL level decreased significantly with increasing the stage of CKD. There was a statistically significant negative correlation between GFR, age, and duration of DM, weight, waist circumference, BMI, DNS, TGs, creatinine and UACR. Also there was statistically significant positive correlation between GFR with ABI, HbA1C and HDL. **Conclusion:** There is a strong association between the degree of renal impairment and DFS. Diabetic patients on dialysis treatment had a high prevalence of DF and most of them had one or more risk factors for developing an ulcer in the future.

Key Words: Diabetic foot, Chronic kidney disease.

Introduction:

Type 2 DM (formerly known as non-insulin dependent DM) is the most common form of DM characterized by hyperglycemia, insulin resistance, and relative insulin deficiency. (1)

People living with type 2 DM are more vulnerable to various forms of both short and long-term complications, which often lead to their premature death. This tendency of increased morbidity and mortality is seen in patients with type 2 DM because of the commonness of this type of DM, its insidious onset and late recognition, especially in resource-poor developing countries like Africa. (2)

CKD can be caused by numerous underlying processes. In general, causes can be grouped into glomerular diseases (such as diabetic nephropathy or lupus nephritis), vascular diseases (such as hypertension), tubulointerstitial diseases (such as obstructive uropathy), and cystic diseases. The most common causes are diabetes mellitus and hypertension, which together account for over two thirds of cases. (3)

Diabetic kidney disease is usually a clinical diagnosis made based on the presence

of albuminuria and/or reduced GFR in the absence of signs or symptoms of other primary causes of kidney damage. The typical presentation of diabetic kidney disease is considered to include a long standing duration of diabetes, retinopathy, albuminuria without haematuria, and gradually progressive loss of e GFR. [4]

Diabetic nephropathy is now the most common cause of chronic kidney disease (CKD). Both types of diabetes can lead to chronic kidney disease and eventually ESRD, but there is much higher prevalence of type 2 diabetes than type 1, often patients with ESRD have type 2 diabetes. The overall incidence 20 years after diagnosis is approximately 4 to 17% and after 30 years is about 16%. [5]

Neuropathies are among the most common long term complications of diabetes, affecting up to 50% of patients. [6]

The neuropathies associated with diabetes mellitus represent insidious and progressive processes for which a disconnection exists between pathological severity and the development of symptoms which are heterogeneous, originating in different parts of the nervous system and resulting in diverse clinical manifestations. By far the most common diabetic neuropathies are chronic sensorimotor distal symmetric polyneuropathy (DPN) and cardiac autonomic neuropathy (CAN). [7]

Risk factors for the development of PN include diabetes duration, degree of hyperglycemia, hyperlipidemia, hypertension, and height. [8] Retinopathy and nephropathy are highly associated with PN, occurring in type 2 diabetic patients by 55% and 32%, respectively. [9]

The definition of the diabetic foot has been described as infection, ulceration and/or destruction of deep tissues associated with neurological abnormalities and various degrees of peripheral vascular disease.[10]

As indicated by this description, one or more of these conditions may coincide, and they often do. Diabetic foot ulcer is the general term to describe a full thickness wound below the ankle in a patient with diabetes, the major adverse outcomes of diabetic foot problems are foot ulcers and amputations, and foot problems account for more hospital admissions than any other long term complications of diabetes, and also result in increasing morbidity and mortality.[11]

Subjects and Methods:

The present study was conducted on 300 patients who were classified into five stages according to estimated glomerular filtration rate (e GFR). Patients of fifth stage (e GFR less than 15) were classified into dialysis group and no dialysis group. [12]

Clinical assessment of the patients included blood pressure, body mass index (BMI), waist circumference (WC) , the severity of peripheral neuropathy by diabetic neuropathy score (DNS) , skin color , hair and nails ,ulcers(size ,depth ,site ,discharge ,margins ,floor, type), checking appropriate foot wear. Also, musculoskeletal assessment of gait, joint flexibility and deformities. [13]

Neurological examination was done by tuning fork, deep tendon reflex of the ankle joints. Vascular examination was done by assessment of ankle brachial pressure index (ABPI) (12), laboratory investigations were done by checking HbA1C, lipid profile, serum creatinine, eGFR (was calculated according to CKD-EPI formula), Urinary albumin creatinine ratio (UACR), complete urine analysis and ultrasound abdomen. [12]

Results:

Regarding demographic data of the study population, the mean age of the cases was 58.2±5.47 years and there were 114 males (38%) and 186 females (62%). The

anthropometric measures of the study population; the mean body weight of the cases is 91.16 ± 9.43 kg, the mean waist circumference is 112.7 ± 27.03 cm and the mean BMI is 27.51 ± 4.87 kg/m2.

Also, there were (18%) current smokers and ex-smokers (19.3%). The median duration of DM among the cases was 12.4 years with range between 3 and 25 years. According to the treatment regimen for DM, insulin was the most commonly used medications alone (38.3%) or in combination with metformin or sulfonylurea. Sulfonylurea is used alone in (29%) and metformin is used alone in (4.7%) while combined regimen (19.3%).

As regarding the past medical history of the cases HTN was present in (72%). CKD (40%), cerebrovascular stroke (12.7%) and coronary artery disease (39.3%). there were (10.7%) with previous hemodialysis, (13.3%) with previous amputation and (6%) with previous foot ulcers. Figure (1)

The cases of study were distributed into 5 groups according to eGFR calculated in ml/min/m2 and demonstrated in the Figure (2).

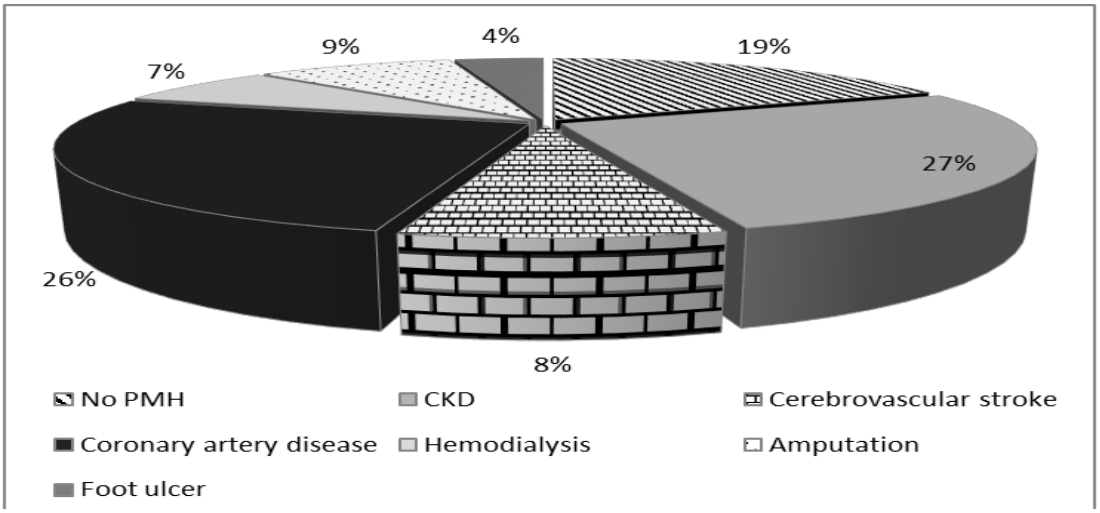

Figure 1: Past medical history

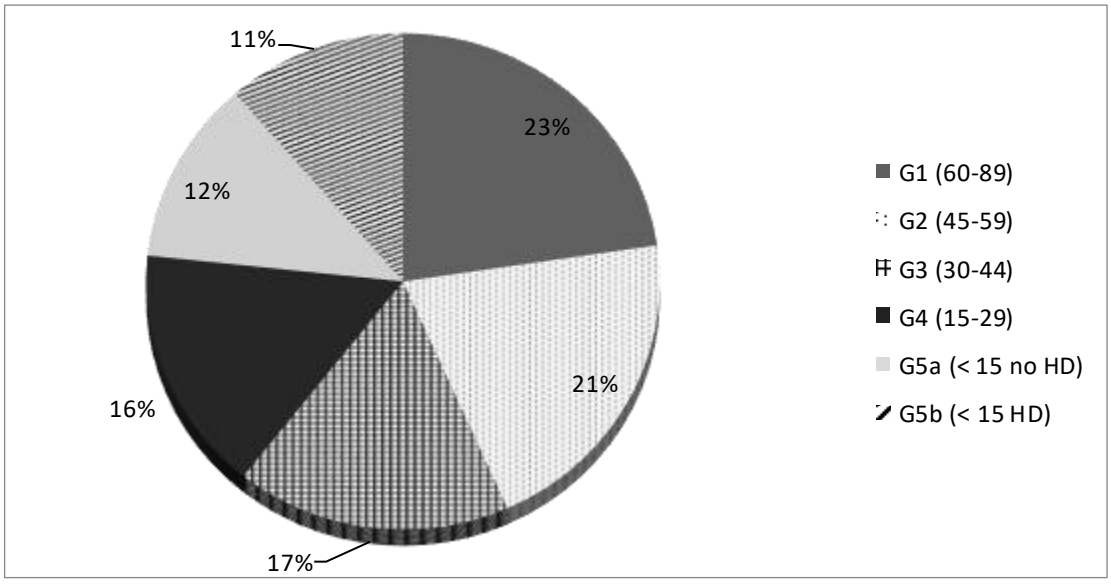

Figure 2: Cases distribution into groups

There was no statistically significant difference in the mean age and sex distribution of the cases within the different subgroups. There was a statistically significant difference between the different subgroups in the mean weight, waist circumference and BMI (p= 0.003, 0.021 and 0.010). There was a significant increase in the weight, waist circumference and BMI with progression of the stage of CKD. There was no statistically significant difference in the prevalence of smoking among the cases in the study (p=0.328).

There was a statistically significant difference in the duration of DM among the cases in the different subgroups (p=0.001). The cases with stage 5B CKD have the longest duration of DM. The cases in the different subgroups have statistically significant longer duration of DM as compared with the cases in group 1.

The prevalence of HTN was high in the cases with stage 5A (94.4%) and stage 5B (94.1%) with statistically significant difference as compared with other groups. Also, the prevalence of HTN was statistically significant higher in cases in stage 3 and 4 CKD as compared with the cases in stage 1 and stage 2 CKD.

Correlating the diabetic neuropathy score (DNS) between the cases within the different groups in the study, there was high statistically significant difference between the different study groups (p<0.001). The DNS was significantly higher in cases in group 4 CKD as compared with G1, in group 5A as compared with group 1 and 2, in group 5B as compared with group 1, 2 and 3. Figure (3)

With comparing the ankle brachial index (ABI) between the cases within the different groups in the study, there was high statistically significant difference between the different study groups (p<0.001). The ABI was significantly lower in cases in group 2 and 3 CKD as compared with G1. The ABI was significantly lower in group 4 as compared with group 1 and 2, in group 5A as compared with group 1, 2 and 3. In group 5B as compared with group 1, 2, 3 and 4. Figure (4)

The nail changes in the form of fungal infection and thick yellow changes were significantly higher with increasing the stage of CKD. The percentage of cases with cold skin temperature and absent skin hair was significantly higher as compared with increasing the stage of CKD.

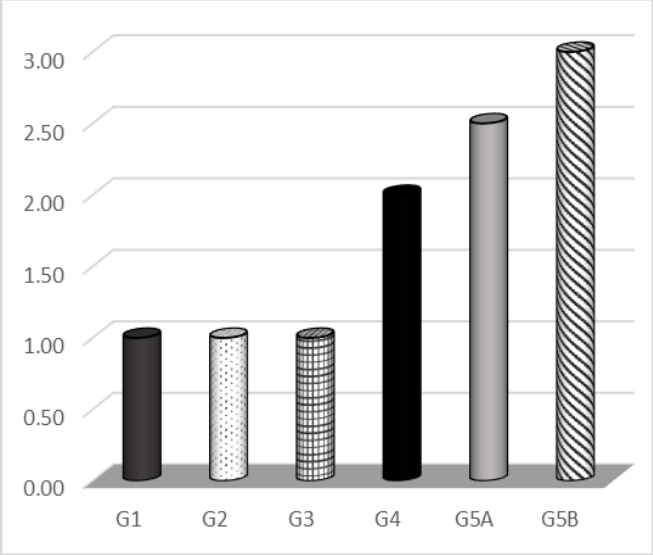

Figure 3: DNS among the cases in the different groups

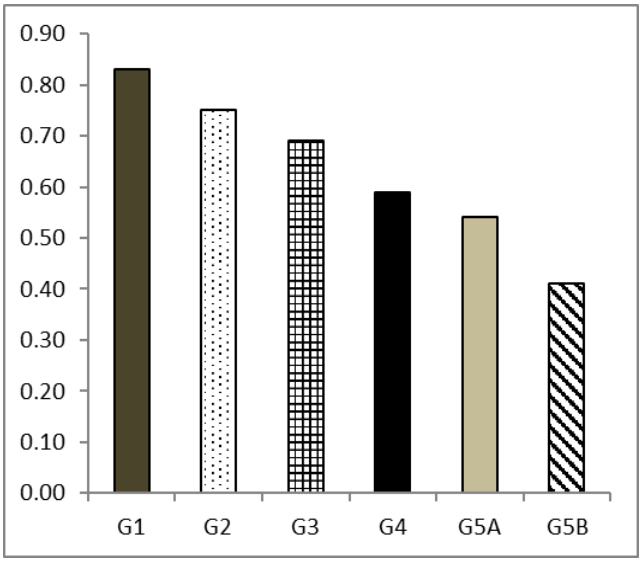

Figure 4: ABI among the cases in the different groups

There was a statistically significant difference in the joint flexibility between the different groups of CKD (P=0.002). There is an increase incidence of limited joint flexibility with increasing the stage of CKD.

The incidence of foot deformities between the different groups of the study (p<0.001) . There is increasing incidence of hallux vulgus and flat foot in group 5A and group 5B. as regards of Charcot joint deformity, 2 cases in group 1 and 4 cases in group 4 CKD had Charcot joint deformity that didn't appear in other groups.

