# Evaluation of Some Fibrinolytic Factors for Assessment of Lower Extremity Arterial Disease (LEAD) in Diabetic Patients

Mahmoud A. Ashour[1]; Hisham Mohamed Omar [2]; Ola Aly Hussein[2] and Nanis A. Salah [2]

[1]Department of Internal Medicine, [2]Department of Clinical and Chemical Pathology, Faculty of Medicine, Zagazig University, Egypt.
kamel_ashour2050@hotmail.com

**Abstract:** This study was carried out in the Clinical Pathology and Internal Medicine Departments, Faculty of Medicine, Zagazig University Hospitals. The study included 57 subjects classified into 3 groups. Group I: It included 13 apparently healthy subjects. Group II: It included 22 insulin-dependent diabetic patients. They were sub-classified into 15 NON Lower Extremity Arterial Disease (LEAD) and 7 LEAD. Group III: It included 22 non-insulin-dependent diabetic patients. They were sub-classified into 15 NON LEAD and 7 LEAD. All patients and control subjects were subjected to the followings: 1- Full history taking. 2-Complete clinical examination 3-Complete blood picture (CBC). 4-Prothrombin time (PT) and Partial thromboplastin (PTT). 5-C reactive protein (CRP). 6-Liver and kidney functions tests fasting and two hours postprandial serum glucose.7-Lipid profile (total cholesterol, triglycerides, HDL- cholesterol).8- Specific laboratory investigations: Glycosylated hemoglobin (HbA1c). Assay of fibrinogen. Assay of tissue plasminogen activator (t-PA). The results revealed the following: - There was no significant difference between all studied groups as regard age of subjects and duration of clinical diabetes. There was a significant increase in BMI in group III compared to groups I and II but no significant difference was found between group II and group I. t-PA was significantly increased in group II compared to group I but significantly decreased in group III compared to groups I and II. There was a significant increase in HbA1c in group II and group III compared to group I with no significant difference was found between group II and group III. There was a significant increase in fibrinogen in groups II and III compared to group I with no significant difference was found between group II and group III. There was a significant increase in CRP in in groups II and III compared to group I. CRP was also significantly increased in group III compared to group II. There was a significant decrease in the levels of cholesterol In groups II and III compared to group I and significantly lower in group II compared to group III. The level of triglyceride was significantly higher In groups II and III compared to group I, also triglyceride in group III was significantly higher compared to group II. The level of HDL-cholesterol was significantly higher in group II compared to group I and significantly lower in group III compared to groups I and II. There was no significant difference between NON LEAD and LEAD in group II as regards t-PA but in the group III, t-PA was significantly higher in LEAD compared to NON LEAD. There was statistically positive correlation between t-PA and age and diabetic duration in total, NON LEAD and LEAD group II. There were statistically positive correlations between t-PA and age in total, NON LEAD and LEAD group III. There was a statistically positive correlation between t-PA and HbA1c in total group III. There were statistically positive correlation between t-PA and diabetic duration in total, NON LEAD and LEAD group III.

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## 1. Introduction

Diabetes mellitus is a group of disorders characterized by hyperglycemia and associated with microvascular (i.e., retinal, renal, neuropathic) and macrovascular (i.e., coronary, peripheral vascular) complications. Hyperglycemia results from lack of endogenous insulin or resistance to the actions of insulin in muscle, fat and the liver. In addition to an inadequate response by the pancreatic beta cell **(Wolfs *et al.*, 2009).**

Diabetes leads to a hypercoagulable state. It is associated with the increased production of tissue factor by endothelial cells and vascular smooth muscle cell (VSMC), as well as increased plasma concentrations of factor VII. Hyperglycemia is also associated with a decreased concentration of antithrombin and protein C, impaired fibrinolytic function, and excess production of PAI-1 **(Beckman *et al.*, 2004).**

The etiology of atherosclerosis, including lower extremity arterial disease (LEAD), is multifactorial. Major risk factors are hyperglycemia, smoking and hypertension **(Dieter *et al.*, 2002).**

The fibrinolytic system includes a broad spectrum of proteolytic enzymes with physiological and pathophysiological functions in several processes

such as hemostatic balance, tissue remodeling, tumor invasion and angiogenesis **(Fay *et al.*, 2007).** The main enzyme of the plasminogen activator system is plasmin, which is responsible for the degradation of fibrin into soluble degradation products. The activation of plasminogen into plasmin is mediated by two types of activators, urokinase–type plasmingen activator (uPA) and tissue-type plasminogen activator (t-PA).The activity of both is regulated by specific plasminogen activator inhibitors (PAIs) **(Esther *et al.*, 2008).**

