

# Utility of Tissue Inhibitor Metalloproteinase-1 and Osteopontin as Prospective Biomarkers of Early Cardiovascular Complications in Type 2 Diabetes

Doaa Samir Salah El-Din\*, Ahmed Ibrahim Amin, Ahmed Osman Egiza

Chemistry, Faculty of Science, Cairo University, Giza 12613, Egypt

## Abstract

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\*Correspondence: Doaa Samir Salah El-Din, Chemistry, Faculty of Science, Cairo University, Giza 12613, Egypt. E-mail: [sdoaa@sci.cu.edu.eg](mailto:sdoaa@sci.cu.edu.eg)

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**AIM:** This work investigated associations between tissue inhibitor metalloproteinase-1 and diabetic cardiovascular diseases in type 2 diabetic patients; also it investigated the role of osteopontin in the diagnosis of type 2 cardiovascular diabetes complications.

**SUBJECTS AND METHODS:** These were examined on eighty subjects, divided into three groups as follows: twenty volunteer healthy control subjects, thirty type 2 diabetes mellitus (DM) patients, and thirty cardiovascular, diabetic patients. Full clinical measurements were carried out, and the expression level of tissue inhibitor metalloproteinase-1 in blood samples was analysed by real-time PCR, using gene-specific primer pairs. Also osteopontin concentrations had been measured by the enzyme-linked immunosorbent assay. Data were tested statistically by parametric tests.

**RESULTS:** The concentrations of osteopontin and the expression levels of tissue inhibitor metalloproteinase-1 were significantly increased in diabetic and cardiovascular diabetic groups compared to control group also they were significantly increased in the cardiovascular diabetic group compared to the diabetic group.

**CONCLUSION:** Tissue inhibitor metalloproteinase-1 and osteopontin concentrations were significantly increased in diabetic patients with cardiovascular complications than other groups.

## Introduction

The expanding commonness of type 2 diabetes mellitus (T2DM) calls for creating corresponding screening systems for early recognisable proof of high-risk people for the disease or its complexities [1]. The condition is comorbid with macrovascular disorders, for example, coronary artery disease, peripheral arterial disease, and stroke. Diabetes mellitus is a major risk cause for cardiovascular disease (CVD) which is the main cause of death among the diabetic patients. Diabetes mellitus intensifies atherosclerosis and heart failure mechanisms. Regrettably, these mechanisms are not sufficiently modulated by therapeutic strategies focusing only on the glycemic control with currently available drugs or approaches [2], underscoring the requirement for forceful CVD risk factor management.

The diagnosis and screening of diabetes

mellitus have been changed since the earlier scientific statement, with the incorporation of glycated haemoglobin (HbA1c) in the diagnostic gauges of type II diabetes of at least 6.5% [3]. Specifically, HbA1c was used as a preferable standardised assay over glucose, a better indicator for overall glycemic exposure, and as less subject to pre-analytic instability, prandial status, biological variability, and intense stress [4].

While insulin and glucose are the most entrenched biomarkers, progresses in the “omics” technologies have recognised the extent of molecules including genetic variants, small metabolites, RNA transcripts, and proteins that could serve as indicators of diabetes risk [5].

Circulating markers of extracellular matrix turnover, such as matrix metalloproteinases (MMP) and their tissue inhibitors (TIMP), are essential to the vascular alternations of atheromatous vascular disease. TIMP-1 was considered as an endogenous MMP inhibitor that might include in the vascular matrix

fibrosis [6]. Also, it has a role in diastolic dysfunction and left ventricular hypertrophy by diminishing cardiac collagen type I turnover and expanding cardiac stiffness and mass [7].

In diabetes, TIMP-1 and MMP-9 levels are significantly increased [8], also in this condition, peripheral and central artery stiffness is increased [9]. Amelioration in the metabolic control leads to decreasing in TIMP-1 levels, and this raises TIMP-1 probability as a marker of vascular composition in diabetes mellitus [10], a potential purpose of mediation.

Phosphoprotein osteopontin (OPN), also called secreted phosphoprotein 1 (SPP1), urinary stone protein, early T lymphocyte activation 1, and bone sialoprotein is found in various cell types [11]. In prevalent patients, a significant association was observed between atherosclerotic plaque development and plasma OPN levels, independently of conventional risk factors [12]. Moreover, high glucose and advanced glycation end product may lead to upregulation of OPN expression in the vascular wall of diabetic animal models and diabetic patients [13].

In this study, the hypothesis that there may be a relationship between elevated both OPN levels and TIMP-1 expression levels and the presence of CVD in type 2 diabetic patients were tested.