There was a statistically significant difference in the absent tuning fork between the different groups of CKD (p<0.001). There is and increase incidence of abnormal tuning fork with increasing the stage of CKD. Figure (5)

There was a statistically significant difference in the ankle reflex between the different groups of CKD (p<0.001). There is and increase incidence of absent ankle reflex with increasing the stage of CKD. Figure (6)

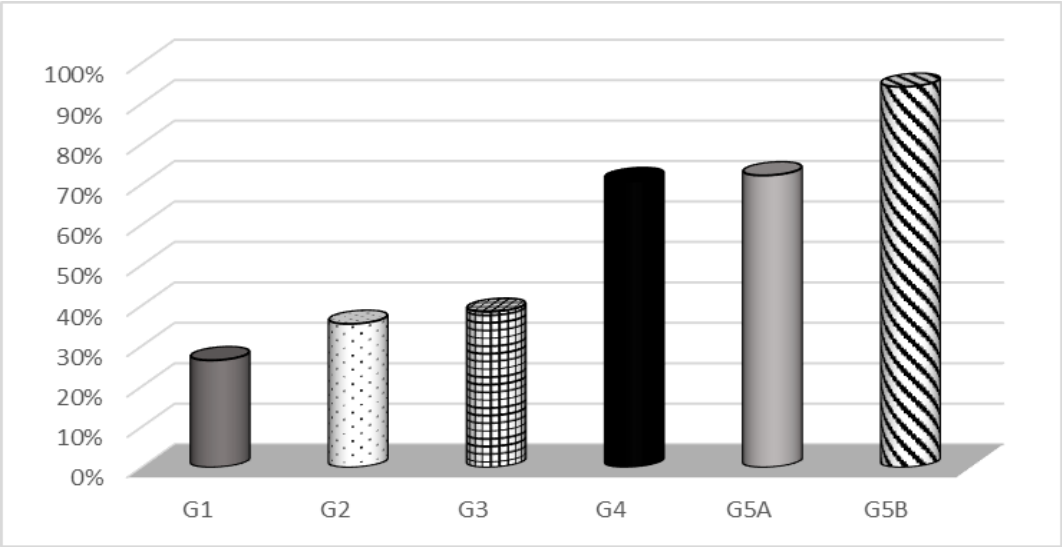

Figure 5: Absent tuning fork test among the cases in the different groups

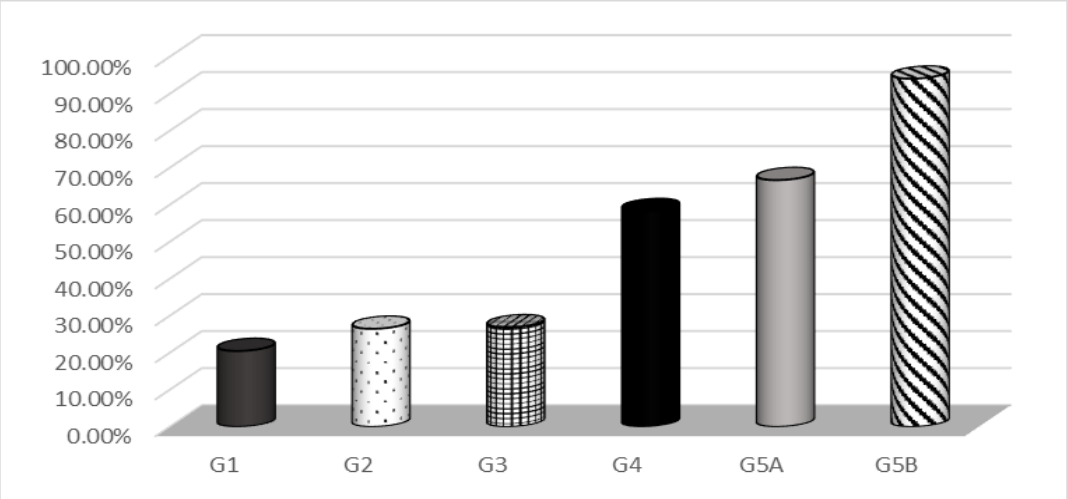

Figure 6: Absent ankle reflex among the cases in the different groups

There was statistically significant difference in the HbA1c, TGs level, HDL, creatinine and UACR between the cases in the within the different study groups. The mean serum cholesterol, mean TGs level, mean LDL level, mean creatinine level and UACR increased with increasing the stage of CKD while the mean HbA1c and HDL level decreased with increasing the stage of CKD.

The ultrasound changes in the kidneys within the cases in the different groups, all cases within group 1 had normal kidneys and (52.9%) of group 5B had grade IV nephropathy. increased cases of grade III nephropathy among group 4,5A,5B.

There is a statistically significant negative correlation between GFR, age, duration of DM, weight, waist circumference, BMI, DNS, TGs, creatinine and UACR. Also, there is statistically significant positive correlation between GFR with ABI, HbA1c and HDL. The multilinear regression analysis, duration of DM and eGFR reveal statistically significant prediction ability with ABI. Table (1)

Table 1: Multinomial regression analysis model for ankle brachial index

	a (constant)	b (regression coefficient)	t	P value
Age	.002	.004	.050	0.960
Duration of DM	.003	-.356	-4.512	<0.001*
Weight	.025	.183	.097	0.923
Height	.014	-.022	-.053	0.958
Waist circumference	.001	-.019	-.111	0.912
Body mass index	.080	-.212	-.119	0.906
HbA1C	.012	-.152	-1.710	0.090
S.Cholesterol	.002	.002	.023	0.982
S.TG	.006	-.064	-.627	0.532
LDL	.001	-.057	-.670	0.504
HDL	.003	.140	1.766	0.080
S.CREATININE	.030	-.001	-.005	0.996
e.GFR ml/min	.001	.342	2.330	0.021*
urinary albumin creatinine ratio (UACR)	.004	-.089	-.597	0.552

$y = a + bx$ where 'y' is the value of the outcome variable, 'x' is the value of the explanatory variable, 'a' is the intercept of the regression line and 'b' is the slope of the regression line

Discussion:

In the present study female were more common than males, In agreement with study of Akbari et al., the present study showed that HTN was present in (72%) of cases. Also, there were 45 current smokers (18%) and 58 ex-smokers (19.3%). [6] The median duration of DM among the cases was 12.4 years. Our results were supported by study of Dòria et al., as they found that the mean diabetes duration was 22.3 years (SD = 12.2 years). 83% of them had HTN. [14]

The current study showed that according to the treatment regimen for DM, insulin was the most commonly used medications alone in 115 cases (38.3%) and as regard the past medical history of the cases, CKD was present in 120 cases (40%), cerebrovascular stroke in 38 cases (12.7%) and coronary artery disease in (39.3%) cases, there were (10.7%) cases with previous hemodialysis, (13.3%) with previous amputation and (6%) with previous foot ulcers.

Our results were supported by He et al., study of More than 90% of patients received insulin therapy to control blood glucose. During the follow up period, (39.3%) of patients suffered from one or more cardiac and (or) cerebrovascular events. Among them, cardiac events occurred in 84 (acute heart failure, 57; acute coronary syndrome, 12; acute coronary syndrome combined with acute heart failure, 9; sudden cardiac death, 6), stroke in 42 and both occurred in 5 patients. Over 50% of the patients with reduced eGFR suffered cardiac events, whereas only less than 30% of patients in the normal eGFR group had cardiac events. [15]

In the study of Wolf et al., forty-six patients (5.1%) of their collective have active or a history of DFS (Diabetic foot syndrome) (defined as acute or previous ulcers or amputation because of DFS). [16]

There was a statistically significant difference in the duration of DM among the cases in the different subgroups (p=0.001). Cases with stage 5B CKD have the longest duration of DM. The prevalence of HTN was high in the cases with stage 5A (94.4%) and stage 5B (94.1%), in cases in stage 3 and 4 CKD.

In the study of Shaheena et al., group 1 included 80 patients with an active foot ulcer, mean age 57.5 ± 7.2 years that was significantly higher than the mean age of group 2, which was 43.2 ± 7.5 years. [17] No significant difference was found between both the groups with respect to BMI. However, Rani et al. found statistical significance were observed in age (P<0.001), gender (P<0.005) duration of diabetes (P<0.001) and duration of hypertension (<0.001) between subgroups. [18]

According to the International Working Group on the Diabetic Foot (IWGDF), DF is defined as the ulceration, infection and/or destruction of deep tissues below ankles in patients with diabetes and/or peripheral arterial disease. Complications affecting the lower limbs are among the most common manifestations of diabetes. It was reported that 15% of T2DM patients will eventually suffer from foot ulceration during their lifetime, and these complications are the frequent cause of hospitalization and disability. [19]

The present study showed that with comparing parameters of DFS as diabetic neuropathy score (DNS), the ankle brachial index (ABI), absent tuning fork, absent ankle reflex, nail changes, cold skin temperature, absent skin hair and limited joint flexibility between the cases within the different groups in the study, there was high statistically significant difference between the different study groups (p<0.001) and increase incidence with increasing the stage of CKD.

Our results were supported by study of Dòria et al., as they reported that the prevalence of foot complications was, from the highest to the lowest, PN (89.1%), moderate or severe PAD (64.2%), foot deformities (54.3%), previous ulcer (19.6%), DF (17.4%), and amputations (16.3%). finally, based on the IWGDF classification, out of 83 patients explored, 87% had some risk grade for suffering DF in the future. [14]

Our results were supported by study of Rani et al. as they demonstrated that neuropathy was higher in group I (34.5 %) followed by group III (33 %) and group II (12 %) respectively (Group I: T2DM with CKD and DFI, Group II: T2DM with CKD, Group III: T2DM with DFI and without CKD and Group IV- T2DM without any complications). [18]

Our results were in contrary with study of Shaheena et al., as they reported that there no statistically significant differences between both the groups with respect to the presence of retinopathy, the presence of ischemic heart disease, mean ankle-brachial index. [17]

The showed current study, the mean serum cholesterol, mean TGs level, mean LDL level, mean creatinine level and UACR increased with increasing the stage of CKD while the mean HBA1C and HDL level decreased with increasing the stage of CKD.

Our results were supported by study of Shaheena et al., as they showed that patients with DFU had a high significant difference with respect to fasting blood glucose, 2 h postprandial blood glucose, HbA1c. [17] Similarly, Wolf et al. concluded that type 2 DM with diabetics' foot syndrome were significantly higher HbA1c and had a longer duration of diabetes compared with type 2 DM without diabetic's foot syndrome. [16]

In the study of Mrozikiewicz-Rakowska et al., the univariate logistic regression analysis showed that CKD risk factors were the following variables: mean creatinine level, mean body weight, mean hips circumference, ischemic heart disease, hypertension and diabetic retinopathy. each mg/dl more in creatinine serum level was increasing the risk of CKD development by 4.5%. The risk of CKD development was increased by 3.7% per each additional kg in body mass. There was also observed an increase in CKD development risk by 6.3% for each additional centimeter in hips circumference. Moreover, CKD risk was increased by the coexistence of ischemic heart disease, hypertension and diabetic retinopathy, over 2.7-fold, 7.3-fold and 4.4-fold, respectively. [20]

As regard the US changes in the kidneys within the cases in the different groups, the present study showed that all cases within group 1 had normal kidney on us examination, grade IV nephropathy was only present in group 5B and increased grade III nephropathy among group 4,5B,5A .

There is a statistically significant negative correlation between GFR with age, duration of DM, weight, waist circumference, BMI, DNS, TGs, creatinine and UACR. Also, there is statistically significant positive correlation between GFR with ABI, HBA1C and HDL. With multi-linear regression analysis, duration of DM and GFR reveal statistically significant prediction ability with ABI. With multi-linear regression analysis, duration of DM and HDL reveal statistically significant prediction ability with DNS.

In the study of Shaheena et al., reported that in a comparison of renal function and urine albumin/creatinine ratio of the study groups. there was a high significant increase in serum creatinine (2.3 ± 0.93 vs. 1.5 ± 0.73) and no significant difference in albumin/creatinine ratio in urine (235.5 ± 274.5 vs. 219.3 ± 112.3) in group 1 versus group 2, and a considerable decrease was seen in

eGFR in group 1 versus group 2 (40.3 ± 24.5 vs. 62.4 ± 23.4) (P < 0.001). [17]

In the study of Ninomiya T, et al., they found that the risk for each outcome increased linearly with lower eGFR levels. Every 10 fold increment in baseline UACR, which corresponds approximately to a change from one clinical stage of albuminuria to the next was associated with a 1.6-fold, two-fold, and 3.3-fold higher, multivariable-adjusted risk of cardiovascular events, cardiovascular death, and renal events, respectively. [21]

In Lepantalo, et al. study it was believed that the essential factor for DFU is a loss of renal function. Disturbance of glucose metabolism and production of glycogen is caused by damaging insulin binding to receptors that cause tissue-insulin resistance, particularly in skeletal muscles. [22]

In the study of Rani, et al. present highlighted that in patients with DFI there is a reduction in eGFR and when the infection subsides the eGFR improved, whereas in patients with preexisting CKD the decrease in eGFR was observed in all the follow-up periods. [18] Among patients with both CKD and DFI, fall in eGFR was similar to that of DFI patients, however the eGFR continues to fall and fails to improve after the DFI subsides.

Wolf, et al., demonstrated that compared to type 2 patients without DFS those with DFS were significantly older (P < 0.005), had a longer duration of diabetes (P < 0.005), higher serum creatinine levels (P < 0.005) and a lower eGFR (P < 0.005). Patients who smoked did not have DFS more frequently than non-smokers. There was a significant negative correlation between the Wagner stages and eGFR (r = −0.104, P < 0.01) as well as Armstrong stages and eGFR (r = −0.125, P < 0.01) in all patients with type 2 diabetes. Non smokers had a similar significant negative correlation between Armstrong and Wagner stages and eGFR. Not surprisingly, Wagner and Armstrong stages showed a highly significant positive correlation in patients with type 2 diabetes (r = 0.698, P = 0.01). Multivariate logistic regression analysis revealed a significant negative association between a 10 ml/min change in eGFR and DFS as well as diastolic blood pressure. [16]

Conclusion:

There was a strong association between the degree of renal impairment and DFS. diabetic patients in dialysis treatment had a high prevalence of DF, and most of them had one or more risk factors for developing an ulcer in the future.

Hypertension is the most common comorbidity of diabetes; insulin is the most common used anti diabetic medication followed by sulphonylurea.

The most common macrovascular complications are coronary artery disease and cerebrovascular stroke. Deterioration of eGFR with the longer duration of diabetes causing Diabetic neuropathy score (DNS) and Ankle brachial pressure index (ABI) to decrease with eGFR decrease.

Fungal infection and foot deformities high risk with increase stages of CKD.

Ultrasound abdomen was normal at early stages of CKD also HbA1c, HDL decrease in delayed stages of CKD.TGs, creatinine and UACR increase in delayed stages of CKD.

Recommendations:

Diabetic patients with CKD should be considered as a high-risk group for the development of DFS and should therefore be regularly screened for DFS during every office visit. Early lesions need consequent management, such as offloading, antibiotic therapy and local wound care.

Ongoing support from a designated podiatrist/chiropodist, an orthotist or shoemaker, and a diabetes or renal physician, depending on available personnel. Routine and systematic assessment for neuropathy, PAD, callus, foot deformity, bed sores, pre ulcerative lesions, ulcers and infection or gangrene in all patients, with the foot rendered completely bare (i.e., no socks or shoes).

Randomized clinical trials and the demonstration of a common mechanism that causes CKD and failure of the skin to heal are required.

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Original Article

Study of Circulatory Level of Serum Adiponectin in Patients with Type 2 Diabetes Mellitus: Effect of Antidiabetic Drugs and Association with Chronic Diabetic Complications

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Abstract

Background. The aim of this work was to study circulatory level of serum adiponectin in patients with type 2 diabetes mellitus and any possible effect of antidiabetic drugs or diabetic complication on serum level of adiponectin. **Subjects and methods.** This study involved 120 diabetic patients with type 2 diabetes mellitus. These patients were recruited from the outpatient clinic at Alexandria Main University Hospital, in addition to 60 non-diabetic healthy control group with matched age and sex. All participants were subjected to full history taking and detailed drug history, complete physical examination including,(body weight and height), waist circumference, vital signs (heart rate and arterial blood pressure), neurological and fundus examination. Blood was drawn for fasting serum glucose, insulin level for HOMA-IR assessment, glycated haemoglobin (HbA1C), total cholesterol, High density lipoprotein cholesterol (HDL-C), Low density lipoprotein cholesterol (LDL- C), triglycerides and adiponectin level by using ELISA. Urinary albumin creatinine ratio and serum creatinine for calculation of eGFR and staging of chronic kidney disease were done. **Results**. The mean serum adiponectin levels was significantly higher in the diabetic group compared to the control group.There was significant positive correlation between adiponectin and age among diabetic group, significantly lower adiponectin in subjects with positive history of smoking, coronary artery disease and dyslipidemia but no significant difference in adiponectin among diabetic group as regard history of hypertension (HTN) or patient with or without peripheral neuropathy, but significantly lower adiponectin among diabetic group with diabetic retinopathy. No significant correlation with systolic blood pressure but negative correlation with diastolic blood pressure was present. There was positive correlation between serum adiponectin and estimated glomerular filtration rate (eGFR) but negative correlation with urinary Albumin/ Creatinine ratio (A/C ratio). Significant increase in adiponectin in patient using glitazon, dipeptidyl peptidase-4 inhibitors (DPP4) and statins was present, on the other hand there was numerical increase in adiponectin with renin-angiotensin enzyme blocking agents (ACEI), beta blockers (BB) and diuretics than in patients using angiotensin receptor blockers (ARBs), and calcium channel blockers (CCB) but this was not significant. **Conclusions:** The quantitative changes in adiponectin provide insight into how adiponectin is important while choice of different lines in management of type 2 diabetes and associated comorbidity.