The fibrinolytic system is primarily an interaction between plasminogen activators, and inhibitors and one response to vascular injury is an activation of t-PA. Increased t-PA-activity may therefore be a potential indicator of an early ongoing vascular damage and, possibly, a compensatory mechanism. Both t-PA and PAI-1 mass levels have been suggested as indicators of vascular damage **(David Sahli *et al.*, 2009).**

In diabetic patients, vascular endothelial cells exposed to high glucose level leading to elevation of t-PA in the plasma accompany impaired fibrinolysis **(Maiello *et al.*, 1992).**

Tissue plasminogen activator level elevates in the plasma of the diabetic patients with lower extremity arterial disease (LEAD) and can be use as an early marker for diagnosis of these cases **(Raffetto *et al.*, 2005).**

The **aim** of the present study is to evaluate the plasma level of tissue plasminogen activator (tPA) as an early predictor marker of asymptomatic lower extremity arterial disease (LEAD) in patients with diabetes mellitus. Also correlation of this marker with diabetic atherosclerosis was done.

## 2. Subjects and methods

### Subjects:

This study was carried out at Clinical Pathology and Internal Medicine Departments, Faculty of Medicine, Zagazig University Hospitals.

Fifty seven subjects were included in the study (13 healthy controls and 44 diabetic patients). They were divided into three groups as follows:

**Group I:** It included 13 apparently healthy subjects. They were 7 males and 6 females, with a mean age 45.8 ± 8.1 years. They matched well with patients as regard age and sex.

**Group II:** It included 22 insulin-dependent diabetic patients. They were 10 males and 12 females. Their ages ranged from 30-70 years with a mean of 48.1 ± 5.7 years. They were sub-classified into 15 NON LEAD and 7 LEAD.

**Group III:** It included 22 non- insulin-dependent diabetic patients. They were 10 males and 12 females. Their ages ranged from 30-70 years with a mean 50.3 ± 8.0 years. They were sub-classified into 15 NON LEAD and 7 LEAD.

**All patients were subjected to the followings:**

1- Full systemic history of hypertension, cardiovascular disease, tobacco use and diabetic duration.
2- Full history of arterial disease (atherosclerosis) taking along a spectrum of severity ranging from no symptoms, intermittent claudication, rest pain, and finally to non-healing wounds and gangrene.
3- Physical local examination by inspection of the foot and palpation of peripheral pulses thorough:
   - Inspection for signs of vascular insufficiency such as dependent rubor, pallor on elevation, absence of hair growth, dystrophic toe nails and dry fissured skin. Also interdigital spaces inspected for fissures, ulcerations, and infections.
   - Assessment of pulsation of femoral, popliteal and pedal vessels.
4-Determination of Ankle Brachial Index (ABI): is the ratio of the systolic blood pressure in the ankle divided by the systolic blood pressure at the arm.
5- Determination of Body mass index (BMI): is a measure of body weight based on a person's weight and height.

### Methods:

All members of this study were subjected to the following:

**1. Routine laboratory investigations:**

- Complete blood picture (CBC) by (Sysmex-KX 21N – Sysmex Corporation).
- Prothrombin time (PT) and Partial thromboplatin (PTT) by (Sysmex –CA 1500, Japan).
- C reactive protein (CRP) by (Cobas-Integra).
- Liver and kidney functions tests, fasting and two hours post prandial serum glucose by dimension RXL MAX autoanalyser (Seimens Medical Solution Diagnostics, UL, USA).
- Lipid profile (total cholesterol, triglycerides, HDL-cholesterol) by dimension RXL MAX autoanalyser (Seimens Medical Solution Diagnostics, UL, USA).

**2. Specific laboratory investigations:**

- Assay of Glycosylated hemoglobin (HbA1c).
- Assay of Fibrinogen by fibrin-timer BFT II analyzer (Siemens, Germany).
- Assay of tissue plaminogen activator (t-PA) by ELISA processor II (Dade Behring, USA).

**Specimen collection:**

After 12 hours, 10 ml venous blood sample was collected from all subjects by venipuncture aseptically and divided as follow:

1-1.5 ml of blood was delivered into a tube containing 1.2 mg/ml EDTA used for complete blood picture and glycosylated hemoglobin (HbA1c).

2-3.6 ml of blood was transferred into a tube containing 0.4 ml Na citrate with a ratio of 9:1 used for PT, PTT, fibrinogen and tissue plaminogen activator (t-PA).

