Elevated Serum Levels of Pregnancy-Associated Plasma Protein-A in Type 2 Diabetics Compared to Healthy Controls: Associations with Subclinical Atherosclerosis Parameters

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Received: 22 Oct. 2013; Received in revised form: 7 Jul. 2014; Accepted: 22 Nov. 2014

Abstract- Type 2 diabetes mellitus is associated with increased inflammation and accelerated atherosclerosis. The association of the pro-inflammatory and potentially pro-atherosclerotic molecule, pregnancy associated plasma protein-A (PAPP-A) with diabetes and vascular diseases remains to be further established. A total of 107 patients with type 2 diabetes and 101 healthy controls participated in this study. Serum levels of PAPP-A was measured by Enzyme-linked Immunosorbent Assay (ELISA). We also evaluated the lipid profile, aortic augmentation index, coronary calcium score, ankle brachial index, flow mediated dilation, and carotid intima media thickness. Serum level of PAPP-A was significantly higher in patients with diabetes compared to controls (P<0.001). In the multivariable regression analysis, PAPP-A was positively correlated with diabetes (P<0.001), aortic augmentation index (P=0.021) and was negatively associated with coronary calcification (P=0.050). In conclusion, serum levels of PAPP-A were significantly higher in diabetics compared to healthy controls and correlated with aortic augmentation index and coronary calcification. Our study results suggest that PAPP-A can be a marker of subclinical atherosclerosis in patients with diabetes.

Acta Med Iran 2015;53(7):395-402.

Keywords: Pregnancy associated plasma protein-A; Diabetes mellitus; Subclinical atherosclerosis; Augmentation index; Coronary calcium score

Introduction

Pregnancy associated plasma protein A (PAPP-A) was first identified in the sera of pregnant women (1). PAPP-A was known to be secreted from placental syncytiotrophoblasts into maternal circulation during pregnancy (2). Later, it was shown that PAPP-A is a zinc metalloproteinase that is involved in local proliferative processes (3). It cleaves insulin-like growth factor binding protein-4 and releases Insulin-like growth factor-I (IGF-I) (3). In atherosclerotic plaques, IGF-I stimulates smooth muscle cells, macrophages, and endothelial cells and accelerates atherosclerosis (4). According to this theory, Bayes-Genis et al. suggested that PAPP-A is a marker of acute coronary syndromes (5).

Although cardiovascular death accounts for about one fifth of all deaths in some developed countries and results in heavy costs and burden, new methods for diagnosis and risk stratification are not well developed (6). Currently, the diagnosis of acute coronary syndromes is made based

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on the markers of myocardial necrosis (i.e. troponin I and T); however, 30–50% of these patients experience sudden and fatal outcomes during the early manifestations (6,7). In addition, about 70% of deaths from acute coronary syndromes occur out of the hospital (7). This is because patients with severe atherosclerosis may be asymptomatic and have no elevated cardiac troponin or ECG changes (6). Therefore, early diagnosis of high-risk atherosclerosis by identification of new markers rather than the markers of myocardial necrosis can reduce adverse cardiovascular events (6). Regarding the potential role of PAPP-A in atherosclerosis, several studies have shown its association with adverse cardiovascular events even in patients with stable coronary heart diseases (8,9).

Patients with type 2 diabetes are at increased risk of developing cardiovascular complications. Therefore, Diabetes Mellitus (DM) is considered as an equivalent of coronary artery disease (10). Patients with DM have about three-fold increase in cardiovascular mortality compared to age matched controls (10). Consequently, early identification of at risk patients and diagnosis of troponin negative cardiovascular events in this vulnerable group is of great value. It has been shown that PAPP-A is associated with increased risk of developing adverse cardiovascular events among patients with diabetes (11). To our knowledge, only two studies have compared PAPP-A serum levels between patients with diabetes and healthy controls, which have reported controversial results (12,13). We performed this study to compare the serum levels of PAPP-A between patients with type 2 diabetes and controls, and to assess its association with subclinical atherosclerosis parameters.

