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Visfatin and Apelin Are New Interrelated Adipokines Playing Role in the Pathogenesis of Type 2 Diabetes Mellitus Associated Coronary Artery Disease in Postmenopausal Women

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***Abstract*—**Visfatin and apelin are two new adipokines that recently gained a special interest in diabetes research. This study was conducted to study the interplay between these two adipokines and their correlation with other inflammatory and biochemical parameters in type 2 diabetic (T2D) postmenopausal women with CAD. Visfatin and apelin were measured by enzyme-linked immunoassay (ELISA). Visfatin was found to be significantly higher in the following groups: T2D patients without CAD, non-obese and obese T2D patients with CAD when compared to control group. Apelin was found to be significantly lower in non-obese and obese T2D patients with CAD when compared to control group. Visfatin and apelin were found to be significantly associated with each other and with other biochemical parameters. The current study provides evidence for the interplay between visfatin and apelin through the inflammatory milieu characteristic of T2D and their possible role in the pathogenesis of CAD complication of T2D.

***Keywords*—**Apelin, Coronary artery disease, Inflammation, Type 2 diabetes, Visfatin

I. Introduction

DIABETES MELLITUS (DM) is a chronic metabolic disorder that affects more than 150 million people annually and is expected to reach 552 million by the year 2030 increasing exponentially especially in the developing countries [1]. Coronary artery disease (CAD) is the most dangerous complications of DM, where it has been estimated that 75% of the deaths in diabetic patients may be attributed to CAD [2].

Many mechanisms explaining the pathogenesis of vascular complications in diabetic patients have been proposed which enhance the formation of atherosclerotic plaques associated with either high risk of rupture or prolonged ischemia [3]. Interestingly, adipose tissue (AT) which was long thought of as a mere storage of lipids, has been proved to be source of several bioactive mediators, termed adipokines, with various functions [4]. One of these adipokines that recently gained a special interest in DM research is visfatin. Visfatin is an insulin-mimetic adipokine that was originally discovered in liver, skeletal muscle and bone marrow as a growth factor for B lymphocyte precursors (whence its alternative name, pre-B cell-colony enhancing factor (PBEF)) [5]. Furthermore, it has also been implicated in plaque destabilization leading to carotid and coronary atherosclerosis [6].

The other adipokine of interest in this study is apelin, which was first isolated from bovine stomach as the endogenous ligand of the 7-transmembrane G-protein coupled receptor [7]. Apelin was first recognized for the role of apelin/apelin receptor in the regulation of the cardiovascular system [8]. Additionally, low plasma apelin levels were noted in patients with T2D and CAD such as macroangiopathy due to possible defect in their expression [9]. However, the role of apelin in CAD complications of diabetes needs further elucidation.

All of the above data suggested a potential role of these adipokines in the CAD diabetic complications. However, their circulating levels in T2D patients with and without CAD have not been adequately studied. Also, their correlation with T2D, obesity and CAD needs further elucidation. Therefore, the current study was designed to identify the changes in CRP, visfatin and apelin levels in obese/non-obese T2D postmenopausal women, with and without CAD compared to healthy age matched control subjects. In addition, we also tried to investigate the possible association of the above markers with each other and with T2D associated hyperglycemia and dyslipidemia in an attempt to explain how these potential players interrelate in the pathogenesis of the CAD complications in T2D patients. As far as our knowledge, the correlation between these three players has not been explored before this work and will provide further insight into the complex process of CAD development in T2D.

II. Materials and Methods

A. Patients Database

The study was approved by the committee of Medical ethics of Ain Shams University hospital and informed consent was obtained from each patient. The study was carried out in accordance with regulations and recommendations of the declaration of Helsinki. Eighty subjects aging from 60 to 68 years were enrolled in the study. The groups were classified as follows: Group I included 20 healthy non-diabetic control subjects. Group II included 20 obese T2D patients not suffering from CAD. CAD (either angina or acute myocardial

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infarction (AMI) within the first month of infarction) was previously diagnosed by lab analysis, and angiogram. The 40 T2D patients suffering CAD were further classified into non-obese group {Body mass index (BMI) <30 kg/m2}; group III and obese group {BMI > 30 kg/m2}; group IV. Height and weight were measured and BMI was calculated as an index of the weight (in kilograms) divided by the square of the height (in meters) measured on the same day of sample withdrawal.