3-The remainder of blood was transferred into a plain tube, allowed to clot at 37 °C followed by centrifugation at 3000 r.p.m. for 15 minutes the serum was collected and used for determination of liver function, kidney function, lipid profile and fasting serum glucose level. Another blood sample was withdrawn from Groups II and III 2 hours postprandial for determination of postprandial serum glucose level.

**Assay of glycosylated hemoglobin (HbA1c)**

**Principle:**

Whole blood was mixed with a lysing reagent containing a detergent and borate ions. Elimination of the labile Schiff's base was thus achieved during the hemolysis.

A preparation of hemolysed whole blood was mixed with a weakly binding cation–exchange resin. The non-glycosylated hemoglobin (HbA0) binds to the resin leaving (HbA1) free to be removed by means of resin separator in the supernate. The percent of HbA1 was determined by measuring the absorbance values at 415 nm of the HbA1 fraction and of the total Hb fraction **(Nuttall, 1998).**

**Reagents:**

1. Glycohemoglobin ion–exchange resin contained: cation – exchange resin (borate 150 mmol/l), imidazole buffered at pH 7.6.
2. Glycohemoglobin lysing reagent contained: 1 mol/l borate and detergents 0.25%.
3. Glycohemoglobin standard (freeze dried hemoglobin): 1 vial prepared from packed human erythrocytes.

**Reagent preparation:**

- One ml distilled water was added to reconstitute the standard vial and allowed standing for minutes at room temperature. The vial was shaked gently to hasten the reconstitution.
- Glycohaemoglobin ion–exchange resin and Glycohaemoglobin lysing reagent are ready to use

**Procedure:**

**Hemolysate preparation**

- 0.5 ml of lysing reagent was pipetted into tubes labeled standard (s) and unknown (u).
- 0.1ml of each well mixed blood sample was pipetted into appropriately labeled tube and mixed and leaved for 5 minutes.

**Glycohemoglobin separation and assay:**

- 0.1ml of the prepared hemolysate was pipetted into appropriately labeled resin tubes.
- A resin separator was positioned in tube and rubber sleeve was approximately 1-2 cm above liquid level then mixed on hematology rocker for 5 minutes.
- A resin separator was pushed into tube until resin was firmly packed in bottom of this tube.
- Each supernatant was directly poured into cuvette for absorbance reading.
- Absorbance of standard and unknown was read with water blank at 415 nm within 60 minute.

**Total hemoglobin assay:**

- 5.0ml of deionized water pipetted into tubes labeled standard (s) and unknown (u).
- 0.02ml of hemlysate was pipetted into approximately labeled tube, mixed well and transferred to cuvette for absorbance reading with water blank at 415nm.

**Calculation of the HbA1c content**

**Factor F Determination by Use of (STD):**

The glycohemoglobin percentage **(**% HbA1c (STD)**)** is stated on the label under %.

Atotal Hb (STD) X % HbA1c (STD)

**F**= AHbA1 (STD)

**Glycohemoglobin Content of the sample:**

$$\% \text{ HbA1c sample} = F \times \frac{\text{A Hb1c sample}}{\text{A total Hb sample}}$$

**Clinical Interpretation:**

Patients with controlled metabolism or stabilized diabetics

HbA1c 4.5 - 7.0 %

Diabetics, insufficiently controlled or with metabolic Imbalance HbA1c ≥ 8.5 %

**Assay of fibrinogen**

**Principle of the method:**

Quantitative measurement by bringing citrated plasma to full coagulation with a large excess of thrombin. So, the coagulation time will depend largely on the fibrinogen content of the specimen and can be measure by fibrin timer **(Cooper and Douglas, 1991).**

**Preparation of Reagents:**

Multifren dissolved with 2 ml of distilled water.

**Procedure:**

1-Multifren brought into a test tube warmed to 37°C.

2-One hundred µl of sample was pipette in reaction tube cuvette.

3-Sample was incubated for 60 seconds at 37°C.

4- Two hundred µl of Multifbren (37°C) was pipette in reaction tube cuvette.

5-The coagulation time was determined by fibrin timer.

**Calculation of the results:**

The results evaluated with the enclosed value table.

**Expected Values:**
180-350 mg/L.

**Assay of tissue plasminogen activator (t-PA)**

**Principle:**

In a first step, the diluted tested plasma is introduced into a microwell coated with a highly purified monoclonal antibody specific for human t-PA. When present, this protein is captured into the solid phase. Following a washing step, the immunoconjugate, which is a monoclonal antibody coupled to horse radish peroxidase (HRP), is introduced, and bound to another free epitope of immobilized t-PA. Following a new washing step, the peroxidase substrate, Tetramethylbenzidine (TMB) in presence of hydrogen peroxide (H2O2), is introduced and a color develops. The amount of color developed is directly proportional to the concentration of human tPA-Ag in the tested sample **(Stein *et al.*, 1997)**.