## Subjects and Methods

### *Patients' data*

T of 80 subjects was recruited for that study and divided into three groups. Twenty volunteer healthy control subjects (G1), thirty type 2 diabetes mellitus (DM) patients (G2); disease duration  $5 \pm 3$  years, and thirty type 2 diabetic patients with cardiovascular (coronary heart) diseases (G3); disease duration  $3 \pm 1$  years. Type II diabetic patients were those consecutively referred during routine medical care visits from an outpatient diabetes clinic (National Institute of Diabetes in Egypt). If patients had any known systemic diseases other than diabetes that can influence the needed results such as epidemiological diseases, chronic periodontitis, nephropathy, retinopathy, neuropathy, gallbladder, liver, and lung diseases, they were excluded in this study. Participation in this study is completely voluntary. Patients decide to participate, can stop participating at any time and may decide not to answer any specific question. Participants' decision not to continue participating will not influence the nature of their relationship with a researcher or with a staff of this study either now or in the future. If a patient withdraws from the study, all associated data collected will be immediately destroyed wherever possible.

### **Laboratory markers of diabetes mellitus**

Blood samples were collected from all fasting participants in this study, Lipemic and hemolyzed samples were excluded, samples were analysed for HbA1c by cation - exchange resin method [14]. Total cholesterol (TC) and triglycerides (TG) in serum were measured by colourimetric enzymatic method [15]. Also, high-density lipoprotein cholesterol (HDL cholesterol) was measured [16], and low-density lipoprotein cholesterol (LDL cholesterol) was evaluated by Friedewald formula [17]. Moreover, heart function biomarkers such as creatine phosphokinase-MB (CK-MB), creatine phosphokinase (CK), and lactate dehydrogenase (LDH) were measured by enzymatic method [18].

Tissue inhibitor of metalloproteinases-1 (TIMP-1) analysis TIMP-1 expression levels were determined using Real Time PCR (qPCR) which performed with SYBR Green (5X HOT FIREPol@EvaGreen@ qPCR Mix Plus) for tenfold diluted cDNA samples in a triplet set using specific primers pairs which synthesized in HVD Life Sciences co., Vienna, Austria; forward primer sequence (CTGGCTTCTGGCATCCTGTTG) and reverse primer sequence (GTCTGGTTGACTTCTGGTGTCC) (NG\_012533.1, NM\_003254.2) against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a reference gene; forward primer sequence (CAGCCTCAAGATCATCAGCAATG) and reverse primer sequence (CAGTCTTCTGGGTGGCAGTGA) (NG\_007073.2, Variant1: NM\_002046.5, Variant 2: NM\_001256799.2, Variant 3: NM\_001289745.1, Variant 4: NM\_001289746.1). Blood RNA was extracted by EZ-10 column blood RNA purification kit (cat.# BS82313) according to the manufacturer's instructions, and it was eluted from the membrane in the presence of RNase - free water. First cDNA strand was synthesised immediately after RNA extraction. Reaction components were mixed as follows: 5 µg RNA, 0.5 µl (10 µmol) Oligo dT and 0.5 µl (10 µmol) random primer, then the volume was adjusted to 12.5 µl using diethyl pyrocarbonate (DEPC) treated water. The reaction tubes were mixed well and incubated 5 min at 60°C to remove the RNA secondary structure, then chilled on ice. Finally, the following components were added to the reaction mixture: 2 µl (10 mM) dNTPs, 4 µl (5 X) Moloney murine leukaemia virus (MMLV) buffer, 0.5 µl ribolock (40 u/µl) and 1 µl reverse transcriptase enzyme (MMLV-RTase) (20 u/µl). As a termination step, the final reaction mixture (20 µl) was spun down and incubated at room temperature for 10 min, then at 42°C for 1hour followed by 10 min at 70°C. cDNA samples were stored at - 20°C for the amplification steps which performed on Bio-Rad CFX Manager™ software, version 3.1. The qPCR was performed according to the following program: Initial denaturation and polymerase activation step for 10 min at 95°C, denaturation step at 95°C for 30 sec, annealing step for 30 sec at 57°C for GAPDH and TIMP-1 and finally, the elongation step for 45 sec at 72°C. The denaturation,

the annealing and the elongation steps were performed at 50 cycles.

### Osteopontin (OPN) analysis

Osteopontin concentrations were determined using commercially available enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's protocol (BOSTER Biological Technology Co., Inc., USA; cat.# EK0482). All samples were analysed in duplicate. Horseradish peroxidase was conjugated with the secondary antibody, and tetramethyl benzidine (TMB) was used as a substrate. The absorbance was measured at 450 nm. Osteopontin concentration was expressed as pg/ml.