Key Words: Adiponectin, Type 2 diabetes, Antidiabetic drugs, Diabetic complications

Introduction

Diabetes mellitus (DM) is a metabolic disorder characterized by the presence of hyperglycemia resulting from defects in insulin secretion, insulin action, or both.[1] Type 2 DM

is the most common form of DM characterized by hyperglycemia, insulin resistance, and relative insulin deficiency. (2) Obesity has been found to contribute to approximately 55% of cases of type 2 DM. (3)

Adipose tissue is considered as endocrine structures because of their wide variety of chemical secretions (adipokines), which affect many diverse physiological functions and related pathological processes of the body, like metabolism of carbohydrates and lipids. (4) Adiponectin is one of the important adipokines which has remarkable insulin sensitizing property as well as antiatherogenic action thereby playing an important role in delaying and suppressing the metabolic derangements, which result in IR, T2DM, and metabolic syndrome. (5)

The chronic hyperglycemia of diabetes is associated with relatively specific long-term microvascular complications affecting the eyes, kidneys and nerves, as well as an increased risk for macrovascular disease including ischemic heart diseases, cerebrovascular diseases, and peripheral arterial diseases. (1) Adiponectin by its insulin sensitizing property and antiatherogenic action can affect complications of diabetes including vascular and cardiac ones. (5)

Serum adiponectin levels can be affected by different lines of management of type 2 diabetes and may be negatively influenced by lifestyle, such as sedentarism, a high-fat diet causing obesity, or excessive smoking. (6) Some antidiabetic drugs are associated with increased adiponectin such as sulphonylureas (7)and thiazolidinediones (TZDs), (8) also incretin-based therapies, GLP-1 agonists, and DPP4 inhibitors can promote secretion of adiponectin. (9,10) In management of associated comorbidity with DM like hypertension and dyslipidemia, different antihypertensive drugs may have quite different effects on adiponectin, despite very similar or equivalent effects on blood pressure, (11-3) Also there is an association between statin therapy and adiponectin levels which vary upon statin type and dose. (14-6)

The aim of this work was to study circulatory level of serum adiponectin in patients with type 2 diabetes mellitus and any possible effect of antidiabetic drugs or diabetic complication on serum level of adiponectin.

Subject and Methods:

The study included 180 subjects classified into two groups, group I:120 type 2 diabetic patients who visit diabetes clinic at Alexandria Main University Hospital.,and group II: 60 non-diabetic healthy control group with matched age and sex with group I. Ethical approval was granted by ethics committee of Alexandria Faculty of Medicine. All participates were given written informed consent after explaining the nature and the aim of the study. The healthy control group included those without any chronic cardiovascular or metabolic disease and not receiving any long-term medication for both conditions. Exclusion criteria: Ischemic cardiovascular event in previous 3 month, severe liver or renal impairment, recent history of major trauma or surgery, hematological disorders or malignancy, chronic inflammatory or autoimmune diseases, as well as patients with recent history of sever significant infection at study entry, Alcoholics, HIV, HBV, HCV, under-weight (BMI $\leq$ 18.5 Kg\ m2). All participants were subjected to: Full history taking including: detailed analysis of different cardio-metabolic risk factors (family history of premature CAD, smoking, diabetes, hypertension or dyslipidemia) and detailed drug history, complete physical examination including :Body weight and height were measured in order to calculate body mass index (BMI), waist circumference, vital signs (heart rate and arterial blood pressure), neurological examination for detection of diabetic peripheral neuropathy and fundus examination was done for detection of diabetic retinopathy.

Laboratory investigations: Blood withdrawn for metabolic, biochemical and hematological parameters after a 10-12 hours overnight fasting and the following were estimated : fasting serum glucose, serum insulin level and Homeostasis Model Assessment 2 (HOMA2) calculator was used to estimate steady state beta cell function (%B) and insulin resistance (%S) (HOMA-IR) according to the updated computer based HOMA2 mode in subjects with normal or impaired glucose tolerance, glycated

haemoglobin (HbA1C),total serum cholesterol, HDL-C, LDL- C, serum triglycerides and serum level of adiponectin by using ELISA. Urinary A/C ratio and serum creatinine for calculation of e GFR and staging chronic kidney disease were done.

Statistical analysis of the data: data were fed to the computer and analyzed using IBM SPSS software package version 20.0. (Armonk, NY: IBM Corp)(297) Qualitative data were described using number and percent. The Kolmogorov-Smirnov test was used to verify the normality of distribution. Quantitative data were described using range (minimum and maximum), mean, standard deviation and median. Significance of the obtained results was judged at the 5% level .

The used tests were Chi-square test for categorical variables, to compare between different groups, Fisher's Exact or Monte Carlo correction for chi-square when more than 20% of the cells have expected count less than 5, student t-test for normally distributed quantitative variables, to compare between two studied groups and Mann Whitney test for abnormally distributed quantitative variables, to compare between two studied groups.

Results

The study included 120 patients and 60 subjects as control group. There was no significant difference between the 2 groups as regard gender, age or smoking state. Patients had significant higher BMI, waist circumference, systolic and diastolic blood pressures, fasting plasma glucose, HbA1c., serum insulin and HOMA2-IR, total cholesterol, LDLC and triglycerides but lower HDLC eGFR were significantly lower and Urinary A/C ratio were significantly higher in patients in comparison to control group. Diabetic retinopathy was present in 23.3% of diabetics and peripheral neuropathy was present in 21.7% among them (Table I, II).

Table (I): Demographic data of the studied subjects

	Control (n = 60)		Patients (n = 120)		Test of Sig. χ^2	p
	No.	%	No.	%		
Sex						
Male	22	36.7	58	48.3		0.138
Female	38	63.3	62	51.7	2.205	
Smoking						
Non-smoker	50	83.3	92	76.7	1.067	0.302
Smoker	10	16.7	28	23.3		
Diabetic duration in years						
≤5			72	60.0		
>5			48	40.0		
History of						
Coronary artery disease (CAD)			12	10.0		
Hypertension (HTN)			64	53.3		
Dyslipidemia			56	46.7		
Family history(FH)						
CAD	4	6.7	24	20.0	5.414*	0.020*
HTN	12	20.0	50	41.7	8.316*	0.004*
DM	16	26.7	88	73.3	35.709*	<0.001*
Peripheral neuropathy (PN)			26	21.7		
Fundus examinatio						
Normal			92	76.7		
Retinopathy			28	23.3		
Non proliferative			18	15.0		
Proliferative			10	8.3		

χ^2: Chi square test
t: Student t-test
p: p value for comparing between the studied groups
*: Statistically significant at $p \leq 0.05$

Table (II): Laboratory and some clinical data of studied subjects in relation to adiponectin

Measures	Control (n = 60) Mean ± SD.	Patients (n = 120) Mean ± SD.	Test of sig.	P
BMI (kg/m^2)	24.94 ± 2.14	32.11 ± 3.26	t=17.664*	<0.001*
Waist circumference (cm)	94.83 ± 7.83	110.4 ± 10.21	t=11.321*	<0.001*
Systolic blood pressures(mmHg)	125.5 ± 10.36	137.4 ± 9.37	t=7.761*	<0.001*
Diastolic blood pressures(mmHg)	80.33 ± 4.10	82.93 ± 5.30	t=3.625*	<0.001*
FPG (mg/dl)	Mean ± SD.	86.77 ± 6.37	t=12.073*	<0.001*
Insulin (µIU/ml)	9.33 ± 3.01	13.37 ± 10.67	U =2584.0*	0.002*
HOMA –IR	1.35 ± 0.48	5.26 ± 3.26	U =414.0*	<0.001*
HbA1c (%)	5.06 ± 0.29	7.75 ± 1.66	t =17.313*	<0.001*
Total cholesterol (mg/dl)	173.6 ± 19.43	212.7 ± 49.79	t =7.526*	<0.001*
Triglycerides (mg/dl)	118.2 ± 16.49	175.0 ± 77.59	U =1238.0*	<0.001*
LDL (mg/dl)	85.77 ± 10.09	107.7 ± 33.65	t =6.574*	<0.001*
HDL (mg/dl)	57.97 ± 5.30	56.20 ± 6.44	t =1.835*	<0.001*
eGFR (ml/min/1.73 m^2)	94.37 ± 3.86	85.40 ± 11.26	U=1216.0*	<0.001*
A/C ratio (µgm/mg)	3.90 ± 1.55	35.62 ± 52.08	U=286.0*	<0.001*
Adiponectin (µg/ml)	4.07 ± 1.01	2.28 ± 1.72	U=958.0*	<0.001*

t: Student t-test
p: p value for comparing between the studied groups
*: Statistically significant at $p \leq 0.05$
U: Mann Whitney test

The mean serum adiponectin levels were statistically significant higher in the diabetic group compared to the control group (Fig 1)

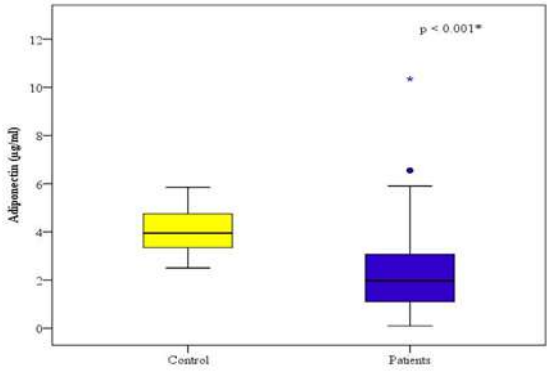

Fig. (1): Comparison between the two studied groups according to adiponectin

There was statistically significant positive correlation between serum adiponectin and the age among diabetic group but no statistically significant difference as regard gender, on the other hand there was statistically significant lower serum adiponectin levels, in subjects with positive history of smoking, coronary artery disease and dyslipidemia but no statistically significant difference in serum adiponectin levels among diabetic group as regard history of hypertension (HTN). No significant difference in serum adiponectin levels among diabetic group with or without peripheral neuropathy, but there were statistically significant lower serum adiponectin levels among diabetic group with diabetic retinopathy. No significant correlation among diabetes with systolic blood pressure (BP) but there was statistically significant negative correlation between serum adiponectin and diastolic BP (Table III, Fig.2- 5)

Table (III): Relation between adiponectin and clinical parameters of studied subjects

	N	Adiponectin (µg/ml)		U	p
		Mean ± SD.	Median		
Sex (control)					
Male	**22**	3.81 ± 0.94	3.90	328.0	0.167
Female	**38**	4.23 ± 1.02	4.0		
Smoking(control)					
Non-smoker	**50**	4.35 ± 0.87	4.25	4.0*	<0.001*
Smoker	**10**	2.70 ± 0.18	2.65		
FH (control)					
CAD					
No	**56**	4.05 ± 1.0	3.95	100.0	0.722
Yes	**4**	4.43 ± 1.24	4.43		
HTN					
No	**48**	4.12 ± 1.05	3.95	260.0	0.604
Yes	**12**	3.88 ± 0.81	4.05		
Dyslipidemia					
No	**44**	4.12 ± 1.01	4.13	302.0	0.403
Yes	**16**	3.94 ± 1.02	3.85		
Sex (Patients)					
Male	**58**	2.37 ± 1.64	2.35	1658.0	0.462
Female	**62**	2.20 ± 1.80	1.95		
Smoking(Patients)					
Non-smoker+++	**92**	2.56 ± 1.78	2.0	682.0*	<0.001*
Smoker	**28**	1.39 ± 1.09	0.70		
History (Patients)					
CAD					
No	**108**	2.49 ± 1.68	2.05	2.0*	<0.001*
Yes	**12**	0.41 ± 0.22	0.45		
HTN					
No	**56**	2.10 ± 1.29	1.73	U= 1696.0	0.613
Yes	**64**	2.45 ± 2.01	2.0		
Dyslipidemia					
No	**56**	3.57 ± 1.70	3.10	4.0*	<0.001*
Yes	**64**	1.16 ± 0.54	1.15		
FM (Patients)					
CAD					
No	**96**	2.28 ± 1.82	1.98	1062.0	0.555
Yes	**24**	2.31 ± 1.23	1.95		
HTN					
No	**70**	2.35 ± 1.99	2.0	1700.0	0.790
Yes	**50**	2.19 ± 1.25	1.90		
Dyslipidemia					
No	**32**	2.27 ± 1.37	2.30	1334.0	0.660
Yes	**88**	2.29 ± 1.83	1.93		
PN (Patients)					
No	**94**	2.31 ± 1.80	1.95	1210.0	0.939
Yes	**26**	2.18 ± 1.38	2.10		
Fundus (Patients)					
Normal	**92**	2.78 ± 1.66	2.48	0.00*	<0.001*
Retinopathy	**28**	0.65 ± 0.28	0.70		

U: Mann Whitney test

p: p value for association between Adiponectin and different parameters

*: Statistically significant at $p \leq 0.05$

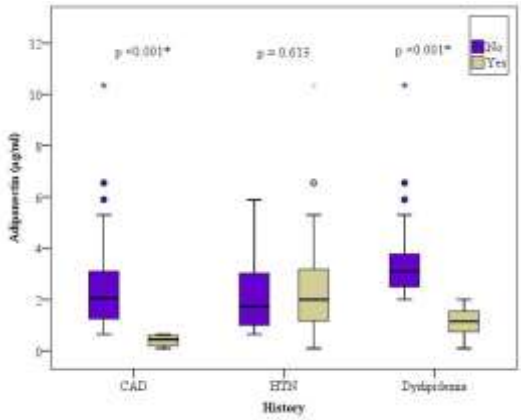

Fig. (2): Relation between adiponectin and history of CAD, HTN and dyslipidemia in patients group (n = 120)

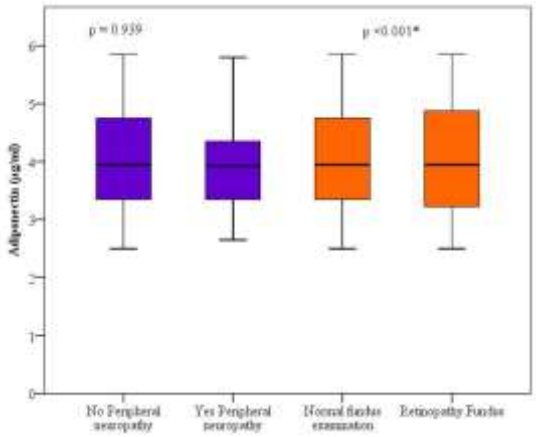

Fig. (3): Relation between adiponectin peripheral neuropathy and fundus examination in patients' group (n = 120)

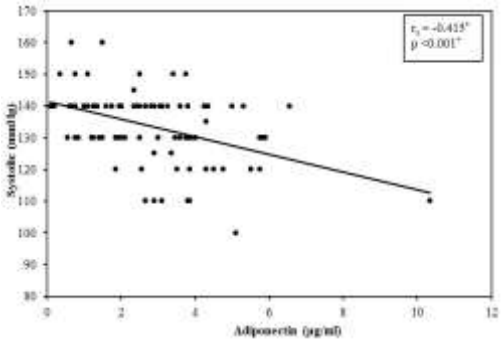

Fig. (4): Correlation between adiponectin and systolic BP

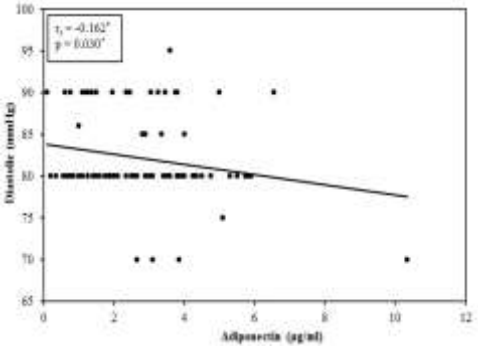

Fig. (5): Correlation between adiponectin and diastolic BP in total group.