**Reagents Preparation:**

- Control I: 1 vial (plasma control I High) restored with 1 ml distilled water.
- Control II: 1 vial (plasma control II Low) restored with 1 ml distilled water.
- Tissue plasminogen activator (t-PA) standard: 1 vial was dissolved with1 ml distilled water.
- The immunoconjugate: (a monoclonal antibody coupled to HRP): each vial was dissolved with 7.5 ml of conjugate diluent.
- Wash Solution: incubated 15-30 min. in a water bath at 37°C until complete dissolution of solids, the vial then diluted 1:20 in distilled water.
- F-Sample Diluent: ready to use.

**Specimen collection:**

Blood sample collected on Na citrate anticoagulant, plasma is sequestrated following a 20 min. centrifugation or stored frozen at –20°C or below until to use, and thawed for 15 min. at 37°C just before use.

**Preparation plasma or sample or controls:**

- The sample diluted two fold (1:2) by the F-Sample Diluent. For expected t-PA concentrations > 20 ng/ml, plasma or samples can be tested at a higher dilution 1:5, or 1:10 or more**.**
- Controls I and II diluted two fold (1:2) with F-Sample Diluent.
- t-PA Standard diluted as follows:

| STD | Dilution guide | t-PA concent. (ng/ml) |
|---|---|---|
| **1** | 1 ml t-PA standard | C |
| **2** | 0.5 ml t-PA standard + 0.5 ml F-Sample Diluent. | C/2 |
| **3** | 0.25 ml t-PA standard + 0.75ml F-Sample Diluent. | C/4 |
| **4** | 0.1 ml t-PA standard + 0.9 ml F-Sample Diluent. | C/10 |
| **5** | 0.05 ml t-PA standard +0.95 ml F-Sample Diluent. | C/20 |
| **6** | 1 ml F-Sample Diluent. | 0 |

**Procedure:**

1- The strips were removed from the aluminum pouch. Then the strips were put in the frame.
2- Two hundred µl from each of 6 tubes of t-PA Standard, diluted controls and diluted samples (1:2) were pipetted in the corresponding micro ELISA plate well.
3- The plate was covered and incubated for 1 hour at room temperature.
4- After incubation, the plate washed 5 times by 300 µl wash solution in each well.
5- Two hundred µl of immunoconjugate was pipetted into each well and the plate was covered, incubated for 1 hour at room temperature. Then the plate washed 5 times by 300 µl wash solution in each well.
6- Two hundred µl of Tetramethylbenzidine (TMB) is delivered immediately after the washing in each well.
7- The plate was incubated 5 min. exactly at room temperature.
8- The reaction was stopped by adding 50 µl of stopping reagent to each

Well; absorbance was read at 450 nm after 10 minutes.

**Calculations of results:**

A standard curve was constructed by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis on log-log graph and a best fit line was drawn through the points on the graph.

**Expected range:**

The t-PA:Ag concentration in normal human plasma is usually < 10 ng/ml.

**Statistical analysis:**

Data were entered, checked and analyzed using SPSS for windows version 10.

## 3. Results

This study included three groups : group I (13 normal individuals as control), group II (22 patients with insulin-dependent diabetes, they were sub-classified into 15 NON LEAD and 7 LEAD) and group III (22 patients with non-insulin-dependent diabetes, they were sub-classified into 15 NON LEAD and 7 LEAD). The results statistically analyzed and came to the following:

Demographic characteristics as regard sex, male and female account respectively for 53.8% and 46.2% in group I, 45.5% and 54.5% in group II and 45.5%,

and 54.5% in group III. No statistically significant difference was found between the three studied groups ($X^2$=0.28, $p$=0.86).

As regard smoking (tobacco use), it is positive in 38.5% in the group I, 27.3% in group II and 27.3% in group III. No statistically significant difference was found between the three studied groups ($X^2$=0.6, $p$ =0.74).

As regard hypertension, it is positive in 30.8% in the group I, 59.1% in group II and 68.2% in group III. No statistically significant difference was found between the three studied groups ($X^2$=4.7,$p$ =0.1).

As regard cardiovascular disease, it is positive in 13.6% in group II and 18.2% in group III. No statistically significant difference was found between the three studied groups ($X^2$=2.56, $p$ =0.27).

There was a significant difference between the three groups as regards SBP ($p$ <0.05).

There was a very highly significant difference between the three groups as regards BMI and Ankle Brachial Index ($p$ <0.001).