Before inclusion, all the study subjects underwent careful physical examination, detailed history, and laboratory investigations to exclude any condition that may interfere with glucose tolerance. The exclusion criteria included: type 1 diabetes, renal or hepatic disease, acute or chronic inflammatory diseases, thyroid dysfunction, pituitary disorders, malignancy, autoimmune disease, acute or chronic infections. The control subjects were not suffering any health problems and were not receiving any medications.

B. Sample Collection

10mls blood was collected from all 80 subjects after an overnight fasting. Plasma was separated after collection of blood samples into Na fluoride vaccutainer tubes for measurements of fasting plasma glucose (FPG), and post-prandial plasma glucose (PPG). Whole blood was collected into disodium EDTA vaccutainer tubes for the analysis of glycated hemoglobin (HbA1c %). Serum was obtained by collecting blood into plain vaccutainer tube for assessment of lipid profile including: triglycerides (TG), total cholesterol (TC), low density lipoprotein cholesterol (LDLc), high density lipoprotein cholesterol (HDLc), CRP, and prothrombin time (PT). Also, visfatin and apelin levels were detected in serum. Whole blood was stored at 4-8oC till assay, usually within a week. Samples were assayed on the same day, otherwise, stored at -80oC till time of assay.

C. Laboratory Assessment

The FPG and PPG, HbA1c %, PT, together with lipid profile: TG, TC, HDLc , LDLc, and CRP; all were measured by using standard enzymatic techniques using appropriate kits, following the manufacturer's instructions, using semi automated photometer 5010. Determination of both serum visfatin and apelin was done using ELISA kits from (Phoenix Pharmaceuticals, Burlingame, CA), with sensitivity = 2.38ng/ml and 0.04 ng/ml, respectively. All ELISA procedures were carried out according to the manufacturers' instructions, using Hy-prep automated ELISA system (Hyperion Inc., USA).

D. Statistical Assay

Results are expressed as mean ± standard error of mean (M ± S.E.M). Kolmogorov- Smirnov was done to evaluate the distribution of variables. Different groups were compared by analysis of variance (ANOVA) and post Hoc Bonferonni was applied to compare individual groups. The general linear modeling was used to control for potential confounders (e.g., age, and BMI). Any skewed data were logarithmically transformed before performing simple and multiple linear stepwise regression analyses to study the association between each of CRP, visfatin and apelin with other biochemical parameters and to study the association between visfatin and apelin adjusted for the effects of other covariates; generally in the multiple linear stepwise regression analysis, the independent variables included demographic factors (e.g. age), metabolic parameters (as BMI, FPG , PPG, HbA1c %, TG, TC, HDLc, LDLc, and PT) and variables found to be significantly associated with the examined dependent variable (CRP, visfatin or apelin) in univariate analysis. All statistical analyses were performed using windows-based SPSS statistical package (SPSS version 17.0, SPSS Inc, Chicago, IL). P-values < 0.05 were considered to be significant.

III. Results

The clinical characteristics of the studied groups are shown in Table I. Concerning CRP levels, as a well established inflammatory marker, they were found to be significantly elevated in both non-obese and obese T2D patients with CAD (group III and IV) when compared to the control group at $p<0.05$.

Concerning visfatin levels, they were found to be significantly elevated in obese T2D patients without CAD (group II), and in both non-obese and obese T2D patients with CAD (group III and IV), as compared to the control group; Table I. Moreover, it was further elevated significantly in obese T2D patients with CAD (group IV) at $p<0.05$ as compared to their non-obese counterparts (group III) and obese T2D patients without CAD (group II). Interestingly, as shown in Table I, even after adjustment for the effects of covariates as age and BMI, visfatin levels remained to be significantly different among the studied groups.