Materials and Methods

Study design and subject selection

This case-control study was conducted at Shariati hospital, affiliated to Tehran University of Medical Sciences, Tehran, Iran. Our 107 cases were selected from about 3000 diabetic patients on a simple sequential basis, from September 2010 to August 2011. The patients were regularly visited and followed up at diabetes outpatient clinics at Shariati hospital. The control group consisted of 101 healthy subjects who were relative in law of the selected diabetic patients. The participants were men and non-pregnant women aged between 30 to 65 years with the diagnosis of DM after 30 years of age. Patients with renal failure (Glomerular Filtration Rate<90 mL/min), cirrhosis, malignancy, diabetic nephropathy, peripheral neuropathy, history of myocardial infarction, angioplasty, coronary artery bypass grafting, stroke, reno-vascular disease, and abnormal ECG changes were excluded from the study. A written informed consent was taken from each participant at the beginning of the study. The Institutional Review Board of Tehran University of Medical Sciences reviewed and approved the study protocol.

The diagnosis of DM was made according to American diabetes association guideline 2009 as fasting plasma glucose (FPG) ≥ 126 mg/dl or two-hours post-load glucose ≥ 200 mg/dl or taking oral hypoglycemic agents (14). Hypercholesterolemia was defined as serum total cholesterol levels ≥ 200 mg/dl, serum Low-density Lipoprotein (LDL) ≥ 150 mg/dl, or treatment with cholesterol lowering drugs. Hypertension was defined as systolic blood pressure ≥ 140 mmHg or diastolic blood pressure ≥ 90 mmHg or taking anti-hypertensive medications. Nephropathy was defined as the albumin to creatinine ratio ≥ 30 mg/g in a random urine sample (15). Neuropathy was defined as impairment in the vibratory or pressure sensation in the extremities (16). We calculated the homeostasis model assessment of insulin resistance (HOMA-IR) by multiplying the fasting plasma glucose level (mmol/L) by fasting plasma insulin level (μIU/mL), divided by 22.5 (17).

Clinical and demographic data collection

Sex, age, drug history, duration of DM, and history of cardiovascular, renal, and liver diseases were collected for all patients using a pre-designed questionnaire approved by two specialists. Height and weight were measured with light clothing and without shoes. Body Mass Index (BMI) was calculated as weight in kilograms divided by height per square meter (kg/m2). After at least 10 minutes resting in sitting position, blood pressure was measured twice on each arm using a standard sphygmomanometer. The interval time between each couple of measurements was about five minutes and the highest recorded value was considered as subject's blood pressure. To evaluate the presence of neuropathy, we applied the combination of vibration perception using a 128-Hz diapason test and 10-g monofilament pressure sensation test in the lower extremities (16).

Laboratory measurements

Venous blood samples were taken from patients after 12 hours overnight fasting. Serum levels of PAPP-A was measured using Enzyme-linked immunosorbent assay kit (Quantikine, R&D Systems, Minneapolis, MN, USA). Colorimetric methods (Pars Azmoon kit) with an auto-analyzer (Hitachi 902, Boehringer Mannheim Germany) were used to measure the fasting plasma levels of glucose,

total cholesterol, LDL cholesterol, HDL cholesterol, triglyceride, high sensitivity C-reactive Protein (hs-CRP) , and creatinine. Fasting plasma Insulin levels were determined by enzyme-linked immunosorbent assay method (Monobind kit, USA). Percentage of HbA1c was detected using high performance liquid chromatography (Knauer, Germany). All of these measurements had intra- and inter-assay coefficients of variation less than 4% which are within the accepted ranges of coefficients of variation. A morning midstream urine sample was taken from each participant for the evaluation of nephropathy.