There was statistically significant negative correlation between serum adiponectin BMI, waist circumference, fasting plasma glucose, HbA1c., serum insulin and HOMA2-IR, total cholesterol, LDL cholesterol and triglycerides but significant positive correlation with HDL cholesterol. No significant correlation between serum adiponectin and e GFR, also the same was observed among patients with diabetes with urinary A/C ratio but there was statistically significant positive and negative correlation between serum adiponectin and eGFR and also with urinary A/C ratio among the total group respectively (Table IV, Fig 6,7)

Table (IV): Correlation between adiponectin and different parameters in each group

	Adiponectin (µg/ml)					
	Control		Patients		Total sample	
	r_s	P	r_s	p	r_s	p
Age (years)	-0.125	0.340	0.235*	0.010*	0.047	0.527
DM since years	-	-	0.021	0.821	0.021	0.821
BMI (kg/m^2)	0.003	0.980	-0.240*	0.008*	-0.569*	<0.001*
Waist circumference (cm)	0.062	0.637	-0.226*	0.013*	-0.518*	<0.001*
Systolic (mmHg)	0.034	0.799	-0.163	0.075	-0.415*	<0.001*
Diastolic (mmHg)	-0.089	0.499	0.002	0.979	-0.162*	0.030*
FPG (mg/dl)	-0.124	0.345	-0.339*	<0.001*	-0.608*	<0.001*
Insulin (µIU/ml)	0.088	0.503	-0.096	0.296	-0.146*	0.049*
HOMA – IR	0.225	0.084	-0.343*	<0.001*	-0.566*	<0.001*
HbA1c (%)	0.089	0.497	-0.274*	0.002*	-0.582*	<0.001*
Total cholesterol (mg/dl)	0.040	0.760	-0.173	0.058	-0.386*	<0.001*
Triglycerides (mg/dl)	-0.033	0.802	-0.306*	<0.001*	-0.487*	<0.001*
LDL (mg/dl)	-0.088	0.505	-0.222*	0.015*	-0.356*	<0.001*
HDL (mg/dl)	0.069	0.599	0.247*	0.007*	0.204	0.006*
eGFR (ml/min/1.73 m^2)	-0.105	0.425	-0.054	0.555	0.276*	<0.001*
A/C ratio (µgm/mg)	-0.048	0.715	-0.132	0.151	-0.515*	<0.001*

r_s: Spearman coefficient

*: Statistically significant at $p \leq 0.05$

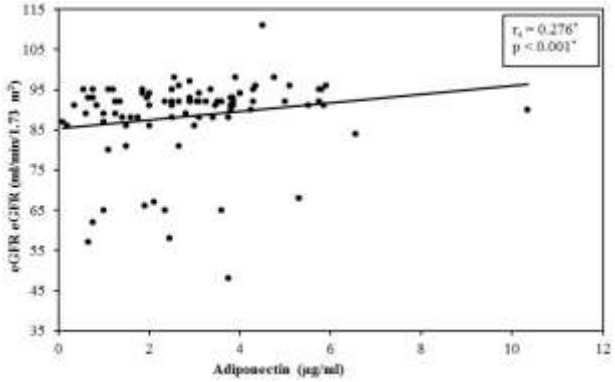

Fig. (6): Correlation between adiponectin and eGFR in total group

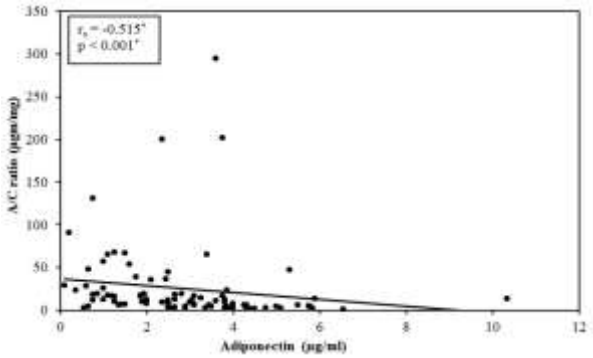

Fig. (7): Correlation between adiponectin and A/C ratio in each group

As regard the relations between serum levels of adiponectin and antidiabetic drugs in patients' group, there was statistically significant increase in serum adiponectin levels in patient using glitazon and DPP4, but there was no statistically significant difference in those using or not using metformin, sulphonylureas or insulin. On the other hand, regarding the relations between serum levels of adiponectin and antihypertensive drugs, there was increase in serum adiponectin levels among diabetic group using ACEI, BB and diuretics than in patients using ARBs, and CCB but this was not statistically significant. There was statistically significant increase in serum adiponectin levels among diabetic group using statins in comparison with those who didn't use statin (Table V, Fig 8-9).

Table (V): Relation between adiponectin and drug history in patients group (n = 120)

Drug history	N	Adiponectin (µg/ml)			U	p
		Min. – Max.	Mean ± SD.	Median		
Metformin						
No	**14**	0.65 – 2.65	1.84 ± 0.79	2.10	660.0	0.502
Yes	**106**	0.10 – 10.35	2.34 ± 1.80	1.95		
Sylphnylurea						
No	**56**	0.10 – 3.80	1.96 ± 1.16	1.98	1566.0	0.234
Yes	**64**	0.55 – 10.35	2.56 ± 2.05	1.95		
Glitazon						
No	**108**	0.10 – 10.35	2.0 ± 1.52	1.85	24.0*	<0.001*
Yes	**12**	3.80 – 6.55	4.87 ± 1.16	4.85		
DPP4						
No	**98**	0.10 – 10.35	2.16 ± 1.73	1.85	776.0*	0.040*
Yes	**22**	0.55 – 6.55	2.81 ± 1.58	2.50		
Insulin						
No	**92**	0.10 – 10.35	2.23 ± 1.83	1.88	1074.0	0.184
Yes	**28**	0.60 – 5.30	2.46 ± 1.31	2.40		
ACE.I						
No	**95**	0.10 – 10.35	10.35 ± 10.35	10.35	1032.50	0.316
Yes	**25**	0.10 – 10.35	2.73 ± 2.21	2.35		
ARBS						
No	**100**	0.10 – 10.35	2.35 ± 1.82	2.0	916.0	0.554
Yes	**20**	0.35 – 3.75	1.94 ± 1.02	1.93		
CCB						
No	**103**	0.10 – 10.35	2.33 ± 1.80	1.95	814.50	0.646
Yes	**17**	0.35 – 3.80	1.97 ± 1.08	2.0		
BB						
No	**112**	0.10 – 10.35	2.21 ± 1.57	1.98	364.0	0.377
Yes	**8**	0.10 – 10.35	3.25 ± 3.13	2.55		
Diuretics						
No	**91**	0.10 – 10.35	2.22 ± 1.78	1.85	1139.50	0.270
Yes	**29**	0.35 – 6.55	2.48 ± 1.50	2.35		
Statin						
No	**68**	0.10 – 3.80	1.88 ± 1.09	1.95	1375.0*	0.037*
Yes	**52**	0.65 – 10.35	2.81 ± 2.19	2.10		

U: Mann Whitney test p: p value for association between Adiponectin and drug history

*: Statistically significant at $p \leq 0.05$

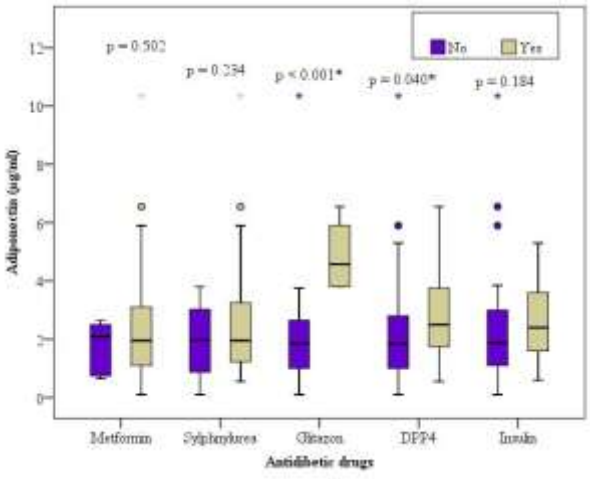

Fig. (8): Relation between adiponectin and antidibetic drugs.

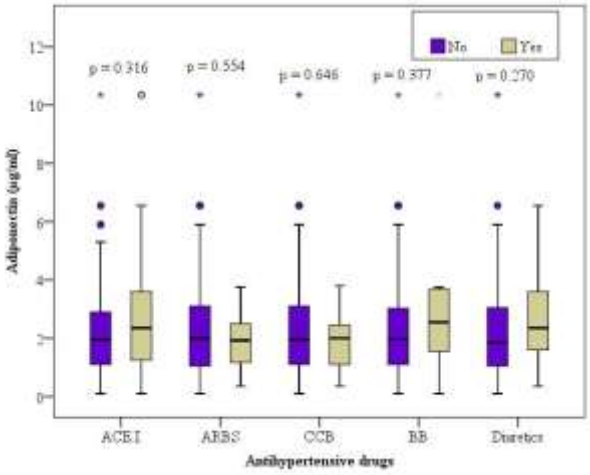

Fig. (9): Relation between adiponectin and antihypertensive drugs history.

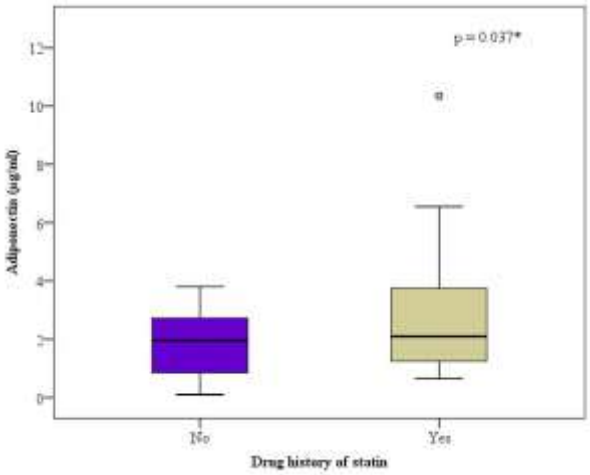

Fig. (10): Relation between adiponectin and drug history of statin.

Discussion

Central obesity appears to play an important role in metabolic syndrome and insulin resistance. [17] In the present study, type 2 diabetics had significantly ($p<0.001$) higher BMI and WC than non-diabetics, also statistically high significant increase in FBG levels, HBA1c and Insulin resistance, measured as HOMA-IR ($p<0.001$) in comparison with control group.

Hypertriglyceridemia, in conjunction with increased small dense LDL C and low HDLC levels, is an important contributor to accelerated atherosclerosis in DM and insulin-resistant conditions. [18] The results of lipid profile in the present study, showed that there was a significant ($P <0.0001$)* increase in serum triglyceride, total serum cholesterol and LDLC but a significant ($P <0.0001$) decrease in HDLC levels in diabetic patients in comparison with its values in the control group.

Adiponectin which is a bioactive adipocytokine exclusively secreted by mature adipocytes in adipose tissue possesses anti-inflammatory, antiatherogenic, and insulin-sensitizing properties. It is the most abundant adipocytokine synthesized by adipocytes. [19–21] So, adiponectin plays an important role in pathogenesis of DM and its complications. In the present study, lower level of serum adiponectin was seen in diabetics (Median =1.98 µg/ml) in relation to control subjects (Median=3.95 µg/ml), which was statistically significant ($p <0.001$) and serum adiponectin levels in diabetic patients had a negative correlation with HbA1c and fasting blood glucose. Also, serum levels of adiponectin were strongly correlated with insulin sensitivity and low adiponectin was found in patients with IR which is supported by the previous studies that found adiponectin to be a major modulator of insulin action and insulin resistance. [22] Jung CH.et al, [23] showed in concordance with the present study significant negative correlations with serum fasting insulin, and HOMA-IR and serum adiponectin levels.

The association between low adiponectin levels and obesity have been documented. [5] In the present study, there was a negative correlation between adiponectin levels and both BMI and WC. Also, there was a negative correlation with total cholesterol, LDLC, triglycerides and positive correlated with HDLC. In agreement with the results of the present study, Jung CH.et al [23] found that serum adiponectin levels was negatively correlated with total cholesterol, LDLC, triglycerides and positively correlated with HDLC.

Adiponectin can affect diabetic complications through its multiple anti-inflammatory and antiatherogenic effects which can involve vascular endothelial cell survival. [19–21]

In the present study, diabetic subject with hypertension had numerically higher serum adiponectin levels but it was non-significant. Mallamaci et al [24] et al showed similar results had significantly higher plasma adiponectin levels in hypertensive men than normotensive ones and he mentioned that plasma adiponectin levels may be affected by arterial pressure, but may also be affected by total body fat, hormones and so on. On the other hand, Murakami et al. [25] reported that low adiponectin levels can only be present in insulin resistant hypertensive patients.

Lower serum levels of adiponectin was detected among patient with positive history of CAD in the present study and this relation was statistically significant. In concordance with this, Hotta et al. [26] found that plasma levels of adiponectin in diabetic individuals with CAD were lower than in diabetic patients without CAD, while, Nakamura et al. [27] noted significantly lower adiponectin plasma levels in patients with acute myocardial infarction and unstable angina pectoris but not with stable angina pectoris, compared to the control group.

Adiponectin, were positively correlated with eGFR and negatively correlated with UACR in the present study, which are well known markeres of diabetic kidney disease (DKD). [28] In disagreement with these results, CH.et al. [23] found the mean levels of adiponectin were significantly lower in patients with DKD. While, Galovicova et al. [29] (who studied 120 patients with type 2 DM found higher plasma adiponectin levels in those with macroalbuminuria, compared to those who had normoalbuminuria,

microalbuminuria, as well as compared to controls (patients with normoalbuminuria had the lower levels of adiponectin). He concluded that diabetic nephropathy potentially plays a very important role in increasing the synthesis and secretion of adiponectin. So, adiponectin may be lower in early stages of DKD but higher in advanced stages.

Adiponectin was significantly lower in patients with diabetic retinopathy (DR) compared to those with normal fundus examination in the present study. Hatef ZS et al. [30] showed similar results among diabetic patients with retinopathy than those without. On the other hand, Jung CH.et al. [23] serum adiponectin levels were significantly higher in type 2 diabetes patients with retinopathy. A number of studies have shown that adiponectin is upregulated in damaged tissues. For example, adiponectin mRNA is detected in the liver of a mouse model of hepatic injury. So, an explanation for the increased concentration of adiponectin in DR is a possible role in tissue injury and repair. [31] Consequently, the relationship between adiponectin and DR may be different from early and advanced stages DR and it is possible that adiponectin is a marker for retinal injury, mediates angiogenesis, or elevated adiponectin may represent a state of adiponectin resistance.

Peripheral neuropathy (PN) in the present study in relation to adiponectin was positively correlated but this associations were not significant. In concordance with the present study, Cha J J et al. [32] found that high adiponectin levels were associated with increased risk of PN, but the significance of this association was lost after adjusting for confounding factors, while, Jung CH.et al [23] found that higher serum adiponectin levels were associated with increased risk of PN. In a study by Kato et al. [33] serum adiponectin was not correlated with neuropathy. So, the relationship between diabetic neuropathy and serum adiponectin, is controversial.

Serum adiponectin levels can be affected by different lines of management of type 2 DM and associated comorbidity. In the present study, a regard anti-diabetic drugs thiazolidinediones and DPP4 inhibitors were associated with significant increase serum adiponectin levels, while serum adiponectin levels were numerically increased but not significant in patients on metformin and sulphonylureas.