### Statistical analysis

SPSS 17.0 software was used to execute the statistical analyses, and the results were represented by the mean ± SD. Student's t-test was used to make a comparison between the groups and correlations were assessed by Pearson's correlation test using P < 0.05 as considered to be significant.

### TIMP-1 expression findings in the diabetic patients

The resulted data of the normalized expression ratio of TIMP-1 to GAPDH as a reference gene was represented in Table 2, and Fig. 1, A. Also individuals' data were statistically analyzed from the point of view as ± S.D and S.E. Then the t-value results were checked on student's t-test to detect the significance level (p-value).

**Table 2: Statistical analysis of TIMP-1 expression levels and osteopontin concentrations in control and different tested groups**

|                                     | Control group                         | Diabetic group | Cardiovascular diabetic group |                    |
|-------------------------------------|---------------------------------------|----------------|-------------------------------|--------------------|
| Statistical analysis of TIMP-1      | Avg. dC <sub>T</sub>                  | 7.03           | 2.27                          | 1.39               |
|                                     | ddC <sub>T</sub>                      | 0.0            | -4.77                         | -5.65              |
|                                     | FC than control group                 | 1.0            | 27.20                         | 50.07              |
|                                     | FC than cardiovascular diabetic group | 0.12           | 0.54                          | 1.0                |
|                                     | ±S.D                                  | 0.75           | 0.9                           | 0.72               |
|                                     | S.E                                   | 0.17           | 0.16                          | 0.13               |
|                                     | F-value (ANOVA)                       | -              | 327.0                         | -                  |
|                                     | T-value                               | G1             | 20.3 <sup>**</sup>            | 26.4 <sup>**</sup> |
|                                     | G2                                    | -              | 4.2 <sup>**</sup>             |                    |
| Statistical analysis of osteopontin | OPN conc. range                       | 6000-8500      | 9000-14700                    | 9550-22000         |
|                                     | OPN conc. mean                        | 7140           | 10765                         | 14421              |
|                                     | ±S.D                                  | 619.8          | 1588.3                        | 3411.6             |
|                                     | S.E                                   | 138.6          | 290.0                         | 622.9              |
|                                     | F-value ANOVA                         | -              | 59.6                          | -                  |
|                                     | T-value                               | G1             | 9.7 <sup>**</sup>             | 9.4 <sup>**</sup>  |
|                                     |                                       | G2             | -                             | 5.3 <sup>**</sup>  |

<sup>\*\*</sup>P ≤ 0.001.

## Results

### Clinical findings in the diabetic patients

Fasting blood sugar (FBS), glycosylated Hb (HbA1c%), Lipid profile; total cholesterol (TC), triglyceride (TG), low density lipoprotein cholesterol (LDL-C), LDL-C/HDL-C ratio, cardiac biomarkers; creatine phosphokinase (CK), creatine phosphokinase-MB isoenzyme (CK-MB), and lactate dehydrogenase (LDH) activities were significantly higher in the cardiovascular diabetic groups than that in the control and the diabetic group, while high-density lipoprotein cholesterol (HDL-C) concentrations were significantly lower in the cardiovascular, diabetic groups than that in the control and the diabetic group as clarified in Table 1.

**Table 1: Some clinical data in control and different tested groups**

|               | Normal range | Control group              | Diabetic Group             | Cardiovascular diabetic group |
|---------------|--------------|----------------------------|----------------------------|-------------------------------|
| FBS (mg/dl)   | 60-110       | 84.0 ± 8.9                 | 268.6 ± 94.2               | 347.0 ± 66.9                  |
| HbA1c (%)     | < 6.5        | 4.9 ± 0.7 <sup>**</sup>    | 9.1 ± 2.0 <sup>**</sup>    | 10.3 ± 1.1                    |
| TC (mg/dl)    | < 200        | 105.8 ± 9.1 <sup>**</sup>  | 157.9 ± 28.9 <sup>**</sup> | 262.7 ± 19.8                  |
| TG (mg/dl)    | 35-140       | 75.6 ± 8.7 <sup>**</sup>   | 123 ± 22.8 <sup>**</sup>   | 387.8 ± 71.9                  |
| HDL-C (mg/dl) | >55          | 59.9 ± 4.6 <sup>**</sup>   | 52.9 ± 6.8 <sup>**</sup>   | 22.9 ± 4.7                    |
| LDL-C (mg/dl) | < 150        | 30.6 ± 1.0 <sup>**</sup>   | 80.4 ± 30.1 <sup>**</sup>  | 162.2 ± 21.6                  |
| LDL-C/HDL-C   | < 2          | 0.5 <sup>**</sup>          | 1.6 <sup>**</sup>          | 7.4                           |
| CK (U/L)      | Up to 120    | 54.5 ± 10.2 <sup>**</sup>  | 42.5 ± 7.8 <sup>**</sup>   | 128.1 ± 23.7                  |
| CK-MB (U/L)   | 0-24         | 21.9 ± 3.9 <sup>**</sup>   | 18.5 ± 3.8 <sup>**</sup>   | 25.2 ± 3.7                    |
| LDH (U/L)     | 160-320      | 207.3 ± 12.5 <sup>**</sup> | 214.6 ± 23.8 <sup>**</sup> | 620.3 ± 101.9                 |