Carotid intima-media thickness

An expert technician measured Carotid Intima Media Thickness (CIMT) using a high resolution B-mode ultrasound scanner with a linear array 13 MHz transducer (MyLab 70 XVision, Biosound Esaote, USA). The method has been described previously (18). At first, a cross-sectional scanning was performed from the proximal part of the common carotid artery to the carotid bifurcation to the distal part of the internal carotid artery to localize possible plaques following a long-axis scanning of the common carotid artery, carotid bifurcation, and internal carotid artery. CIMT was defined as the distance between intima-adventitia interface and lumen-intima interface. Vascular tools 5 software (Medical Imaging Aplications LLC, USA) was used to detect CIMT. Far and near walls of distal 1 cm of common carotid artery, the bifurcation (1 cm in length) and the proximal 1 cm of internal carotid artery were considered to measure CIMT. The average of 12 measurements (6 measurements on the right and 6 measurements on the left) was considered as the subject's CIMT.

Coronary artery calcium score

Coronary artery calcium scanning was done using a Phillips 64 multi detector computed tomography scanner. At first, we obtained antero-posterior and lateral chest scout views for planning. We used 64 × 2.5 mm × 400 ms with 120 KVP and 50-75mAs to completely cover the ascending aorta and the heart. The quantification of the calcium was done using Agatston scoring method (19). An area ≥ 1mm² with 130 HU was defined as positive calcium score. The presence of calcification in coronary arteries was reported as a dichotomous variable (yes or no) by the scanner.

Flow-mediated dilation

Flow mediated dilation was measured following American College of Cardiology guidelines using a high resolution B-mode ultrasound scanner with a linear array 13 MHz transducer (MyLab 70 XVision, Biosound Esaote, USA) (20). Baseline diameter was measured at 5cm above the ante-cubital fossa of the non-dominant arm, and the skin was marked to perform the next measurements at the same side. Pre-cuff diameter was measured dynamically for two minutes and the mean of the maximum diameters of all cardiac cycles was considered as pre-cuff diameter. The diameter of the brachial artery was defined as the distance between anterior and posterior intima-lumen interfaces. A previously placed sphygmomanometer on the forearm was inflated at least 50 mmHg above the systolic blood pressure for five minutes. During five minutes after cuff release, brachial artery diameter was measured dynamically and the mean of the maximum diameter of all cardiac cycles was considered as the post-cuff diameter. An expert technician performed all the measurements in the end-diastolic phase. The brachial flow mediated dilation was defined as the change in the post-occlusion diameter as a percentage of the mean baseline diameter (20).

Aortic augmentation index

Aortic Augmentation Index (AAIx) was measured using tonometric system with validated software (SphygmoCor, AtCor Medical Pty, Sydney, Australia). After the participants rested at least 15 minutes supine position, the point of maximal radial artery pulse was located. The probe was placed on the overlying skin to achieve at least 20 sequential stable waveforms. AAIx was calculated by dividing augmentation pressure by the aortic pulse pressure and expressed as percentage. Augmentation pressure was calculated as the difference between the first and second systolic notches of aortic pressure waveforms. Pulse pressure, systolic, and diastolic blood pressures were obtained from the pulse wave analysis. Pulse pressure was defined as the difference between central systolic and diastolic pressures in the recorded waveforms. The average of two measurements with quality exceeding 90% for each one, was considered as the participant's AAIx (21).

Ankle-brachial index

An expert technician measured the ankle brachial index using a sphygmomanometer and handheld Doppler probe. After five to 10 minutes resting in supine position, systolic blood pressure was measured in both arms and both ankles. To measure brachial systolic blood pressure, the cuff was placed on participant's upper arm. Then the tip of the probe was placed on the

brachial pulse location to hear arterial pulse sounds. Then, the cuff was inflated to 20 mmHg above the point that the brachial artery flow ceased and no pulse sound was heard. At this step, the cuff was slowly deflated at two mm Hg/second speed and the value in which pulse sounds appeared again was recorded. To measure dorsalis pedis and posterior tibialis systolic blood pressure, the cuff was placed on the participant's leg, just above the medial malleolus. The systolic blood pressure of the dorsalis pedis and tibialis posterior arteries was measured in both legs following the mentioned method. The higher blood pressure among the two arms and the highest value of the two ankle measurements was used to calculate the participant's ankle brachial index. Ankle systolic pressure was divided by the brachial systolic pressure to calculate the participant's ankle brachial index (22).