In agreement with the results of the present study, Phillips SA et al [34] observed no change in serum adiponectin with metformin but troglitazone treatment increased serum adiponectin levels nearly threefold. Also in a systematic review which summarizes the evidence of the effect of thiazolidinediones on circulating adiponectin levels was performed, a significant increase in adiponectin (80-178%) after thiazolidinediones treatment was observed in all included studies. [8] In ten RCTs, evaluating DPP4i (sitagliptin and vildagliptin) versus placebo or an active control drug in type 2 diabetic patients including 1495 subjects. DPP4i (sitagliptin and vildagliptin) treatment were associated with significantly elevated adiponectin levels. [9] AmetovAS and Gusenbekova DG [10] studied the effect of sitagliptin in combination with metformin as well as of metformin monotherapy. After24 weeks of therapy, adiponectin content in blood increased by 27.06% in the group receiving sitagliptin and metformin combination, and by 7.16% in the group receiving metformin monotherapy. In disagreement with the results of the current study, a meta-analysis was done to investigate and determine the role of metformin on serum adiponectin levels in patients with type 2 DM, post-treatment serum adiponectin levels were higher than pre-treatment levels. [35] Also, Tsunekawa T et al. (7) reported a highly significant elevation in plasma adiponectin concentration after 8 weeks of glimepiride treatment. By contrast, the control group treated with glibenclamide did not show change in plasma adiponectin concentration.

Different types of antihypertensive drugs which are used for control of hypertension may have different effects on adiponectin. ACEI and angiotensin converting enzyme inhibitors was found to increase adiponectin levels and improve insulin sensitivity without affecting the degree of body adiposity,[36] Calcium channel blockers may impair insulin release, but this effect on glucose metabolism appears to be balanced by their action to increase peripheral glucose uptake [12,13] on the other, hand beta-blockers

differ in terms of their mechanism of action and their effects on glucose and lipid metabolism. [37]

In the present study, there was numerical increase in serum adiponectin levels among diabetic group using ACEI, BB and diuretics than in patients using ARBs, and CCB but this was not statistically significant. YILMAZ MI et al. [38] observed that plasma adiponectin concentrations significantly increased with ramipril and valsartan. Derosa, G., et al (2010) [39] Within CCBs, candesartan, but not Olmesartan therapy, over the period of a year resulted in increased adiponectin and insulin sensitivity in T2DM hypertensive patients, even though BP lowering was similar in both treatment groups. Piecha, G., et al. [11] In a comparison of enalapril, metoprolol, amlodipine, and indapamide, no changes in adiponectin level were seen with enalapril, amlodipine, or metoprolol, whereas a reduction in adiponectin was seen with indapamide. This reduction in adiponectin with the thiazide-like diuretic correlated with increased insulin resistance. Patients treated with nebivolol and carvedilol were found to had a trend toward more improvement in insulin sensitivity and glucose tolerance. Nebivolol and carvedilol therapy significantly improved glycemic profile, which may improve prognosis. [40] Nebivolol and metoprolol were shown to have similar reductions in blood pressures but differ in their effects on plasma adiponectin levels. Nebivolol improved insulin resistance and oxidative stress while there were no significant alterations in the metoprolol group. [40] Also, Hara Y et al. [41] showed that, there was a significant worsening of insulin resistance in patients receiving atenolol.

In the present study, there was statistically significant increase in serum adiponectin levels among diabetic group using statins. The association between statin therapy and adiponectin levels vary upon statin type and dose.[14-6]

Chrusciel P et al. [14] showed in the results of their meta-analysis significant increase in plasma adiponectin levels following statin therapy and the pleiotropic adiponectin-elevating effect of statins may be explained, at least in part, the putative benefits of these drugs in reducing cardiovascular risk in diabetic patients. Atorvastatin and pravastatin were more effective (numerically) than other statins in increasing plasma adiponectin concentrations. Simvastatin and atorvastatin were found to improve insulin sensitivity in diabetic patients; [15] However, others have reported that simvastatin either did not change or worsened insulin sensitivity in diabetic patients. [16,42] So, statin in most of studies improve adiponectin levels.

Conclusion

The quantitative changes in adiponectin provide insight into how serum adiponectin levels can be affected by different lines of management of type 2 diabetes and associated comorbidity. Thus, medications that improve insulin sensitivity and glucose control, as well as blood pressure control, have a significantly beneficial role beyond blood glucose or blood pressure control.

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Original Article

Thrombomodulin-Protein (C): Relation to Cardiovascular Risk in Chronic Kidney Disease and Hemodialysis Patients

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Abstract

Background: Patients receiving maintenance hemodialysis (HD) present with hemostatic abnormalities, which may be aggravated by comorbid conditions. Thrombomodulin (TM), which is an initiator of the anticoagulant protein C pathway, was assessed as a marker for vascular pathology in these patients category.

Methods: : The study included 90 subjects divided into 3 groups; Group I consisted of 30 patients on maintenance hemodialysis for more than 6 months recruited from the dialysis unit from El Moasaa hospital. These patients undergo bicarbonate HD 3 times/week, 4 hours/session using polysulphone dialyzer. Group II consisted of 30 patients with CKD stage 3-5 not yet on dialysis and were recruited from outpatient clinic or admitted in Alexandria Main University hospital and group III consisted of 30 age and sex matched individuals and were taken as a control group. Thrombomodulin and protein C were compared in the three groups.

Results & Conclusion TM, Protein C and CRP were significantly higher in group I and II than group III, with no significant differences between group I and II. Thrombomodulin was significantly related to creatinine, LVESD and LVEDD, while CRP was significantly related to eGFR, albumin/ creatinin ratio, LDL and HDL. Protein C was significantly related to eGFR and albumin/creatinin ratio. So TM is associated with HD and CKD patients.

Key words: Chronic Kidney Disease, Coagulation, inflammation, soluble thrombomodulin, Protein C, CRP

Introduction

Cardiovascular diseases are the most frequent cause of morbidity and mortality in patients with chronic kidney disease (CKD). The adult population with impaired renal function is characterized by high incidence of classical cardiovascular risk factors. Moreover, other factors such as endothelial dysfunction (ED) and oxidative stress are considered as causes of cardiovascular disease. Initially, ED was studied in the most advanced stages of CKD (dialyzed patients) and a reduction in endothelial-dependent vasodilatation of the brachial artery was demonstrated. Recent studies supported the concept that ED is present at early stages of chronic kidney disease.[(1)]

Noninvasive methods for ED assessment include evaluation of shed endothelial surface layer components—soluble thrombomodulin (TM) and syndecan-1. TM is a transmembrane glycoprotein built from 5 domains. Its anticoagulant effect is mediated by the binding of thrombin and activation of protein C.[(2)]

Active protein C inhibits the coagulation cascade through degradation of active factors V and VIII and inactivation of tissue plasminogen activator inhibitor. Thrombomodulin also has an anti-inflammatory activity.[(3)] In case of endothelial cell damage, its transmembrane

portion is released and may be identified as a soluble TM (sTM) and the TM molecule loses its vasoprotective properties.[4]

The aim of the current study is to study thrombomodulin and protein c level in CKD and hemodialysis patients and correlate their level with other cardiovascular risk factors.

Material and Methods

The study was approved by the ethics committee of Alexandria University.

Subjects

The study enrolled 90 subjects divided into three groups; Group I consisted of 30 patients on maintenance hemodialysis for more than 6 months recruited from the dialysis unit of El Moasaa hospital. These patients undergo bicarbonate HD 3 times/week, 4 hours/session using polysulphone dialyzer. Group II consisted of 30 patients with CKD stage 3-5 not yet on dialysis and were recruited from outpatient clinic or admitted in Alexandria Main University hospital and group III consisted of 30 age and sex match individuals and were taken as a control group.

Any patient who was a smoker or had a history of diabetes, hepatic, cardiac or cerebrovascular disease was excluded from the study. After collection of patients, the study was conducted according to the local ethical committee of Alexandria Faculty of medicine and according to declaration of Helsinki for studies on humans an informed consent that was from all participants in the study.

Methods

All studied population were subjected to full history taking stressing on cardiovascular system complaints and complete clinical examination especially CVS.

Lab investigation for each subject included, Complete blood count (CBC)., Complete lipid profile which consisted of total cholesterol, low density lipoprotein, high density lipoprotein, total triglyceride, Blood urea, Serum creatinine, eGFR by CKD-epidemiology for G II, Urinary protein excretion in CKD group, Serum calcium, phosphorus, iPTH, C reactive protein., Thrombomoduline (TM) by enzyme linked immunosorbant assay (ELISA), Protein c by enzyme linked immunosorbant assay (ELISA).

Other investigations included ; electrocardiography (ECG) stressed on Q wave and ST segment and T wave deviation and echocardiography stressed on left ventricular end diastolic (EDD), end systolic dimension (ESD), Ejection fraction(EF) and Regional wall motion abnormalities(RWM)

Statistics

Continuous data were expressed as mean + SD values or as median and range, according to the distribution, and were analyzed for differences using the Student's *t*-test. Categorical data were presented as numbers, and chi-squared test was used for statistical comparison of percentages. Binary logistic regression analysis was also carried out, and the receiver operating characteristic (ROC) curve was applied to determine the predictive value of endometrial parameters. Discriminant analysis was used to evaluate the predictive ability of all of the endometrial parameters combined. The significance level for all analyses was $P < 0.05$. Statistical analysis was carried out using the Statistical Package for Social Sciences (SPSS Inc, Chicago, IL, USA).

Results

The percentage of males was 50%, 57.7% and 47.7% for group I, group II and group III respectively(p=0.733). The age ranged from 25.0 to 70 in group I, from 23 to 68 in group II and from 25 to 70 in group III. The mean age was 50.23 ± 13.57, 48.10 ± 12.41 and 49.43 ± 11.69 respectively. (p=0.804). There were no significant statistical differences according to age and sex between the three groups. Table I

Table (I): Comparison between the three studied groups according to renal function

Renal function	Group 1 (n = 30)	Group 2 (n = 30)	Group 3 (n = 30)	H	p
Blood urea (mg/dl)					
Min. – Max.	76.0 – 289.0	64.0 – 198.0	18.0 – 45.0		
Mean ± SD.	140.3 ± 46.42	133.8 ± 44.78	29.47 ± 7.50	59.416*	<0.001*
Median	125.5	133.5	29.0		
Sig. bet. grps	p_1=0.809,p_2<0.001*,p_3<0.001*				
Serum creatinine (mg/dl)					
Min. – Max.	5.20 – 14.0	1.6 – 4.50	0.74 – 1.10		
Mean ± SD.	10.08 ± 2.38	2.65 ± 0.95	0.94 ± 0.10	79.168*	<0.001*
Median	10.50	2.40	0.94		
Sig. bet. grps	p_1<0.001*,p_2<0.001*,p_3<0.001*				

H: H for **Kruskal Wallis test,** Pairwise comparison bet. each 2 groups was done using **Post Hoc Test (Dunn's for multiple comparisons test)**

Table (II): Distribution of group II (CKD) cases according to eGFR (n=30)

eGFR	**No.**	**%**
G 3a (45 - 59)	4	13.3
G3b (30 - 44)	10	33.3
G4 (15 - 29)	13	43.3
G5 (<15)	3	10.0

Table (III): Distribution of the studied cases according to duration of dialysis (n=30)

Duration of dialysis	**No.**	**%**
<5 years	4	13.3
5 – 10 years	7	23.3
>10	19	63.3
Min. – Max.	2.0 – 22.0	
Mean ± SD.	13.90 ± 5.92	
Median	15.0	

Table (IV): Descriptive analysis of the studied cases according to URR in group 1 (n = 30)

	Min. – Max.	Mean ± SD.	Median
URR %	42.60 – 74.0	66.95 ± 7.63	69.80

Group 1: Dialysis

Table (V): Descriptive analysis of the studied cases according to eGFR in group 2 (n = 30)

	Min. – Max.	Mean ± SD.	Median
eGFR (ml/min)	9.82 – 56.21	29.81 ± 13.25	26.43

Group 2: CKD

Table (VI): Comparison between the two studied groups according to albumin/ creatinine ratio

Albumin/ creatinine ratio (mg/gm)	**Group 2 (n = 30)**	**Group 3 (n = 30)**	**U**	**p**
Min. – Max.	10.0 – 1000.0	5.0 – 30.0		
Mean ± SD.	260.8 ± 255.5	17.27 ± 6.76	87.0*	<0.001*
Median	225.0	17.50		

U: **Mann Whitney test** **p:** p value for comparing between the **two groups** *: Statistically significant at $p \leq 0.05$

Table (VII): Comparison between the three studied groups according to protein C, CRP, and Thrombomodulin

Protein C (IU/dl)	**Group 1 (n = 30)**	**Group 2 (n = 30)**	**Group 3 (n = 30)**	**F**	**p**
Min. – Max.	17.7 – 85.0	30.6 – 111.0	45.0 – 138.0		
Mean ± SD.	51.01 ± 16.80	56.69 ± 15.89	103.23 ± 21.87	72.916*	<0.001*
Median	52.10	54.35	101.50		
Sig. bet. grps	p_1=0.459,p_2<0.001*,p_3<0.001*				
CRP (mg/dl)	Group 1 (n = 30)	Group 2 (n = 29)	Group 3 (n = 30)	H	p
Min. – Max.	2.0 – 31.0	1.0 – 20.0	1.0 – 3.0		
Mean ± SD.	6.30 ± 7.7	6.0 ± 6.12	2.21 ± 0.67	13.960*	<0.001*
Median	2.0	4.0	2.30		
Sig. bet. grps	p_1=0.238,p_2=0.039*,p_3<0.001*				
Thrombomodulin (ng/ml)	Group 1(n = 30)	Group 2 (n = 30)	Group 3 (n = 30)	H	p
Min. – Max.	0.99 – 2.80	0.89 – 1.38	0.55 – 1.54		
Mean ± SD.	1.35 ± 0.39	1.20 ± 0.10	0.94 ± 0.18	47.755*	<0.001*
Median	1.31	1.18	0.97		
Sig. bet. grps	p_1=0.114,p_2<0.001*,p_3<0.0.001*				

H: H for **Kruskal Wallis test,** Pairwise comparison bet. each 2 groups was done using **Post Hoc Test (Dunn's for multiple comparisons test)**

Table (VIII): Comparison between the three studied groups according to lipid profile

Lipid profile	Group 1 (n = 30)	Group 2 (n = 30)	Group 3 (n = 30)	Test of Sig.	p
Total cholesterol (mg/dl)					
Min. – Max.	118.0 – 300.0	138.0 – 330.0	128.0 – 187.0		
Mean ± SD.	170.2 ± 44.57	174.13 ± 39.95	166.10 ± 14.59	H= 1.473	0.479
Median	157.0	166.0	165.50		
Triglyceride (mg/dl)					
Min. – Max.	110.0 – 198.0	112.0 – 200.0	112.0 – 150.0		
Mean ± SD.	143.4 ± 22.53	138.8 ± 22.57	130.4 ± 11.27	F= 3.412*	0.037*
Median	140.0	136.5	132.0		
Sig. bet. grps	p_1=0.626,p_2=0.031*,p_3=0.229				
LDL (mg/dl)					
Min. – Max.	75.0 – 250.0	65.0 – 280.0	68.0 – 98.0		
Mean ± SD.	112.2 ± 56.94	105.7 ± 51.36	84.53 ± 9.07	H= 7.335*	0.025*
Median	91.0	92.50	86.0		
Sig. bet. grps	p_1=0.633,p_2=0.038*,p_3=0.011*				
HDL (mg/dl)					
Min. – Max.	29.0 – 72.0	37.0 – 72.0	60.0 – 81.0		
Mean ± SD.	55.10 ± 10.03	57.17 ± 7.76	69.03 ± 4.77	H= 46.876*	<0.001*
Median	57.0	58.50	69.0		
Sig. bet. grps	p_1=0.621,p_2<0.001*,p_3<0.001*				