Mean ± SD, vs. cardiovascular diabetic group. \*P ≤ 0.05; \*\*P ≤ 0.001.

The present study showed that; the expression level of TIMP-1 gene significantly increased by 27.2 and 50.07 folds to reference gene; GAPDH, in diabetic and cardiovascular diabetic patients respectively than normal volunteers, so it might be considered as diagnostic marker for DM, and its expression level significantly increased by 1.84 folds in cardiovascular, diabetic patients than the diabetic patients, so it might be considered as diagnostic marker for detection of cardiovascular complications in type 2 diabetes.

### Osteopontin (OPN) concentration findings in the diabetic patients

The individuals' data of osteopontin (OPN) concentration of different tested groups was statistically analysed in Table 2 and evaluated from the point of view as ± S.D and S.E. The result of the t-value is then checked on student's t-test to find out the significance level (p-value).

From the table, it appeared that highly significant difference (p ≤ 0.001) was found in diabetic group (G2) (10765 ± 1588.3 pg/ml), and cardiovascular, diabetic group (G3) (14421 ± 3411.6 pg/ml) as compared with the control group (G1) (7140 ± 619.8 pg/ml), also highly significant difference was appeared in G3 as compared with G2 (Fig. 1, B).

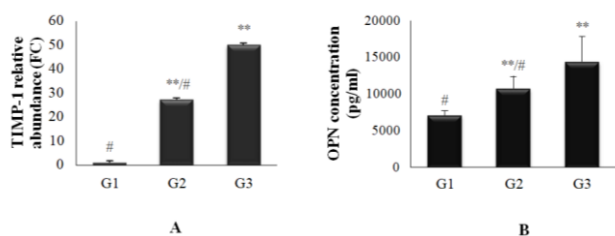


Figure 1: (A) TIMP-1 relative expression level (FC ± S.D). (B) Osteopontin concentrations (Mean ± S.D) in control and different tested groups. \*\*Highly significant different with the control group G1 (p ≤ 0.001), # Highly significant different with cardiovascular, diabetic group G4

Table 3 showed highly significant positive correlation observed between osteopontin with some variables; FBS, HbA1c, cholesterol, triglyceride, LDL-C, CK, and LDH, and between TIMP-1 and HDL-C, while there was low significant positive correlation between osteopontin and CK-MB concentrations.

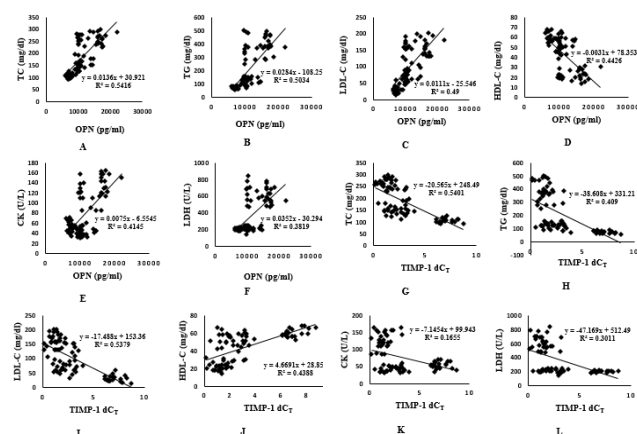


Figure 2: Correlation between osteopontin concentrations and TIMP-1 with some variable

Also there was highly significant negative correlation between osteopontin and HDL-C, also between TIMP-1 and some variables; FBS, HbA1c, cholesterol, triglyceride, LDL-C, CK, and LDH, while there was no correlation between TIMP-1 and CK-MB concentrations. (Fig. 2, A-L).