Regarding apelin levels, they were found to be significantly decreased in non-obese T2D patients with CAD (group III) and surprisingly diminished in their obese counterparts (group IV) as compared to both the control group and to the obese T2D patients without CAD (group II). However, no significant difference was found in apelin levels of T2D patients without CAD (group II) as compared to the control group. Still, after adjustment for the effects of covariates as age and BMI, apelin levels remained to be significantly different among the studied groups.

We next analyzed the correlation between each of CRP, visfatin, and apelin levels with each other and with other parameters. Concerning CRP levels, as shown in Table II, simple linear regression analysis, revealed that they were found to be positively correlated with FPG, PPG, HbA1c %, and visfatin levels. Also CRP was significantly negatively correlated with HDLc, and apelin. On performing multiple linear stepwise regression analysis using CRP as dependent variable together with age, BMI, FPG, PPG, HbA1c%, TG, TC, HDLc, LDLc, PT, visfatin and apelin all as independent variables, only HbA1c% ($\beta=0.452$, $P=0.000$) and apelin ($\beta=-0.459$, $P=0.000$) remained to be significantly associated with CRP.

Concerning visfatin, as shown in Table II, simple linear regression analysis revealed significant positive correlation with age, BMI, FPG, PPG, HbA1c%, TG, TC, LDLc, and

CRP, while significant negative correlation with HDLc and apelin. Whereas On performing multiple linear stepwise regression analysis using visfatin as dependent variable, together with BMI, age, FPG, PPG, HbA1c %, TG, TC, HDLc, LDLc, CRP, PT and apelin all as independent variables, only BMI (β=0.352, P=0.000), HbA1c% (β=0.414, P=0.000), and apelin (β=-0.215, P=0.020) remained significantly associated with visfatin.

With respect to apelin, on conducting simple linear regression analysis as shown in Table II, it was found to be significantly negatively correlated with age, BMI, FPG, PPG, HbA1c%, TG, CRP and visfatin, while significantly positively correlated with HDLc. On performing multiple linear stepwise regression analysis using apelin as dependent variable, together with age, BMI, FPG, PPG, HbA1c%, TG, TC, HDLc, LDLc, CRP, PT and visfatin all as independent variables, only BMI (β=-0.564, P=0.000), HbA1c % (β=-0.256, P=0.006) and CRP (β=-0.804, P=0.000) remained significantly associated with apelin.