There was non-significant difference between the three groups as regards age and DBP ($p$ >0.05).

BMI was significantly increased in group III compared to groups I and II ($p$ <0.001) but no significant difference was found between groups II and I ($p$ >0.05).

SBP was significantly increased in groups II and III compared to group I ($p$ <0.05) with no significant difference was found between group II and group III ($p$ >0.05).

ABI was significantly lower in group II and group III compared to group I ($p$ <0.001) with no significant difference was found between group II and group III ($p$ >0.05).

There was a significant difference between the three groups as regards triglycerides ($p$ <0.05).

There was a highly significant difference between the three groups as regards fibrinogen and CRP ($p$ <0.01).

There was a very highly significant difference between the three groups as regards HbA1c, t-PA, cholesterol and HDL- cholesterol ($p$ <0.001).

HbA1c was significantly higher in group II and group III compared to group I ($p$ <0.001) with no significant difference was found between group II and group III ($p$ >0.05).

t-PA was significantly higher in group II compared to group I ($p$ <0.001), but significantly lower in group III compared to group I and group II ($p$ <0.001).

There was a significant increase of fibrinogen in group II and group III compared to group I ($p$ <0.05 and $p$ <0.001 respectively) with no significant difference was found between group II and group III ($p$ >0.05).

There was a significant increase of CRP in group II and group III compared to group I ($p$ <0.05 and $p$ <0.001 respectively). CRP was also significantly higher in group III compared to group II ($p$ <0.05).

Cholesterol was significantly lower in group II and group III compared to group I ($p$ <0.05) and significantly lower in group II compared to group III ($p$ <0.05).

Triglyceride was significantly higher in group II and group III compared to group I ($p$ <0.05 and $p$ <0.001, respectively)), Also triglyceride in group III was significantly higher compared to group II ($p$ <0.05).

HDL- cholesterol was significantly higher in group II compared to group I ($p$ <0.001), but significantly lower in group III compared to group II and group I ($p$ <0.001).

There was significant difference between LEAD and NON LEAD as regards CRP and cholesterol ($p$ < 0.05).

There was a very high significant difference between LEAD and NON LEAD as regards Ankle Brachial Index ($p$ <0.001). There was non-significant difference between LEAD and NON LEAD as regards age, BMI, SBP, DBP, HbA1c, t-PA, fibrinogen, diabetic duration, triglycerides, HDL cholesterol, FSG and PPSG ($p$ >0.05).

There was significant difference between LEAD and NON LEAD as regards HbA1c, t-PA and Fibrinogen ($p$ < 0.05).

There was a very highly significant difference between LEAD and NON LEAD as regard age, SBP, CRP and Ankle Brachial Index ($p$ <0.001).

There was non-significant difference between LEAD and NON LEAD as regard BMI, DBP, cholesterol, triglycerides, HDL- cholesterol, diabetic duration, FSG and PPSG ($p$ >0.05).

There were statistically positive correlations with age and diabetic duration (r = 83.0, $p$ = 0.000, r = 0.8, $p$ = 0.000) ; (r = 0.84, $p$ = 0.000, r = 0.81, $p$ = 0.001) and (r = 0.92, $p$ = 0.002,r = 0.82, $p$ = 0.02) in total, NON LEAD and LEAD respectively .

There were statistically positive correlations with age (r = 0.77, $p$ = 0.000), (r = 0.8, P = 0.000), (r = 0.73, P = 0.05) in total, NON LEAD and LEAD respectively.

There was statistically positive correlations with HbA1c (r = 0.65, $p$ = 0.001) in total.

There were statistically positive correlations with diabetic duration (r = 0.59, $p$ = 0.004), (r = 0.75, $p$ = 0.001), (r =0.53, $p$ = 0.05) in total, NON LEAD and LEAD respectively.

**Table (1):** Demographic characteristics of the studied groups:

| | Group I n = 13 | Group II n = 22 | Group III n = 22 | $X^2$ | P |
|---|---|---|---|---|---|
| Sex | n | n | n | | |
| M | 7 53.8% | 10 45.5% | 10 45.5% | 0.28 | 0.86 |
| F | 6 46.2% | 12 54.5% | 12 54.5% | | |
| Smoking | n | n | n | | |
| -ve | 8 61.5% | 16 72.7% | 16 72.7% | 0.6 | 0.74 |
| +ve | 5 38.5% | 6 27.3% | 6 27.3% | | |
| Hypertension | n | n | n | | |
| -ve | 9 69.2% | 9 40.9% | 7 31.8% | 4.77 | 0.1 |
| +ve | 4 30.8% | 13 59.1% | 15 68.2% | | |
| Cardiovascular | n | n | n | | |
| -ve | 13 100.0% | 15 86.4% | 18 81.8% | 2.56 | 0.27 |
| +ve | 0 | 3 13.6% | 4 18.2% | | |