H: H for **Kruskal Wallis test,** Pairwise comparison bet. each 2 groups were done using **Post Hoc Test (Dunn's for multiple comparisons test)**

Table (IX): Comparison between the three studied groups according to ECG parameters

	Group 1 (n = 30)		**Group (n = 30)**		**Group (n = 30)**		**χ^2**	**p**
	No.	**%**	**No.**	**%**	**No.**	**%**		
Q wave								
Absent	21	70.0	19	63.3	30	100.0	13.243*	0.001*
Present	9	30.0	11	36.7	0	0.0		
Sig. bet. grps	p_1=0.584,p_2=0.002*, p_3<0.001*							
ST segment								
Depressed	24	80.0	23	76.7	0	0.0	49.253*	<0.001*
Isoelectric	6	20.0	7	23.3	30	100.0		
Sig. bet. grps	p_1=0.754,p_2<0.001*, p_3<0.001*							
T wave deviation								
Normal	10	33.3	7	23.3	30	100.0	41.822*	^{MC}p <0.001*
Tall peaked	3	10.0	3	10.0	0	0.0		
Inverted	17	56.7	20	66.7	0	0.0		
Sig. bet. grps	p_1=0.748,p_2<0.001*, p_3<0.001*							

χ^2: **Chi square test**, MC :Monte Carlo test

Table (X): Comparison between the three studied groups according to LVESD, LVEDD, EF % and RWMA

	Group 1 (n = 30)		**Group 2 (n = 30)**		**Group 3 (n = 30)**		**H**	**p**
LVESD (mm)								
Min. – Max.	33.0 – 55.0		35.0 – 56.0		37.0 – 40.0			
Mean ± SD.	43.50 ± 7.45		42.70 ± 6.19		38.43 ± 0.86		9.098*	0.011*
Median	41.50		41.0		38.0			
Sig. bet. grps	p_1=0.852,p_2=0.007*,p_3=0.012*							
LVEDD(mm)								
Min. – Max.	52.0 – 67.0		50.0 – 62.0		59.0 – 64.0			
Mean ± SD.	57.4 ± 3.40		56.97 ± 3.13		61.10 ± 1.21		41.110*	<0.001*
Median	58.0		58.0		61.0			
Sig. bet. grps	p_1=0.952,p_2=0.007*,p_3=0.012*							
EF %	Group 1 (n = 30)		Group 2 (n = 30)		Group (n = 30)		H	P
Min. – Max.	36.0 – 61.0		40.0 – 68.0		59.0 – 76.0			
Mean ± SD.	54.03 ± 7.31		57.60 ± 6.22		66.50 ± 4.73		49.701*	<0.001*
Median	56.50		58.50		66.0			
Sig. bet. grps	p_1=0.053,p_2<0.001*,p_3<0.001*							
RWMA								
No	5	16.7	6	20.0	30	100.0	53.848*	<0.001*
Yes	25	83.3	24	80.0	0	0.0		
Sig. bet. grps	p_1=0.739,p_2<0.001*, p_3<0.001*							

H: H for **Kruskal Wallis test,** Pairwise comparison bet. each 2 groups was done using **Post Hoc Test (Dunn's for multiple comparisons test)**

Table (XI): Correlations between different parameters for cases (Group 1 + 2) (n=60)

	CRP		**Thrombomodulin**		**Protein C**	
	r_s	**p**	**r_s**	**P**	**r**	**p**
Age (years)	157	0.232	0.122	0.354	-0.029	0.828
Hb (g/dl)	0.030	0.817	0.239	0.066	-0.158	0.229
WBCs (10^3/ul)	-0.149	0.256	0.068	0.608	0.051	0.699
Platelets (10^3/ul)	0.045	0.735	0.020	0.879	-0.183	0.163
Blood urea (mg/dl)	0.586*	<0.001*	0.043	0.745	-0.168*	0.201
Serum creatinine (mg/dl)	0.312*	0.015*	0.327*	0.011*	-0.325*	0.011*
URR %	0.189	0.317	0.345	0.062	-0.167	0.378
eGFR (ml/min)	-0.663*	<0.001*	-0.346*	0.050*	0.915*	<0.001*
Albumin/ creatinine ratio (mg/gm) (n = 30)	0.987*	<0.001*	0.157	0.408	-0.582*	0.001*
Serum calcium (mg/dl)	-0.020	0.880	0.032	0.806	0.054	0.684
Serum phosphorus (mg/dl)	0.187	0.153	0.024	0.855	-0.108	0.413
PTH	0.105	0.426	0.008	0.950	-0.012	0.930
Total cholesterol (mg/dl)	0.257*	0.047*	-0.222	0.088	0.022	0.867
Triglyceride (mg/dl)	0.897*	<0.001*	0.150	0.253	-0.186	0.156
LDL (mg/dl)	0.126	0.338	-0.208	0.110	-0.037	0.779
HDL (mg/dl)	-0.910*	<0.001*	-0.138	0.295	0.138	0.293
LVESD	-0.083	0.529	0.255*	0.049*	-0.114	0.385
LVEDD	-0.078	0.556	0.340*	0.008*	-0.042	0.749
EF %	-0.084	0.524	0.151	0.250	0.134	0.306

r: Pearson coefficient r_s: Spearman coefficient *: Statistically significant at $p \leq 0.05$

Table (XII): Correlations between ECHO parameters for cases (Group I + II) (n=60)

ECHO parameters	Lipid profile								Protein C (IU/dl)		CRP (mg/dl)		Thrombomodulin (ng/ml)	
	Total cholesterol (mg/dl)		Triglyceride (mg/dl)		LDL (mg/dl)		HDL (mg/dl)							
	r_s	p	r_s	p	r_s	p	r_s	p	r_s	p	r_s	p	r_s	p
LVESD (mm)	0.173	0.186	–0.029	0.823	0.190	0.146	0.043	0.744	–0.114	0.385	–0.083	0.529	0.255*	0.049*
LVEDD(mm)	0.160	0.223	0.021	0.873	0.165	0.208	–0.016	0.905	–0.042	0.749	–0.078	0.556	0.340*	0.008*
EF %	0.112	0.395	–0.120	0.363	0.065	0.621	0.127	0.333	0.134	0.306	–0.084	0.524	0.151	0.250

r_s: Spearman coefficient *: Statistically significant at $p \leq 0.05$

Table (XIII): Relation between RWMA and different parameters (n=60)

	RWMA		Test of Sig.	p
	No (n=11)	Yes (n=49)		
Total cholesterol (mg/dl) Min. – Max. Mean ± SD. Median	 142.0 – 220.0 168.8 ± 21.65 167.0	 118.0 – 330.0 172.9 ± 45.48 162.0	U= 238.0	0.547
Triglyceride (mg/dl) Min. – Max. Mean ± SD. Median	 112.0 – 150.0 133.2 ± 13.17 136.0	 110.0 – 200.0 142.9 ± 23.82 140.0	t= 1.30	0.199
LDL (mg/dl) Min. – Max. Mean ± SD. Median	 80.0 – 250.0 106.8 ± 48.02 97.0	 65.0 – 280.0 109.4 ± 55.53 92.0	U= 205.0	0.217
HDL (mg/dl) Min. – Max. Mean ± SD. Median	 52.0 – 67.0 59.18 ± 4.58 59.0	 29.0 – 72.0 55.45 ± 9.56 57.0	U= 220.0	0.343
Protein C (IU/dl) Min. – Max. Mean ± SD. Median	 17.70 – 93.0 49.35 ± 20.66 46.90	 17.90 – 111.0 54.86 ± 15.45 54.60	t= 1.002	0.320
CRP (mg/dl) Min. – Max. Mean ± SD. Median	 1.0 – 18.0 4.73 ± 4.86 3.0	 1.0 – 31.0 6.47 ± 7.30 3.0	U= 238.50	0.545
Thrombomodulin (ng/ml) Min. – Max. Mean ± SD. Median	 1.12 – 1.40 1.26 ± 0.09 1.26	 0.89 – 2.80 1.28 ± 0.32 1.21	U= 224.0	0.385

t: **Student t-test** U: **Mann Whitney test** p: p value for comparing between the two category

Table (XIV): Agreement (sensitivity, specificity) for Thrombomodulin (ng/ml) to predict dialysis cases (vs CKD) (Group I vs II)

	AUC	p	95% C.I	Cut off	Sensitivity	Specificity	PPV	NPV
Thrombomodulin (ng/ml)	0.668*	0.025*	0.527 – 0.809	**>1.245**	60.0	73.33	69.2	64.7

AUC: Area Under a Curve p value: Probability value

CI: Confidence Intervals NPV: Negative predictive value

PPV: Positive predictive value *: Statistically significant at $p \leq 0.05$

Table (XV): Agreement (sensitivity, specificity) for Thrombomodulin (ng/ml) to predict cases (vs control)

	AUC	p	95% C.I	Cut off	Sensitivity	Specificity	PPV	NPV
Protein C	0.949*	<0.001*	0.893 – 1.004	**≤70.1**	95.0	93.3	96.6	90.3
Thrombomodulin (ng/ml)	0.937*	<0.001*	0.867 – 1.006	**>0.993**	95.0	70.0	86.4	87.5
CRP	0.727*	<0.001*	0.624 – 0.831	**>3**	46.67	100.0	100.0	48.4

AUC: Area Under a Curve p value: Probability value

CI: Confidence Intervals NPV: Negative predictive value

PPV: Positive predictive value *: Statistically significant at $p \leq 0.05$

Discussion

As has been known for decades, the leading cause of death in CKD patients and those undergoing hemodialysis (HD) is cardiovascular disease, in which both vascular calcification (VC) and endothelial damage are the crucial underlying process. In VC associated with CKD, serum concentrations of inorganic phosphate (Pi) are believed by some studies to be the most important factor [(1)].

Thrombomodulin (TM) is an endothelial cell membrane-bound glycoprotein containing five distinct domains: an NH2-terminal lectin-like region, a domain with an epidermal growth factor-like structure, an O-glycosylation site-rich domain, a transmembrane domain, and a cytoplasmic tail domain [(2)].TM is ubiquitously expressed on endothelial cells (ECs) and binds to thrombin, activates the protein C pathway, and acts as an anticoagulant. However, several evidences revealed that TM also has protein C- and thrombin-independent physiological functions [(3)]. In addition to being an anticoagulant, TM was found to possess multiple functions such as anti-inflammatory and scavenging actions for high-mobility group box 1, damage-associated molecular patterns, pathogen-associated molecular patterns, and other molecules in the vascular system [(4)]. In recent years, increased plasma concentrations of TM and tissue factor in patients receiving maintenance HD have been shown to be markers of vascular EC injury [(5)]. Nevertheless, whether FGF23 at elevated levels in HD patients exerts direct toxic effects on EC barrier function was still unclear. This study therefore aimed to assess the mechanism by which increased FGF23 levels may influence TM expression in ECs in the presence of α-Klotho.

The first finding of the current study was that TM was significantly higher in CKD and HD patients in comparison to healthy control, however, the level were comparable in HD and CKD patients with no significant differences. Previous studies have indicated that coagulation and inflammation are important processes that contribute to kidney injury and the progress of kidney disease [(6)]. Therefore, the roles of endothelial cells dysfunction and inflammation have been elucidated recently as interactive mechanisms of kidney injury. Soluble TM is the important factor correlated simultaneously with the processes of coagulation and inflammation [(7)]. For this reason soluble TM may play critical roles in the development of kidney diseases.

Our finding was in complete agreement with the previous study of Bao et al[(8)] which aimed to test the serum levels of soluble (TM) in patients with (CKD) 3-5 and to assess their connection with the different stages and severity of disease. They conducted their study on Sixty-seven patients with CKD and nineteen healthy volunteers served as healthy controls. Their conclusion was that soluble TM concentrations significantly increase in the CKD patients and may play critical roles in the development of CKD, as a biomarker of endothelial cells damage, anticoagulation and anti-inflammation.

Tanaka et al[(9)] also agreed to our finding. They found not only TM was significantly higher in CKD and HD patients compared to healthy control, but also other endothelial cell injury markers like Growth Factor-23/α-Klotho. Another study that completely agreed to our results is Dane et al[(10)] as they found that elevated TM level is an essential component of the inflammatory process of the CKD patients.

A logical explanation for this finding is that TM expression was enhanced in order to protect the kidney from thrombotic injuries while its increase was not enough to inhibit thrombosis. The increase in circulating soluble TM might not only reflect endothelial cell damage, but also be a sign of increased synthesis, in order to enhance the anticoagulant property of the endothelial cell surface. Studies demonstrate that raised TM concentrations imply an increased risk of bleeding.[(11)] Therefore, during the progress of CKD TM may be released from injured endothelial cells to prevent ongoing thrombosis.

However, relative insufficiency in TM activity allows thrombus formation. The present study supports the correlation between increased serum soluble TM levels and CKD, and is consistent with the previous reports.[11]

The second observation of our study is that protein C was significantly lower in the CKD as well as HD patients when compared to healthy controls. Nevertheless, the levels in CKD and HD population were similar without significant statistical differences. In renal diseases and uremia, a complex association between bleeding tendency and hypercoagulability have been described [12,13]. The reason for this is not quite clear but several papers dealing with renal disorders and hemostasis have been published [14,15]. The results are somewhat conflicting but some of the consistent findings as elevated factor V and VIII and the impaired fibrinolysis could indicate a central role for protein C in the pathogenesis of thrombosis in renal diseases.

The reason for the low protein C activity in chronic renal failure is not clear. The mechanisms regulating protein C levels are only poorly known. Decreased synthesis is not a likely cause as liver function was not compromised in the patients. Urinary loss of protein C could be negligeable because In nephrotic syndrome known to be associated with thrombotic tendency [16,17] a loss of protein C in the urine could be expected as in the case of antithrombin III [18]. Many studies found normal protein C activity in nephrotic syndrome, so urinary loss of protein C is probably of minor importance. It is tempting to assume that progressive uremic intoxication in some way depresses the protein C activity. This is supported by many authors who observed that C activity rose correspondingly to the fall in serum creatinine after HD [19].

The finding of low protein C in CKD patients was in complete accordance with the previously published results of Sorenson et al [20] who conducted this study on 19 healthy controls and in 52 patients with renal diseases and clinically divided them into three groups I) Nephrotic syndrome, II) Renal insufficiency, III) Terminal uremia, requiring maintenance dialysis. In the nephrotic syndrome they found that protein C levels were normal, but in renal insufficiency and terminal uremia the protein C activity was significantly decreased.

Maruyama et al[21] was another study that agreed to our results completely as they found that protein C levels decreased significantly in HD patients specially with long term dialysis. Matching our finding, Molino et al [22] also found that both CKD and HD patients were associated with low levels of protein C and this may contribute to the hyper-coagulable state of this population.

However, some studies did disagree to our findings like Lai et al[23] who studied the effect of hemodialysis on natural coagulation inhibitors including protein C, protein S (PS), and antithrombin III (AT III) in 20 uremic patients on maintenance hemodialysis immediately before, during, and after dialysis treatment. They found that predialysis plasma PC antigen level and functional activity were not different from those of normal controls. A significant correlation between the antigen level and functional activity of PC, PS, and AT III was demonstrated in healthy controls, but not in hemodialysis patients.