TABLE I
CLINICAL AND LABORATORY CHARACTERISTICS OF THE STUDIED GROUPS

Groups/ Parameters	Group(I) Control (n=20)	Group(II) Obese T2D without CAD (n=20)	Group(III) Non-obese T2D with CAD (n=20)	Group(IV) Obese T2D with CAD (n=20)
Age (Years)	62.35±0.46	63.40±0.53	63.35±0.54	64.70±0.45
BMI(Kg/m^2)	26.10±0.21	35.16±0.63^{a}	26.73±0.29^{b}	37.66±0.40^{a,b,c}
Waist (cm)	90.94±0.10	111.81±0.69^{a}	92.15±0.12^{b}	113.06±0.16$^{a, c}$
Hip (cm)	101.01±0.18	114.89±0.7^{a}	102.24±0.12^{b}	114.97±0.15^{a,c}
WHR	0.90±0.001	0.97±0.003^{a}	0.90±0.001^{b}	0.98±0.001^{a,c}
Systolic BP	126.50±0.96	148.00±1.93^{a}	148.50±1.66^{a}	148.00±1.82^{a}
Diastolic BP	83.33±0.80	92.50±0.92^{a}	92.25±0.92^{a}	94.25±0.83^{a}
FPG(mg/dl)	87.75±2.22	176.95±10.22^{a}	200.70±15.55^{a}	236.40±17.20$^{a, b,c}$
PPG(mg/dl)	102.90±2.01	266.90±16.59^{a}	286.75±19.87^{a}	335.85±21.64^{a,b,c}
HbA_{1c} (%)	4.1±0.075 (21 mmol/mol)	9.7±0.42^{a} (83 mmol/mol)	12.0±0.34 ^{a,b} (108 mmol/mol)	12.5±0.26 ^{a,b} (113 mmol/mol)
TG(mg/ dL)	97.95±5.64	205.65±8.02^{a}	105.65±2.76^{b}	197.20±7.51$^{a, c}$
TC(mg/dL)	176.75±3.78	280.75±10.40^{a}	185.20±5.98^{b}	239.50±14.38$^{a, b,c}$
HDLc(mg/dL)	44.25±1.78	33.90±3.15^{a}	34.35±2.14^{a}	26.50±1.28^{a,b,c}
LDLc(mg/ dL)	112.91±3.79	205.72±10.46^{a}	129.72±5.07^{b}	173.^{56}±14.27^{a,b,c}
PT(sec.)	11.92±0.24	11.96±0.26	13.15±0.52 ^{a,b}	12.66±0.44
CRP(mg/L)	5.00±0.28	4.95±0.35	139.50±7.99^{a,b}	157.50±5.79^{a,b,c}
Visfatin(ng/ml)	16.51±0.94	26.35±0.71^{a}	24.67±0.67 ^{a}	32.07±0.84 ^{a,b,c}
Visfatin#(ng/ml)	16.13±1.44	26.69±1.19^{a}	24.26±1.35^{a}	32.53±1.62^{a,b,c}
Apelin(ng/ml)	34.52±0.14	33.99±0.13	27.93±1.07 $^{a, b}$	2.57±0.25$^{a, b, c}$
Apelin#(ng/ml)	35.28±0.94	33.12±0.78	28.95±0.89^{a,b}	1.66±1.06^{a,b,c}
Type of OHA (MET/ SU / /none)	……..	15/5/0	14/6/0	9/11/0
Antihypertensive treatment (B-blocker / B-blocker+diuretic /none)	……..	7/1/12	8/3/9	9/4/7

Values are expressed in terms of (Mean ± SEM). T2D, type 2 diabetes mellitus; BMI: body mass index, WHR; waist to hip ratio, FPG fasting plasma glucose; PPG post prandial glucose and HbA1c% is glycated hemoglobin, TG triglycerides; TC total cholesterol; HDLc high density lipoprotein cholesterol; LDLc low density lipoprotein cholesterol, CRP C reactive protein, and PT prothrombin time. OHA Oral hypoglycemic agent, MET Metformin, SU Sulfonylurea.

a: Significantly different from control group I at $p<0.05$

b: Significantly different from group II at $p<0.05$

c: Significantly different from group III at $p<0.05$

#: Mean ± SEM by general linear model with adjustment of age and BMI.

TABLE II
SIMPLE LINEAR REGRESSION ANALYSIS USING CRP, VISFATIN, OR APELIN AS DEPENDENT VARIABLE

variable	CRP		Visfatin		Apelin	
	β (r)	P	β (r)	P	β (r)	P
Age	0.207	0.066	0.316**	0.004	-0.369**	0.001
BMI	0.181	0.108	0.670**	0.000	-0.592**	0.000
FPG	0.545**	0.000	0.581**	0.000	-0.507**	0.000
PPG@	0.594**	0.000	0.688**	0.000	-0.498**	0.000
HbA_{1c} (%)	0.719**	0.000	0.701**	0.000	-0.581**	0.000
TG	0.040	0.728	0.580**	0.000	-0.397**	0.000
TC	-0.078	0.491	0.405**	0.000	-0.125	0.269
HDLc	-0.363**	0.001	-0.504**	0.000	0.446**	0.000
LDLc	-0.017	0.883	0.423**	0.000	-0.146	0.196
CRP	---	---	0.542**	0.000	-0.722**	0.000
PT@	0.207	0.065	0.103	0.365	-0.123	0.278
Visfatin/Nampt	0.542**	0.000	----	----	-0.664**	0.000
Apelin	-0.722**	0.000	-0.664**	0.000	------	-------

*: Significant at $p<0.05$
** Significant at $p<0.01$
@: Log transformed values were used. ; BMI: body mass index, FPG fasting plasma glucose; PPG post prandial glucose and HbA1c % is glycated hemoglobin, TG triglycerides; TC total cholesterol; HDLc high density lipoprotein cholesterol; LDLc low density lipoprotein cholesterol, CRP C reactive protein, and PT prothrombin time.