Statistical analysis

Summary statistics were presented as mean ± standard deviation (SD) for continuous variables and percent for dichotomous variables. Kolmogorov-Smirnov test was applied to evaluate normality of the continuous variables. T-test was used to compare means of normally distributed variables between case and control groups. For non-normally distributed variables, we performed Mann-Whitney U test to compare means. Also, $\chi 2$ test was used to compare dichotomous variables between the two groups. To assess the relationship between PAPP-A and other variables, we first pooled the data of the cases and controls and then performed univariable linear regression analysis considering PAPP-A as dependent variable. Independent variables with *P*-values ≤0.2 were considered for entering in the multivariable regression model. In the multivariable analysis, we excluded the variables with *P*-values > 0.05 except some variables of interest based on the data from literature. Finally, we checked for collinearity among the independent variables. *P*-values ≤ 0.05 were considered statistically significant.

Results

Table 1 summarizes the primary characteristics of the case and the control groups.

Table 1. Baseline characteristics of the participants

Variables	Controls (N=101)	Diabetics (N=107)	*P* value
Age	52.30 ± 7.65	53.36 ± 8.12	0.338
Female Sex (%)	50.49	53.27	0.689[1]
Diabetes Duration(years)	------	8.69(6.25)	-----
Body mass index (kg/m^2)	28.65 ± 4.28	27.79 ±4.17	0.149
Systolic blood pressure (mmHg)	121.33 ± 15.73	131.37 ± 18.77	0.006
Diastolic blood pressure (mmHg)	78.50 ± 8.87	80.48 ± 10.88	0.338
Hypertension (%)	8.9	31.8	< 0.001[1]
Smoking (%)	2	8.4	0.073[1]
Fasting Plasma Glucose (mg/dL)	96.57 ± 11.51	164.72 ± 62.23	< 0.001
Fasting plasma insulin (µIU/L)	9.58 ± 6.78	8.84 ± 6.79	0.434
HbA1C (%)	5.29 ± 0. 63	7.94 ± 1.73	<0.001
HOMA_IR	2.32 ± 1.69	3.64 ± 2.89	<0.001
Creatinine (mg/dL)	0.98 ± 0.15	0.96 ± 0.18	0.284
Total cholesterol (mg/dL)	201.69 ± 34.82	174.93 ± 39.77	< 0.001
Triglyceride (mg/dL)	169.65 ± 74.62	195.62 ± 107.98	0.046
HDL-cholesterol (mg/dL)	45.16 ± 10.19	40.89 ± 8.78	<0.001
LDL- cholesterol (mg/dL)	115.46 ± 22.93	95.15 ± 24.06	< 0.001
Hypercholesterolemia (%)	52.5	57	0.373[1]
Statin use (%)	5	39.3	<0.001[1]
Flow mediated dilation (%)	15.15 ± 7.70	12.92 ± 7.18	0.089[1]
CIMT (mm)	0.66 ± 0.14	0.78 ± 0.20	< 0.001
Ankle brachial index	1.17 ± 0.11	1.12 ± 0.12	0.003
Coronary Calcium Score	39.80 ± 108.70	105.87 ± 345.62	0.052[2]
Aortic augmentation index	7.60 ± 4.57	9.43 ± 6.49	0.079
Coronary calcification (%)	0	4.1	0.007[1]
C-reactive protein (mg/dL)	2.20 ± 2.66	3.11 ± 4.55	0.292[2]
PAPP-A (mIU/L)	0.35 ± 0.08	0.61 ± 0.24	< 0.001[2]