Another important finding in our study is that all ECG and ECHO parameters were significantly different in CKD and HD patients when compared to normal control. Most CKD and dialysis patients had abnormal Q wave, ST segment, T wave, EF% and RWMA while all healthy controls were normal. These data shows that ECG abnormalities are common in CKD patients and in our study the most common abnormality was ST depression. We have also found that Echo changes namely EF% were significantly related to urea, creat, eGFR and phosphorus, while RWMA was significantly correlated to age, calcium, phosphorus and duration of dialysis. Chronic kidney disease is

associated with a markedly increased risk for cardiovascular events and mortality [24]. In other studies, abnormal ECG findings were noticed in 50–86% of all patients.[24,25] Disturbances of the cardiac electrical cycle might be detectable in early stages of CKD. Prolonged QT intervals and greater QT dispersion have been reported among uncontrolled case series of long-term hemodialysis patients [26]. In contrast, a recent study found no association of kidney function with QT interval duration among approximately 200 nondialysis patients with earlier stages of CKD [27]. Existing data do not clarify whether electrocardiogram (ECG) disturbances are more prevalent among patients with CKD or which ECG markers might be particularly important.[28]

Gromadziński et al [29] found that significant LV diastolic dysfunction appeared only in CKD stages 4 and 5 and rarely accompanied its increased estimated LV end-diastolic filling pressure. They found also that patients with severe kidney failure did not differ from non-CKD patients as regards systolic and diastolic tricuspid annular velocities. Thus, they stated that kidney dysfunction does not negatively affect RV function.

Shivendra et al[30] aimed to evaluate and analyze the echocardiographic changes in end stage renal disease patients on maintenance hemodialysis. They performed M-mode echocardiography in 35 ESRD patients during interdialytic period after 18 hours of the last session, without obvious clinical evidence of coronary artery disease, valvular heart disease, congenital heart disease and pericardial effusion. They found that Echocardiography revealed LV dilation and diastolic dysfunction in 18 (51.2%), left ventricular hypertrophy (LVH) in 17 (48%), systolic dysfunction and pericardial effusion in 10 (28.57%) and 6 (17.14%) patients respectively. They found also that RWMA was present in 8.5% and valvular calcification was not seen in their patient group. In sub-group of patients with Hb<10 gm%, LVH was present in 71.42% (15) vs 14.28% (2) in patient group with Hb ≥ 10 gm% (p=0.002). Hypertensive patient population also had higher prevalence of LVH (51.85%)] and systolic dysfunction and RWMA was absent in normotensive group.

Echocardiographic findings in other studies have also observed presence of systolic dysfunction in 20% and diastolic dysfunction in 50% patients [31,32]. Agarwal S. et al. had observed diastolic dysfunction in 53.2% and systolic dysfunction in 30% patients with severe CKD (S. Cr. >6 mg %)[33]. In a study conducted by Laddha M et al. in 2014, reported LVH in 74%, systolic dysfunction in 24.3%, diastolic dysfunction in 61.4% and pericardial effusion in 14.35% of ESRD patients on hemodialysis[34]. Zoccali C et al. had reported incidence of LVH and systolic dysfunction of 77% and 22% respectively in ESRD population on hemodialysis[35]. Valvular calcifications are four times more common in dialysis patients compared to general population[36]. None of our patients had valvular calcification probably because of small study population.

An interesting finding of our study is that protein C but not TM was significantly correlated with the severity of the CKD condition. This was matching the results of Sorenson et al[20] who found that there is a significant correlation between decreasing protein C and progressive renal failure. However, this was inconsistent with Bao et al [8] who found that soluble TM concentrations significantly increase in the CKD patients and are associated with the severity of the disease.

To the best of our knowledge, we were the first to show that TM was significantly correlated to three parameters which were creatinine, LVESD and LVEDD. And also we were the first to discuss the sensitivity and specificity of protein C and TM in differentiating patients with kidney function abnormalities from normal kidney function patients.

The CKD population is at increased risk of cardiovascular disease. Until recently, it was

considered that this risk was only increased with GFR < 60 ml/min/1.73 m2, but modern long-term observations indicate that the increase in cardiovascular mortality is already present with a decrease in GFR below 90 ml/min/1.73 m^2[38]. In adult patients with CKD, the cluster of CV risk factors (hypertension, diabetes, hypercholesterolemia, and smoking) is common. Traditional risk factors, however, cannot explain the high prevalence of cardiovascular disease in pediatric population with CKD, as many factors affecting ED such as diabetes, smoking, effect of ageing, long lasting HT as a cause of CKD, and CV comorbidities are not present in this population, which allows for more accurate evaluation of the impact of oxidative stress and uremia on ED.

Accelerated atherosclerosis in patients with chronic kidney disease is responsible for increased morbidity and mortality due to cardiovascular causes[39]. Endothelial dysfunction is postulated to be the main cause of atherosclerosis before the onset of clinically manifested atherosclerotic plaques[40,41]. Endothelial cell function impairment also plays a role in the progression of atherosclerotic lesions and their clinical complications[42]. Cachofeiro et al. described oxidative stress, endothelial dysfunction, and inflammation as the key triad in the development and progression of atherosclerosis[43]. Thrombomodulin is an anticoagulant cell surface proteoglycan that is cleaved from the endothelial cell surface layer by neutrophil-derived enzymes [44,45].

In the study by Krzanowski et al., the authors partialy agreed to our results as they found a significant correlation between thrombomodulin and serum creatinine, as well as inflammation and endothelial dysfunction markers in CKD. Adult CKD patients with severe radial artery calcification had higher concentrations of TM than patients with less advanced lesions. Thrombomodulin concentrations did not correlate with age and classical cardiovascular risk factors (SCORE) which could indicate that thrombomodulin determines vascular injury and advanced calcification in patients with impaired renal function.[46]

The role of thrombomodulin as a vasoprotective agent may also be confirmed by the results of Eguchi et al., who have shown that the supply of recombinant TM reverses FK506-induced ED. Recombinant TM is registered in Japan for DIC treatment [47].

The main limitation to our study is the relatively small size of cohort which may affects the level of the evidence we provide, nevertheless, we provide a suggestion not a strong recommendation. The other limitation may be not following patients for a sufficient period for determining the actual CVS events and its incidence in each group.

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Original Article

Prophylactic Targeting of Non-Alcoholic Fatty Liver Disease by Sirt-1 Activator (Resveratrol) and 1, 25 Dihydroxy-Vitamin D3 in Rats

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Abstract

Introduction: Non-alcoholic fatty liver disease (NAFLD) is considered to be the hepatic component of the metabolic syndrome (MS), several studies tried to prevent it. The current study aims is to study the possible effect of resveratrol (RSV), and 1, 25 Dihydroxy-vitamin D3 on NAFLD in rats model. **Methods:** Forty male albino rats were assigned into four groups, group I (normal control) that were fed normal diet (n= 10), group II (n=10) received high-fat diet(HFD) for 16 week, group III (n=10) received HFD, and RSV (30mg\kg) daily by orogastric catheter for 16 week, group IV (n=10) received HFD, and Vitamin D3 (5ug\kg body weight), intraperitoneal injection, twice per week for 16 week. **Results:** Resveratrol significantly decreases liver enzymes levels, lipid profile, increased liver superoxide dismutase (SOD), decreased liver malondialdehyde (MDA), and increased liver SIRT1 activity, good improvement in hepatic steatosis. Vitamin D3 also decreases liver function markers, lipid profile, increased liver SOD, decreased liver MDA, and increased SIRT1 activity, slight improvement in hepatic steatosis. **Conclusion:** Resveratrol may be a potential therapeutic target for treating NAFLD, and vitamin D had a hepatoprotective effect that could be mediated through antioxidant and anti-inflammatory effects.

Key words: NAFLD, NASH, Resveratrol, SIRT1, 1, 25 dihydroxy vitamin D3, oxidative stress.

Introduction

Non-alcoholic fatty liver disease (NAFLD) refers to a condition defined by ectopic fat accumulation in the form of triacylglycride (TG) in the liver. (1)

The major risk factors for NAFLD are the same as the components of the metabolic syndrome (MS): central obesity, T2DM, dyslipidemia and insulin resistance (IR). Today, NAFLD is considered to be the hepatic component of the MS. (2)

The clinical spectrum of NAFLD ranges from simple fatty change to nonalcoholic steatohepatitis (NASH), which is characterized by cytolytic changes in hepatocytes (i.e. ballooning degeneration, Mallory bodies, and lobular inflammation). Although NAFLD is mostly benign, 20–30% of the patients develop NASH. (3)

The homeostasis of fat and energy in hepatic cells is regulated by mitochondrial activities, including beta-oxidation of free fatty acids (FFAs), electron transfer and production of adenosine triphosphate (ATP), and reactive oxygen species (ROS). Mitochondrial abnormalities alter the balance between pro-oxidant and antioxidant mechanisms, leading to

the blockade of fatty acid beta-oxidation and the consequent induction of ROS production. (3)

Activation of SIRT1 could serve as an effective therapeutic approach for preventing the development of fatty liver diseases at all stages, including the onset, progression and complication, resveratrol was identified as direct SIRT1 activator for the first time. (5)

Resveratrol (RSV) (3,5,4′-trihydroxy-trans-stilbene) is a stilbenoid, a type of natural phenol, and a phytoalexin produced by several plants in response to injury or, when the plant is under attack by pathogens such as bacteria or fungi. (6)

Through the activation of AMP-activated protein kinase (AMPK), SIRT1 and alternative routes including anti-inflammatory and anti-oxidant actions, RSV may inhibit the development or progression of steatosis and steatohepatitis.(10)

Vitamin D is a fat-soluble steroid hormone involved in many functions of mineralization, cellular growth, and decreasing the risk for chronic illnesses such as diabetes, cardiovascular disease, cancer, obesity, and autoimmune disease.(11)

Aim of the Work

The aim of present work was to assess the possible protective effect of SIRT-1 activator (resveratrol) and 1, 25-dihydroxy vitamin D_3 in a rat model of non-alcoholic fatty liver disease (NAFLD).

Materials and Methods

This study was carried out on 40 male Wistar albino rats with body weight 150-200 grams. The animals were housed in standard cages, ten per cage, at room temperature, with a 12 h light-dark cycle. All procedures involving the animals were conducted in accordance with the protocol approved by the ethics Committee, Faculty of Medicine, Alexandria University.

The duration of this study was 16 weeks for all groups. Rats were divided randomly into 4 experimental groups; each of it included ten rats:

Group 1 (control group): fed on standard rat chow containing carbohydrates 51%, protein 16%, vitamins and minerals 4%, and lipids 3%, the standard diet contained 2.9 Kcal per 1g of diet for 16 week.(16)

Group 2 (NAFLD group): fed on high-fat chow from the first week, containing carbohydrate 24.55%, protein 14.47%, fat 60.98%, presenting a total of 5.28 Kcal per 1 g of diet, for 16 week.(17)

Group 3 (NAFLD+Resveratrol group): fed on high-fat chow from first week as previous group and received Resveratrol (obtained from Sigma company) 10mg daily by oral route through an orogastric catheter, Resveratrol was diluted in 1ml of distilled water for 16 week.(16)

Group 4 (NAFLD+Vitamin D3 group): fed on high-fat chow from the first week and treated with 1, 25(OH) 2D3 (obtained from Minapharm in Egypt) 5μg/kg body weight, intraperitoneal injection, twice per week from the first week to 16th week. (17)

After 16 weeks of experiment, all animals were fasted for 14 hour. Then animals were sacrificed and blood was collected into non-heparinized tubes then serum was separated by centrifugation at 3000 rpm for 15 minutes. The serum was separated and stored at -20^{0}C until biochemical parameters assay:

Serum liver enzymes; alanine aminotransferase (ALT) and aspartate aminotransferase (AST) by colorimetric activity.(18)

1- Fasting plasma total cholesterol and triglyceride (TG) levels.(19)

The liver tissues were quickly removed, washed with saline and cut into pieces. One gram of liver was homogenized with 9 volumes of phosphate buffer (0.1 M, PH7.9) and then centrifuged at 400 Xg for 15 minutes and the supernatant was stored to be used as a liver homogenate for determination for:

- Hepatic tissue level of superoxide dismutase (SOD).[20]

- Hepatic tissue level of Malondialdehyde (MDA) as an indicator of lipid peroxidation by TBARS.[20]

- Hepatic tissue level of SIRT1 by ELISA assay.[21]

The right lobe of liver from control, NAFL group and treated groups was excised and then fixed in 10% formalin solution then processed to be stained routinely with Hematoxylin and Eosin. The prepared slides were examined under light microscope using objective lens power (X100) for histological examination.

Results

Table (I): Serum levels of aspartate aminotransferase (AST)(U\L) in the different studied groups

AST (U/L)	**Group1 (Control) (n = 10)**	**Group2 (NAFLD) (n = 10)**	**Group3 (NAFLD+ Resveratrol) (n = 10)**	**Group4 (NAFLD + Vit D3) (n = 10)**	**F**	**P**
Min. – Max.	37.8 – 55	104.2 – 159.7	43.1 – 60	80.1 – 100	123.924*	<0.001*
Mean ± SD.	45.47 ± 5.34	126.35 ± 19.38	50.6 ± 5.19	92.45 ± 6.1		
$p_{control}$		<0.001*	0.716	<0.001*		
Sig. bet. grps.		p_1<0.001*,p_2<0.001*,p_3<0.001*				

H: H for **Kruskal Wallis test,** Pairwise comparison bet. each 2 groups was done using **Post Hoc Test (Dunn's for multiple comparisons test)**

P: p value for comparing between the different groups

Pcontrol: p value for comparing between **control** and each groups

p_1: p value for comparing between NAFLD and NAFLD + Resveratrol

p_2: p value for comparing between NAFLD and NAFLD + Vit D

p_3: p value for comparing between NAFLD + Resveratrol and NAFLD + Vit D

*: Statistically significant at $p \leq 0.05$

Table (II): Serum levels of alanine amino transferase (ALT) (U\L) in the different studied groups

ALT (U/L)	Group1 (Control) (n = 10)	Group2 (NAFLD) (n = 10)	Group3 (NAFLD+ Resveratrol) (n = 10)	Group4 (NAFLD + Vit D3) (n = 10)	F	P
Min. – Max.	30.1 – 50	65.6 – 95.1	50.1 – 63	51.2 – 73	45.244*	<0.001*
Mean ± SD.	40.4 ± 6	78.56 ± 10.59	55.34 ± 4.42	60.33 ± 7.16		
$p_{control}$		<0.001*	<0.001*	<0.001*		
Sig. bet. grps.		p_1<0.001*,p_2<0.001*,p_3=0.443				

H: H for **Kruskal Wallis test,** Pairwise comparison bet. each 2 groups was done using **Post Hoc Test (Dunn's for multiple comparisons test)**

P: p value for comparing between the different groups

Pcontrol: p value for comparing between **control** and each groups

p_1: p value for comparing between NAFLD and NAFLD + Resveratrol

p_2: p value for comparing between NAFLD and NAFLD + Vit D

p_3: p value for comparing between NAFLD + Resveratrol and NAFLD + Vit D

*: Statistically significant at $p \leq 0.05$

Table (III): Serum level of triglycerides (TG) (mg\dl) in the different studied groups

TG (mg/dl)	Control (n = 10)	NAFLD (n = 10)	NAFLD+ Resveratrol (n = 10)	NAFLD + Vit D (n = 10)	F	P
Min. – Max.	112.7–198	189–246.1	160.1–191	169.0 – 205.5	14.788*	<0.001*
Mean ± SD.	160.18±25.58	214.11±19.74	179.69±11.96	186.68±12.65		
$p_{control}$		<0.001*	0.100	0.014*		
Sig. bet. grps.		p_1=0.001*,p_2=0.010*,p_3=0.829				

F: **F for ANOVA test**, Pairwise comparison bet. each 2 groups was done using **Post Hoc Test (Tukey)**