IV. DISCUSSION

Over the last decade, adipokines were found to play role, not only in onset of diabetes, but also, in its complications especially diabetes associated CAD [10]. Understanding of the diverse effects of distinct adipokines as well as the interplay between these bioactive mediators is still incomplete, and if fully elucidated, would provide much better understanding for the molecular basis of T2D and its complications. This inspired us to conduct this study to explore two recent adipokines; visfatin and apelin, in conjunction with CRP, a well-established inflammatory marker, in T2D postmenopausal women with and without CAD.

Interestingly, the overall "adipokines-cytokines cocktail" favors inflammatory milieu in T2D to the extent that T2D is considered a low grade inflammatory disease [11]. In accordance with that, in the current study, higher levels of CRP were observed in both non-obese and obese T2D patients with CAD as compared to the control group. Actually, inflammation is considered a very important link between IR, T2D, obesity and CAD [12]. However, the precise events and mechanisms leading to the initiation of the inflammatory response in T2D and CAD remain incompletely understood. The cause of deterioration due to hyperglycemia is associated with ROS, which lead to chronic inflammatory condition together with production of advanced glycated end products, and these events increase the CRP, Which, in turn, promote pro-atherosclerotic inflammatory processes at CAD [13].

Regarding visfatin levels, they were found to be significantly elevated in patients suffering from T2D and CAD as compared to the control group, with the highest elevation observed in the obese patients suffering T2D and CAD as compared to their non-obese counterparts and as compared to the control group, not only that but also as compared to obese patients suffering T2D without CAD, Which highlights the possible synergistic effect of both obesity and CAD on visfatin levels in T2D patients. Actually, elevated visfatin levels in patients with T2D could have more than one possible explanation: Firstly; this may suggest the impairment of visfatin signaling in target tissues (i.e., visfatin resistance resulting in eventual hypervisfatinemia) [14]. Secondly; being insulin mimetic, the elevated visfatin levels could be a compensatory mechanism in response to hyperglycemia [15]. Thirdly, the discovery of visfatin mediated NAD biosynthesis regulating glucose-stimulated insulin secretion in pancreatic β-cells [16] could explain the elevated levels of visfatin in T2D patients as a compensatory mechanism for β-cell functioning.

Additionally, visfatin was found to be positively correlated with BMI, which could be partially explained by the previous finding that visfatin levels are higher in visceral than in subcutaneous fat [5]. Furthermore, visfatin levels were found to be positively correlated with FPG, PPG, and HbA1c %. This could be explained by the previous finding that visfatin release was found to be enhanced by glucose in cultured human adipocytes in vitro and by hyperglycemia in healthy humans [17].

Moreover, visfatin levels were found to be positively correlated with TG, TC and LDLc, while negatively correlated with HDLc. Previously, visfatin was found to have a possible role in lipid homeostasis [18], however the mechanism is obscure. Actually, being an adipokine, a direct or indirect relationship may exist between visfatin levels and lipid metabolism. Additionally, the relationship between visfatin and lipid profile might represent a compensatory mechanism for diabetic dyslipidemia since visfatin was previously reported to up-regulate PPARγ activity [19], [20].

Furthermore, we found a significant positive correlation between visfatin and CRP levels. In fact, our results concurred with previous studies reporting significant relationships between visfatin and CRP [21]. Interestingly, visfatin was also previously found to be significantly associated with IL-6 in Egyptian T2D patients [22]. So, it's important to point here that this provides further evidence for a potential crosstalk

existing between visfatin and inflammation in mediating both T2D onset and its complications, which may be through inflammatory mediators such as CRP.