**Table (2):** Comparison of clinical and demographic data in the three studied groups as regards age, BMI, SBP, DBP and Ankle Brachial Index.

| | **Group I (n =13) Mean ± SD** | **Group II (n=22) Mean ± SD** | **Group III (n=22) Mean ± SD** | **F** | ***P*** |
|---|---|---|---|---|---|
| **Age (years)** | 45.8± 8.1 | 48.1±5.7 | 50.3±8 | 1.6 | 0.21 |
| **BMI(kg/m$^2$)** | 24.5±2.4 | 25±1.8 | 29.6±3 | 24.7 | 0.000 |
| **SBP(mmHg)** | 128.8±14.4 | 143.8±15.4 | 142.7±18.6 | 3.8 | 0.028 |
| **DBp(mmHg)** | 76.5 ± 7.7 | 78.6±8 | 81.1+7.5 | 1.4 | 0.234 |
| **Ankle Brachial Index** | 1.29±0.28 | 0.96±0.33 | 0.90±0.40 | 5.2 | 0.0001 |

**Table (3):** LSD for comparison of BMI, SBP, and ABI between the three groups.

| | | |
|---|---|---|
| LSD for comparison of BMI | | |
| | Group I | Group II |
| Group II | >0.05 | |
| Group III | <0.001 | <0.001 |
| LSD for comparison of SBP | | |
| | Group I | Group II |
| Group II | <0.05 | |
| Group III | <0.05 | >0.05 |
| LSD for comparison of ABI | | |
| | Group I | Group II |
| Group II | <0.001 | |
| Group III | <0.001 | >0.05 |
| LSD for comparison of CRP | | |
| | Group I | Group II |
| Group II | <0.05 | |
| Group III | <0.001 | <0.05 |
| LSD for comparison of cholesterol | | |
| | Group I | Group II |
| Group II | <0.05 | |
| Group III | <0.05 | <0.05 |
| LSD for comparison of triglyceride | | |
| | Group I | GroupII |

| | | |
|---|---|---|
| Group II | <0.05 | |
| Group III | <0.001 | <0.05 |
| LSD for comparison of HDL- cholesterol | | |
| | Group I | Group II |
| Group II | <0.001 | |
| Group III | <0.001 | <0.001 |
| LSD for comparison of BMI | | |
| | Group I | Group II |
| Group II | >0.05 | |
| Group III | <0.001 | <0.001 |
| LSD for comparison of SBP | | |
| | Group I | Group II |
| Group II | <0.05 | |
| Group III | <0.05 | >0.05 |
| LSD for comparison of ABI | | |
| | Group I | Group II |
| Group II | <0.001 | |
| Group III | <0.001 | >0.05 |

**Table (4):** Comparison of laboratory data in the three studied groups as regards HbA1c, t-PA, fibrinogen, CRP, cholesterol, triglycerides and HDL- cholesterol.

| | Group I (n =13) Mean ± SD | Group II (n=22) Mean ± SD | Group III (n=22) Mean ± SD | F | *P* |
|---|---|---|---|---|---|
| HbA1c (%) | 4.46 ±0.4 | 7.2 ±1.1 | 6.9±1.1 | 33.9 | 0.000 |
| t-PA(ng/ml) | 10.0 ± 3.7 | 14.5±4.0 | 6.4±3.4 | 25.9 | 0.000 |
| Fibrinogen(mg/dl) | 263.9±69.9 | 340.4±85.4 | 357.1±86.2 | 5.52 | 0.01 |
| CRP(mg/l) | 2.7±1.5 | 17.7±6.7 | 33.6±37 | 7.34 | 0.002 |
| Cholesterol(mg/dl) | 181.8±20.5 | 160.6±17.8 | 178.6±36.4 | 33.6 | 0.000 |
| Triglycerides(mg/dl) | 73.2±8.5 | 116.8±70.8 | 156.7±55.1 | 3.5 | 0.03 |
| HDL- cholesterol(mg/dl) | 36.0±4.6 | 42.6±5.3 | 31.1±4.1 | 9.2 | 0.000 |