PAPP-A: pregnancy associated plasma protein-A , HOMAR-IR: Homeostasis Model Assessment for insulin resistance, CIMT: carotid intima media thickness

1. The variables were compared using χ^2 test.
2. The variables were compared using Mann-Whitney U test.

Values are expressed as mean ± SD

There was no significant difference between two groups regarding age, sex, BMI, hypercholesterolemia, mean diastolic blood pressure (DBP), and serum levels of creatinine. Control subjects had higher serum levels of total cholesterol (P<0.001), low density lipoprotein cholesterol (LDL-C) (P< 0.001), high density lipoprotein cholesterol (HDL-C) (P<0.001) compared to patients with diabetes, whereas values for the rate of statin use (P<0.001), mean systolic blood pressure (SBP) (P=0.006), and serum levels of triglyceride (P=0.046) were higher in patients compared to controls. Moreover, serum levels of PAPP-A was significantly increased in patients compared to controls (P<0.001).

Table 2 shows the results of the univariable and multivariable regression analysis of the relationship between PAPP-A and the independent variables. In univariable analysis, most of the variables including DM, calcification, AAIx, CIMT, ABI, sex, HTN, and statin use were correlated with PAPP-A. In contrast, flow mediated dilation, smoking, hypercholesterolemia, age, BMI, and hs-CRP had no significant association with PAPP-A. In the multivariable analysis, only DM (β=0.237, P<0.001), AAIx (β=0.007, P=0.021), and coronary calcification (β= -0.192, P=0.050) remained significantly associated with PAPP-A. There was also a borderline negative association between ABI and PAPP-A (β= -0.06, P=0.09). No collinearity was observed between the included independent variables.

Table 2. Results of univariable and multivariable regression analyses considering PAPP-A as dependent variable

	Univarriate		Multivariable	
	B	***P*-value**	**B**	***P*-value**
Diabetes(yes/no)	0.267	<0.001	0.237	<0.001
HbA1C (%)	0.053	<0.001	--	--
Creatinine (mg/dL)	-0.234	0.014	--	--
HOMA_IR	0.014	0.022	--	--
Aortic augmentation index	0.010	0.007	0.007	0.021
Smoking(yes/no)	-0.023	0.699		
Coronary calcification(yes/no)	-0.306	<0.001	-0.192	0.050
Statin use(yes/no)	0.355	<0.001	--	--
Hypercholesterolemia(yes/no)	0.025	0.429	0.016	0.614
Flow mediated dilation (%)	-0.002	0.331	0.002	0.345
CIMT (mm)	0.319	<0.001	0.047	0.658
Ankle brachial index	-0.382	0.005	-0.060	0.090
hs-CRP(mg/dL)	0.004	0.335	--	--
Age(yr)	0.002	0.293	--	--
Sex (female/male)	0.068	0.026	--	--
Body mass index(kg/m2)	-0.001	0.701	--	--
Hypertension (yes/no)	-0.152	<0.001	--	--

HOMAR-IR: Homeostasis Model Assessment for insulin resistance, CIMT: Carotid intima media thickness
multivariable regression R^2=0.402

Table 2 shows the results of the univariable and multivariable regression analysis of the association between PAPP-A and the independent variables. In univariable analysis, most of the variables including DM, calcification, AAIx, CIMT, ABI, sex, HTN, and statin use were correlated with PAPP-A. In contrast, flow-mediated dilation, smoking, hypercholesterolemia, age, BMI, and hs-CRP had no significant association with PAPP-A. In the multivariable analysis, only DM (β=0.237, P<0.001), aortic augmentation index (β=0.007, P=0.021), and coronary calcification (β= -0.192, P=0.050) remained significantly associated with PAPP-A. There was also a borderline negative association of ABI with PAPP-A (β= -0.06, P=0.09). No collinearity was observed between the included independent variables.