As for apelin levels, they were found to be significantly lower in CAD groups; they were slightly decreased in non-obese T2D patients with CAD, however, they were really diminished in the obese CAD patients. This might be explained as that CAD may be considered as a major determinant of apelin level in diabetic patients. However, the coexistence of obesity and CAD further decreased the apelin levels in diabetic patients, which highlights their potent synergistic effects. It's noteworthy here that previously, low apelin levels were found to constitute an independent determinant of CAD severity in asymptomatic patients with CAD [23]. Actually, accumulating evidence supports apelin involvement in cardiovascular function, but its causative relationship with ischemic heart disease is controversial [24]. In addition, considerable evidence has emerged, indicating the association of reduced apelin with coronary atherosclerosis [25]. All of these findings in literature explain the diminished apelin levels which we found in obese T2D patients with CAD.

Actually, apelin levels in diabetic patients are a matter of controversy. On one hand, basal and two hours post-glucose plasma levels of apelin were previously found to be elevated in T2D subjects and in humans with impaired glucose tolerance (IGT) [26]. On the other hand, circulating apelin levels were found in another study to be lower in newly diagnosed T2D patients [27]. Interestingly, the current study found apelin to be negatively correlated with FPG, PPG, and HbA1c %. Not only that, but it also remained to be negatively correlated with HbA1c % after multiple linear stepwise regression, which indicates that apelin is positively associated with good diabetic control reflected by the levels of HbA1c %. Previously, Dray and coworkers, proved that injecting apelin into diabetic mice could provide a new potential therapy for DM [28]. Taking into consideration the diminished apelin level observed in T2D patients with CAD, apelin might indeed play an additional protective role against CAD complications of DM.

Furthermore, in the current study, apelin was found to be negatively correlated with BMI, TG, and TC. But, it was positively correlated with HDLc. These factors constitute important elements for the development of atherosclerotic plaque development and vulnerability [29]. It's important to point here that apelin, like visfatin is also associated with the inflammatory process, since previously apelin was found to have anti-inflammatory, anti-infection, and inhibitory effects on inflammatory mediators release [30]. This report comes in accordance with our results where apelin was found to be negatively correlated with the inflammatory marker CRP. This model is further supported by the consistent considerable negative regression between apelin and CRP even after multiple regression analysis.

Another interesting finding in the current study is the strong negative association between visfatin and apelin in both simple and multiple linear stepwise regression analyses. Actually, the negative correlation between these 2 markers and with CRP, might explain the diminished level of apelin in obese T2D patients with CAD, where it might be suppressed by two potential inflammatory players as CRP and visfatin. Moreover, the strong association between these three players sheds light on a potential mechanism accounting for the pathogenesis of CAD in diabetes.

However, it's important to point that the current study is an epidemiologic study, capable of identifying correlations between variables and not direct cause and effect. Accordingly further experimental studies are required to unravel the molecular mechanism of the observed associations between visfatin as well as apelin with one another and with various metabolic parameters. Furthermore, although the current study had sufficient power to detect significant associations, yet, it was done on women only, thus, further large scale studies are required to be done on both men and women to detect/ elucidate any difference are required to gain more insight into their role in T2D and to investigate their role in other diabetic complications as well. It's also noteworthy here that the diabetic patients in the current study showed relatively high levels of HbA1c%, indicating that they were poorly controlled, and further large scale studies to investigate the effect of glycemic control on visfatin as well as apelin levels would be of good value.

In conclusion, this study showed that T2D patients with CAD showed elevated levels of visfatin and CRP, indicating an existence of inflammatory state. On the other hand, apelin level was diminished in T2D patients with CAD, which might have predisposed these patients to CAD complications of diabetes. The positive association of CRP with visfatin, together with the negative association between both markers and apelin indicates a potential crosstalk occurring between these markers in the pathogenesis of CAD in T2D. Elevated levels of CRP and visfatin might have impaired apelin levels in T2D patients, which could potentially exacerbate the CAD in these patients.

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