P: p value for comparing between the different groups

Pcontrol: p value for comparing between **control** and each group

p_1: p value for comparing between NAFLD and NAFLD + Resveratrol

p_2: p value for comparing between NAFLD and NAFLD + Vit D

p_3: p value for comparing between NAFLD + Resveratrol and NAFLD + Vit D

*: Statistically significant at $p \leq 0.05$

Table (IV): Serum levels of cholesterol (mg\dl) in the different studied groups

Cholesterol (mg/dl)	Control (n = 10)	NAFLD (n = 10)	NAFLD+ Resveratrol (n = 10)	NAFLD + Vit D (n = 10)	F	P
Min. – Max.	103.5 – 150.2	170.3 – 209.7	109.9 – 185.0	147.0 – 190.0	26.544*	<0.001*
Mean ± SD.	125.9 ± 16.18	188.7 ± 11.69	144.5 ± 22.29	162.61±13.52		
$p_{control}$		<0.001*	0.072	<0.001*		
Sig. bet. grps.		p_1<0.001*,p_2=0.006*,p_3=0.083				

F: **F for ANOVA test**, Pairwise comparison bet. each 2 groups was done using **Post Hoc Test (Tukey)**

P: p value for comparing between the different groups

Pcontrol: p value for comparing between **control** and each group

p_1: p value for comparing between NAFLD and NAFLD + Resveratrol

p_2: p value for comparing between NAFLD and NAFLD + Vit D

p_3: p value for comparing between NAFLD + Resveratrol and NAFLD + Vit D

*: Statistically significant at $p \leq 0.05$

Table (V): Hepatic tissue level of superoxide dismutase (SOD) (u\gm protein)

Liver level of SOD (u\gm protein)	Control (n = 10)	NAFLD (n = 10)	NAFLD+ Resveratrol (n = 10)	NAFLD + Vit D (n = 10)	F	P
Min. – Max.	19.20 – 34.70	12.30 – 19.20	15.10 – 33.0	16.20 – 27.70	18.238*	<0.001*
Mean ± SD.	28.27±4.62	14.89±2.23	26.10±6.29	21.07±3.43		
$p_{control}$		<0.001*	0.691	0.004*		
Sig. bet. grps.		p_1<0.001*,p_2=0.017*,p_3=0.068				

F: **F for ANOVA test**, Pairwise comparison bet. each 2 groups was done using **Post Hoc Test (Tukey)**

P: p value for comparing between the different groups

Pcontrol: p value for comparing between **control** and each group

p_1: p value for comparing between NAFLD and NAFLD + Resveratrol

p_2: p value for comparing between NAFLD and NAFLD + Vit D

p_3: p value for comparing between NAFLD + Resveratrol and NAFLD + Vit D

*: Statistically significant at $p \leq 0.05$

Table (VI): Hepatic tissue level of manoldehyde (MDA) (nmol\gm tissue)

Liver level of MDA (nmol/gm tissue)	Control (n = 10)	NAFLD (n = 10)	NAFLD+ Resveratrol (n = 10)	NAFLD + Vit D (n = 10)	F	P
Min. – Max.	8.6 – 16.9	13.5 – 40	12 – 21.8	13.0 – 32.90	14.117*	<0.001*
Mean ± SD.	12.89 ± 3.1	29.34 ± 9.69	16.9 ± 3.51	24.57 ± 6.31		
$p_{control}$		<0.001*	0.485	0.001*		
Sig. bet. grps.		p_1<0.001*,p_2=0.334,p_3=0.044*				

F: **F for ANOVA test**, Pairwise comparison bet. each 2 groups was done using **Post Hoc Test (Tukey)**

P: p value for comparing between the different groups

Pcontrol: p value for comparing between **control** and each group

p_1: p value for comparing between NAFLD and NAFLD + Resveratrol

p_2: p value for comparing between NAFLD and NAFLD + Vit D

p_3: p value for comparing between NAFLD + Resveratrol and NAFLD + Vit D

*: Statistically significant at $p \leq 0.05$

Table (VII): Hepatic tissue level of SIRT1 (lu\ml) in the different studied groups

Hepatic tissue level of SIRT1 (Iu/ml)	Group1 (Control) (n = 10)	Group2 (NAFLD) (n = 10)	Group3 (NAFLD+ Resveratrol) (n = 10)	Group4 (NAFLD + Vit D3) (n = 10)	F	p
Min. – Max.	2.6 – 6.3	0.8 – 4.2	2.50 – 5.50	2.5 – 5.1	4.452*	0.009*
Mean ± SD.	4.15 ± 1.3	2.55 ± 1.13	4.05 ± 0.98	3.74 ± 0.97		
$p_{control}$		0.013*	0.997	0.839		
Sig. bet. grps.		p_1=0.022*,p_2=0.093,p_3=0.922				

F: **F for ANOVA test**, Pairwise comparison bet. each 2 groups was done using **Post Hoc Test (Tukey)**

P: p value for comparing between the different groups

Pcontrol: p value for comparing between **control** and each group

p_1: p value for comparing between NAFLD and NAFLD + Resveratrol

p_2: p value for comparing between NAFLD and NAFLD + Vit D

p_3: p value for comparing between NAFLD + Resveratrol and NAFLD + Vit D

*: Statistically significant at $p \leq 0.05$

Histological results

Light microscopic examination of liver sections from the control group (Group 1) showed normal liver architecture (Figures 1).

Liver examination of NAFLD group showed, disturbed liver architecture. Bridging fibrosis between portal tracts, Hepatocytes revealed microvesicular, macrovesicular steatosis and hepatocellular ballooning. (Figure 2).

Liver sections from Resveratrol group, showed a good hepatic architecture. Most hepatocytes appeared normal except for few with rarefied cytoplasm, macrovesicular and microvesicular steatosis. (Figure 3).

Liver sections from vitamin D group, showed disturbed liver architecture. Hepatocytes showed microsteatosis, macrovesicular steatosis, hepatocellular ballooning and rarefied cytoplasm. Mallory's bodies were seen within hepatocytes. (Figure 4).

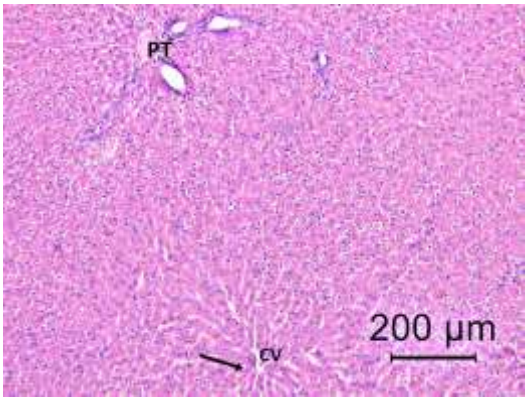

Figure (1): A photomicrograph of rat liver of control group, showing normal liver architecture; cords of hepatocytes (thin black arrow) are seen radiating from the central vein (CV) and separated by blood sinusoids. Notice the portal tract (PT) at the periphery of the hepatic lobule enclosed by a very little amount of connective tissue.

(H&E stain, Mic. Mag. × 100)

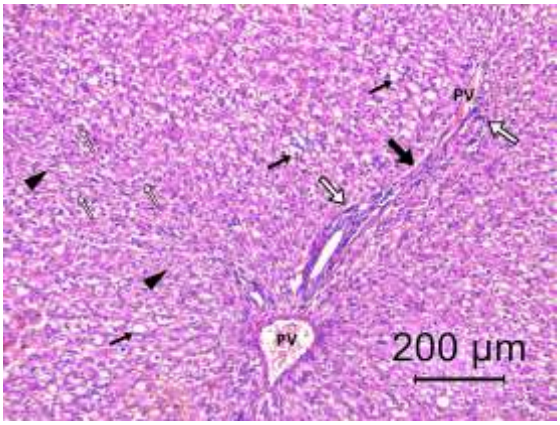

Figure (2): A photomicrograph of a rat liver of NAFLD group, showing bridging fibrosis between portal tracts (thick black arrow).Hepatocytes showing macrovesicular steatosis (black arrow head), hepatocellular ballooning (thin black arrow) and Mallory's bodies (thin white arrow). Notice periportal cellular infiltration (thick white arrow).

(H&E stain, Mic. Mag. × 100)

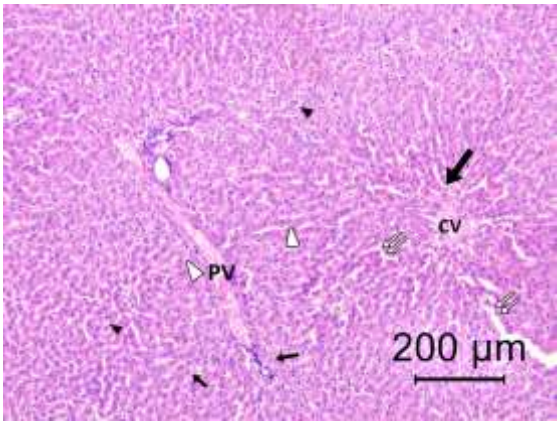

Figure (3) A photomicrograph of a rat liver from Resveratol group, showing good liver architecture; where cords of hepatocytes (thick black arrow) are radiating from central vein (CV). Some blood sinusoids are dilated (double thin white arrow). Microvesicular steatosis (white arrow head), and rarefied cytoplasm (thin black arrow).

(H&E stain, Mic. Mag. × 100)

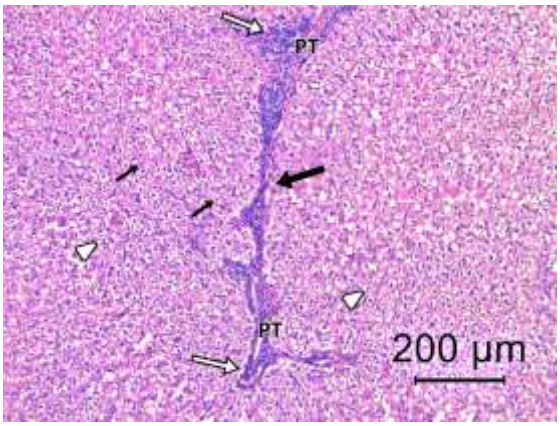

Figure (4): A photomicrograph of a rat liver from Vitamin D group, showing disturbed liver architecture. Hepatocytes show macrovesicular steatosis (white arrow head) and rarefied cytoplasm (thin black arrow). Bridging fibrosis (thick black arrow). Notice periportal inflammatory cellular infiltration (thick white arrow).

(H&E stain, Mic. Mag. × 100)

Discussion

Non-alcoholic fatty liver disease (NAFLD) is the most common form of chronic liver diseases. Two thirds of the patients are asymptomatic. It is characterized by the accumulation of triglycerides in the liver and spans a histological spectrum of liver disease, ranging from simple steatosis to steatohepatitis, fibrosis and rarely to cirrhosis or even hepatocellular carcinoma.[22]

The current work showed a significant increase in serum liver function markers (AST, ALT) in NAFLD group when compared to control rats. This elevation is attributed to lipid deposition in liver causing damage of hepatocytes. [23] Moreover, these changes coincide with the histopathological changes of hepatic tissues observed in the present work which showed loss of the normal liver architecture.

Moreover, rats on HFD received resveratrol caused significant decrease in liver enzymes (AST, ALT). Ren et al, [24] reported that elevated serum level of ALT is a marker of inflammation and oxidative stress and AST is also related to oxidative stress. Therefore, the study may be attributed to the anti-inflammatory and antioxidant effect of RSV. [25]

In the present study, rats received preventive vitamin D3 decrease liver enzymes, this is may be attributed to antioxidant effect of vitamin D3,[27] similar results was found by Zhu et al,[19] as they found that both serum ALT and AST, in rats received vitamin D3 were considerably reduced compared to those in NAFLD.

On contrary, Chachay et al, [28] reported that levels of alanine and aspartate aminotransferases increased significantly in resveratrol group. Hariri et al,[15] showed that no significant effect of Vitamin D on liver enzymes except one which revealed that Vitamin D together with calcium carbonate can reduce liver enzymes.

In current study, there was a significant elevation of serum TG, and cholesterol in NAFLD group when compared to control group. These alterations were described by study done by Meryem et al,[29] as they reported that HFD induces lipid and lipoproteins metabolic disorders, associated with abnormal expression of pathway enzymes lipid storage (lipoprotein lipase (LPL))and lipid mobilization enzyme [hormone-sensitive lipase (HSL)].

Administration of RSV to rats significantly improve serum TG, and cholesterol, Shang J et al,[30] observed that RSV improve in these parameters via AMPK activation and dowenregulation of SREBP-1c and FAS expressions, thus preventing lipid synthesis.

Also, administration of vitamin D3 to rats decreases serum TG, and cholesterol, this is may be attributed the vitamin D3 mediated reduction of serum triglycerides to the increases in serum calcium which enhancing intestinal calcium absorption. [32] Several mechanisms are suggested to explain the effect of calcium on lipids, including its reducing role in fatty acid absorption via the formation of insoluble calcium-fatty complexes in the gut. [33]

Regarding to lipid peroxidation and antioxidant, oxidative stress, beta-oxidation of fatty acids and the resultant oxidants such as ROS are among the important mechanisms in the development of NAFLD, especially for NASH. High hepatic MDA levels and lower SOD levels in NAFLD group, in agreement with other studies. [34]

The antioxidant effects of RSV were demonstrated in our study that RSV leads to enhanced levels of SOD, and reduced level of MDA, which was attributed to the important mechanism, is the complex interaction of SIRT1 with FOXO transcription factors which leads to production of ROS-detoxifying enzymes, including SOD, and catalase. [35]The current results were supported by Bujanda et al. [20]

Administration of vitamin D significantly decreased MDA tissue level compared to NAFLD group. Zhu et al, [19] hypothesized that vitamin D would enhance antioxidant capacity by modulating Nuclear factor-erythroid-2-

related factor 2 (Nrf2) to combat oxidative stress.

As regard the results of the hepatic levels of SIRT1: The data of the present work showed that SIRT1 expression was significantly decreased in the NAFLD group compared with the control group. Previous study, they found that SIRT1 values were significantly lower in fat liver infiltration, although they have no definitive explanation for this expression.[36] This is agreement with study conducted by Mariani et al, [37] showing that the fatty liver infiltration had lower amount of SIRT1 level compared with no NAFLD affected group.

In addition, the present study revealed that RSV supplementation a significantly increased in SIRT1 levels. These finding is running in parallel with a previous study, RSV administration apparently restored SIRT1 levels in liver of HFD fed mice, simultaneously RSV suppressed the expression of the indicated LD associated genes, leading to suppression of hepatic lipid content, so RSV decrease expression of LD associated genes in liver and hepatocytes through the SIRT1-mediated signaling pathway.[38]

The present study given 1,25(OH)3D increases SIRT1 activity .These results suggest that vitamin D might promote fat mobilization and hence decrease intracellular fat accumulation and increase lipolysis, concurrently with an increase of activity in SIRT1.[40]

Supporting these findings Chang et al,[41] revealed that 1,25(OH)3D increased SIRT1 expression and activity, and proposes SIRT1 activation is closely related to the activation of a steroid hormone receptor, vitamin D receptor (VDR), which mediates 1,25(OH)3D-induced genomic changes.

Finally, the result of the present study was confirmed by the histological examination, the HFD diet feeding induced loss of the normal liver architecture. Bridging fibrosis between portal tracts, and lobular inflammatory cellular infiltrates, macro, micro steatosis were noticed. The hepatic steatosis induced by increasing the FFA load imposed on the liver and reducing fatty acid-β oxidation. [49, 50] Also these changes are in agreement with Sampey et al [51]

Administration of resveratrol show most hepatocytes appeared normal except for few with rarefied cytoplasm, macrovesicular and microvesicular steatosis, no fibrosis was noticed.

The current result supported by Zhou et al[38] showed administration of resveratrol strongly attenuated hepatic steatosis this is attributed to that resveratrol suppress the expression of the indicated lipid droplet(LD) associated genes in liver by SIRT1 activity and upregulating SIRT1 expression.

Administration of vitamin D showed a slight improvement when compared to HFD group which was agreement with Barcheta et al [52]. The protective effects of vitamin D may be related to a reduction in oxidative stress and an increase in antioxidant capacity.

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