Table 3: Correlation between osteopontin concentrations and TIMP-1 with other variables

| Osteopontin       |                         |      | TIMP-1                  |                 |
|-------------------|-------------------------|------|-------------------------|-----------------|
| Variable          | Pearson Correlation (r) |      | Pearson Correlation (r) | Sig. (2-tailed) |
| FBS               | 0.605                   | H.S. | -0.740                  | H.S.            |
| HbA1c             | 0.656                   | H.S. | -0.777                  | H.S.            |
| Cholesterol       | 0.736                   | H.S. | -0.735                  | H.S.            |
| Triglyceride      | 0.709                   | H.S. | -0.640                  | H.S.            |
| HDL - cholesterol | -0.666                  | H.S. | 0.663                   | H.S.            |
| LDL- cholesterol  | 0.701                   | H.S. | -0.734                  | H.S.            |
| CK                | 0.644                   | H.S. | -0.407                  | H.S.            |
| CK-MB             | 0.257                   | L.S. | -0.074                  | N.S.            |
| LDH               | 0.618                   | H.S. | -0.549                  | H.S.            |

N.S. P > 0.05; \*P ≤ 0.05; \*\*P ≤ 0.001.

## Discussion

Diabetes mellitus is a complex disease affecting nearly all tissues and organs, with 158 metabolic ramifications extending far beyond impaired glucose metabolism. Biomarkers may reflect the presence and/or severity of hyperglycemia and the vascular complications of diabetes [19].

TIMP-1 is a diagnostic marker for diabetes, in this study, the expression level of TIMP-1 was significantly increased in diabetic and cardiovascular diabetic patients than normal volunteers, also increased significantly in cardiovascular, diabetic patients than other diabetic groups, higher levels of circulating TIMP-1 was connected with the higher risk of diabetes and cardiovascular demise. This finding was consistent with Usmanova [20], furthermore, Lee and his colleagues who found that plasma TIMP-1 concentration was significantly raised in type II diabetic patients [21]. Also in this study, the TIMP-1 expression level was significantly higher in diabetic patients with cardiovascular diseases (CVD) than in those without cardiovascular diseases, these were in agreement with Papazafiropoulou and Tentolouris results [22]. TIMP-1 is an endogenous MMP inhibitor that might be involved in vascular matrix fibrosis [6] and has a role in left ventricular hypertrophy and diastolic dysfunction by reducing cardiac collagen type I turnover, thereby increasing cardiac mass and stiffness [6][23]. Increased Central and peripheral artery stiffness [9] occurs with diabetes [24], and higher TIMP-1 circulating levels complete the hypothesis that altered TIMP-1 activities can be related to arterial stiffness.

Another biomarker studied in that work, was osteopontin (OPN) which is a phosphoprotein found in different types of cells [11]. In this study, Levels of serum OPN increased in all diabetic groups significantly in comparing with the control group, the expression of OPN was highly induced by glucose [25], this agreed with Takemoto and his colleagues [26] who reported an increase of serum OPN in diabetic patients, suggesting that it may be involved in the accelerated atherosclerosis-related to diabetes. Also in this study, its concentration increased in a cardiovascular, diabetic group significantly in comparing with the diabetic group. OPN is a pleiotropic cytokine that is a common and relevant component of many acute and chronic vascular or endothelial responses to injury characterised by inflammation and/or fibrosis, including atherosclerosis, arterial neointimal hyperplasia, and aortic stenosis [27], as well as the vascular damage, accompanied diabetes [28]. OPN has been acting as the main factor in the atherosclerosis development [29][30]. Plasma OPN concentration is elevated in the essential hypertension, coronary artery disease (CAD), and restenosis [31][32][33]. In the cardiovascular system, OPN is one of the main regulators of the vascular disease and the chronic inflammation [34]. In

other aspects of vascular diseases, Yan and his colleagues [35] found that plasma OPN level, parallels with the severity of nephropathy and CAD in diabetes, suggesting that an elevated plasma OPN level can be used as indicator for the diabetic vasculopathy screening.

This study revealed that, the expression levels of TIMP-1 and osteopontin concentrations had highly significant differences between diabetic and cardiovascular diabetic patients in Egypt which appeared in Table 2, so they could be used as molecular biomarkers in the diagnosis of cardiovascular complications in type 2 diabetic patients to predict potential progression and aid in case management.

In the future, we can be hopeful that new blood-based biomarkers will facilitate the discovery, prohibition, and treatment of diabetes and its complications much sooner than overt disease develops.

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