Discussion

In this study, we found elevated serum levels of PAPP-A among patients with diabetes compared to healthy controls, which remained significant after adjustment for other variables. PAPP-A was also positively and negatively associated with AAIx and coronary calcification, respectively. The first study that compared PAPP-A between patients with diabetes and controls reported higher levels of PAPP-A in patients, which is in accordance with our results (12). However,

another study found decreased levels of PAPP-A in these patients compared to controls (13). In fact DM is associated with chronic low grade inflammation, which is believed to be both the cause of DM and an important cause of its complications (23-25). This systemic inflammation is associated with the increase in the serum levels of well-known inflammatory cytokines including CRP, tumor necrosis factor-α, and interleukine-6 (23). PAPP-A is potentially a pro-inflammatory molecule and is involved in local proliferative and inflammatory processes (4). This increased inflammation, can be the cause of increased serum levels of PAPP-A in patients with diabetes compared to healthy controls.

Aortic augmentation index is an indicator of arterial stiffness. Arterial stiffness is a result of mechanisms including vascular smooth muscle cell proliferation, increased collagen and extracellular matrix synthesis, and elastin degradation (26,27). Such mechanisms are mediated by activation of different matrix metalloproteinases (MMP) (26,27). PAPP-A is a MMP which increases the local bioavailability of IGF- I and consequently vascular smooth muscle cell proliferation, matrix synthesis and fibrosis of the vessel wall (4). Some of the previous studies have shown a positive relation between MMPs and arterial stiffness (28,29), while another study has shown an inverse relation (30). Although a recent study proposed that MMP-2 and MMP-9 are not associated with arterial stiffness in patients with diabetes (31), our study showed a positive association between AAIx and PAPP-A which remained significant after adjustment for DM and other included variables. This positive association seems to be biologically plausible as stated above.

Although coronary calcification and PAPP-A are both correlated with increased risk of plaque rupture and death among patients with cardiovascular diseases (5,32,33), their association was negative among our patients. One of the main mechanisms involved in the plaque calcification is apoptosis (32). Apoptotic debries including phospholipid rich membranes, can facilitate calcification of atherosclerotic plaques (32). PAPP-A increases local IGF-I in atherosclerotic plaques which consequently promotes mitogenesis and decreases apoptosis in various cell types (4). This proliferative and anti-apoptotic activity may decrease the rate of calcification and may explain the negative association observed in our study. However, these results should be interpreted with caution until more specific studies are done.

In the present study, PAPP-A and CIMT were significantly associated in the univariable analysis, but this association did not remain significant in the final multivariable analysis (Table 2). Some of the previous studies have shown a positive association between PAPP-A and CIMT (12,34), while others have not reported such association (35-37). In our study, the relationship between PAPP-A and CIMT was not significant after entering the DM in the regression equation. These results suggest that the presence of this association may be confounded by other variables or may be mediated by other cardiovascular risk factors and needs to be further clarified. One of the previous studies has indicated that serum levels of PAPP-A is associated with the increased echogenicity of the plaque, but not with CIMT itself (36). Beaudeux et al, suggested that PAPP-A is positively associated with increased echogenicity of the plaques (type V or grater lesions) which are at increased risk of rupture (36). This results further suggest that PAPP-A may be correlated with other parameters of carotid atherosclerosis rather than CIMT. Also, the association of PAPP-A and hypercholesterolemia and also the effect of statin therapy on PAPP-A remained controversial in the current and previous studies (12,36-40).

In the current study, we found that PAPP-A is elevated in the sera of patients with diabetes compared to healthy controls, which may be explained by the increased inflammation among such patients. PAPP-A was also positively associated with AAIx and negatively associated with coronary artery calcification. The results of our study suggest that PAPP-A can be an indicator of subclinical atherosclerosis in patients with type 2 diabetes.

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