**Table (5):** Comparison of different parameters between NON LEAD and LEAD in group II.

| Group II | NON LEAD (n = 15) | LEAD (n = 7) | t | *P* |
|---|---|---|---|---|
| Age | 47.0±5.3 | 50.7±6.0 | 1.4 | 0.16 |
| BMI | 25.0±2.0 | 25.1±1.5 | 0.16 | 0.87 |
| SBP | 141.3±14.8 | 149.2±16.4 | 1.13 | 0.27 |
| DBP | 80.3±8.5 | 75.0±5.7 | 1.4 | 0.15 |
| HbA1c | 7.0±0.92 | 7.8±1.4 | 1.6 | 0.10 |
| t-PA | 14.3±3.0 | 14.9±5.8 | 0.32 | 0.74 |
| Fibrinogen | 320.6±96.6 | 383.0±26.1 | 1.6 | 0.11 |
| C.R.P | 20.0±4.0 | 12.5±8.7 | 2.7 | 0.03 |
| Ankle Brachial Index | 1.15±0.22 | 0.56±0.06 | 6.6 | 0.000 |
| Cholesterol | 155.4±15.7 | 171.8±17.6 | 2.19 | 0.04 |
| Triglycerides | 133.8±80.2 | 80.4±15.3 | 1.72 | 0.10 |
| HDL- cholesterol | 41.4±5.4 | 45.4±3.9 | 1.75 | 0.09 |
| Diabetic duration | 29.6±3.8 | 32.5±9.7 | 1.0 | 0.32 |
| Fasting serum glucose | 279.1±95.2 | 251±68 | 0.69 | 0.49 |
| Post prandial serum glucose | 310.7±94.8 | 275.2±65.4 | 0.89 | 0.38 |

**Table (6):** Comparison of different parameters between NON LEAD and LEAD in group III

| Group III | NON LEAD ( n= 15) | LEAD (n = 7) | t | *P* |
|---|---|---|---|---|
| Age | 50.0±8.3 | 57.4±4.2 | 2.1 | 0.000 |
| BMI | 30.0±3.3 | 28.8±2.2 | 0.80 | 0.42 |

| | | | | |
|---|---|---|---|---|
| SBP | 138.0±18.8 | 152.8±14.6 | 1.8 | 0.000 |
| DBP | 80.8±8.4 | 83.5±4.7 | 1.0 | 0.3 |
| HbA1c | 6.6±1.01 | 7.7±0.9 | 2.4 | 0.02 |
| t-PA | 5.2±2.6 | 8.9±3.8 | 2.5 | 0.02 |
| Fibrinogen | 329.3±85.6 | 416.7±53.2 | 2.4 | 0.03 |
| C.R.P | 13.1±13.2 | 77.5±33.0 | 6.6 | 0.000 |
| Ankle Brachial Index | 1.1±0.32 | 0.48±0.07 | 5.01 | 0.000 |
| Diabetic duration | 8.0±4 | 8.5±4.5 | 0.2 | 0.7 |
| Fasting serum glucose | 159.0±69.9 | 200.8±69.8 | 1.3 | 0.2 |
| Post prandial serum glucose | 188.9±98.3 | 244.2±87.4 | 1.2 | 0.21 |
| Cholesterol | 170.2±35.7 | 196.7±32.9 | 1.65 | 0.11 |
| Triglycerides | 144.5±57.3 | 182.8±43.1 | 1.57 | 0.13 |
| HDL- cholesterol | 31.2±4.1 | 30.7±4.4 | 0.28 | 0.77 |

**Table (7):** Correlation between t-PA and other clinical and laboratory findings in total, NON LEAD and LEAD in GroupII.

| t-PA Group II | Total (n = 22) | | NON LEAD (n = 15) | | LEAD (n = 7) | |
|---|---|---|---|---|---|---|
| | r | *p* | r | *p* | r | *P* |
| Age | 83.0 | 0.00 | 0.84 | 0.00 | 0.92 | 0.002 |
| BMI | 0.06 | 0.78 | -0.22 | 0.43 | 0.50 | 0.25 |
| SBP | 0.03 | 0.87 | 0.26 | 0.33 | -0.25 | 0.58 |
| DBP | 0.19 | 0.37 | 0.24 | 0.37 | 0.30 | 0.51 |
| HbA1c | -0.14 | 0.54 | -0.13 | 0.64 | -0.20 | 0.65 |
| Fibrinogen | -0.17 | 0.45 | -0.25 | 0.36 | -0.40 | 0.37 |
| C.R.P | 0.22 | 0.32 | 0.15 | 0.57 | -0.39 | 0.38 |
| Ankle Brachial Index | -0.19 | 0.39 | -0.29 | 0.28 | -0.49 | 0.26 |
| FSG | -0.06 | 0.80 | 0.24 | 0.37 | -0.55 | 0.19 |
| PPSG | -0.06 | 0.78 | 0.21 | 0.44 | 0.52 | 0.23 |
| Diabetic duration | 0.8 | 0.00 | 0.81 | 0.00 | 0.82 | 0.02 |
| HDL- cholesterol | 0.11 | 0.62 | -0.27 | 0.33 | 0.72 | 0.06 |
| Cholesterol | 0.001 | 0.99 | 0.000 | 1.00 | -0.07 | 0.87 |
| Triglyceride | -0.07 | 0.73 | -0.21 | 0.43 | 0.8 | 0.03 |

**Table (8):** Correlation between t-PA and other clinical and laboratory findings in total, NON LEAD and LEAD in GroupIII

| t-PA Group III | Total (n = 22) | | NON LEAD (n = 15) | | LEAD (n = 7) | |
|---|---|---|---|---|---|---|
| | r | *P* | r | *P* | r | *P* |
| Age | 0.77 | 0.00 | 0.80 | 0.000 | 0.73 | 0.05 |
| BMI | 0.007 | 0.97 | 0.33 | 0.22 | -0.37 | 0.41 |
| SBP | 0.30 | 0.17 | 0.03 | 0.91 | 0.37 | 0.40 |
| DBP | 0.24 | 0.27 | 0.29 | 0.28 | -0.17 | 0.70 |
| HbA1c | 0.65 | 0.001 | 0.45 | 0.08 | 0.72 | 0.06 |
| Fibrinogen | 0.25 | 0.26 | 0.09 | 0.73 | -0.02 | 0.66 |
| C.R.P | 0.54 | 0.008 | 0.39 | 0.14 | 0.21 | 0.85 |
| Ankle Brachial Index | -0.35 | 0.124 | 0.124 | 0.66 | -0.08 | 0.86 |
| FSG | 0.60 | 0.003 | 0.55 | 0.034 | 0.60 | 0.15 |
| PPSG | 0.58 | 0.005 | 0.53 | 0.04 | 0.57 | 0.17 |
| Diabetic duration | 0.59 | 0.004 | 0.75 | 0.001 | 0.53 | 0.05 |
| HDLcholesterol | -0.17 | 0.45 | -0.5 | 0.04 | 0.39 | 0.38 |
| Cholesterol | 0.08 | 0.71 | -0.29 | 0.28 | 0.19 | 0.68 |
| Triglyceride | 0.05 | 0.80 | -0.16 | 0.54 | -0.09 | 0.84 |

## 4. Discussion

Diabetes mellitus is defined as the dysregulation of glucose metabolism characterized by chronic hyperglycemia resulting from defects in insulin secretion, decreased insulin sensitivity or a combination of both **(Jochen and Werner, 2006).**

Diabetes leads to a hypercoagulable state. It is associated with the increased production of tissue factor by endothelial cells and VSMC, as well as increased plasma concentrations of factor VII. Hyperglycemia is also associated with a decreased concentration of antithrombin and protein C, impaired fibrinolytic function, and excess production of PAI-1 **(Beckman *et al.*, 2004).**

The fibrinolytic system is primarily an interaction between plasminogen activators and inhibitors and one response to vascular injury is an activation of t-PA. Increased t-PA-activity may therefore be a potential indicator of an early ongoing vascular damage **(David Sahli *et al.*, 2009).**

Tissue plasminogen activator level elevates in the plasma of the diabetic patients with lower extremity arterial disease (LEAD) and can be use as an early marker for diagnosis of these cases **(Raffetto *et al.*, 2005).**

Peripheral arterial disease is a major risk factor for lower-extremity amputation, especially in patients with diabetes. Moreover, even for the asymptomatic patient, PAD is a marker for systemic vascular disease **(Nathaniel, 2003).**

Diabetes mellitus increases the risk for PAD via deleterious effects on the vessel wall e.g., (derangement of nitric oxide bioavailability in endothelial cell) as well as effects on blood cells e.g., (enhanced platelet aggregation and hypercoagulable state) and rheology e.g., (increased blood viscosity and fibrinogen levels) **(American Diabetes Association, 2003).**

Tissue plasminogen activator (t-PA) is synthesized and released by vascular endothelial cells into the circulating blood as the single-chain form and is the predominant activator in plasma. The main function of t-PA is in the dissolution of fibrin in the vasculature, helping to maintain vessel patency. Tissue plasminogen activator (t-PA) acts by forming a ternary complex with fibrin and plasminogen and catalyzes the conversion of inactive plasminogen to plasmin (**Suzanne *et al.*, 2006).**

Increased t-PA occurs in association with endothelial cell dysfunction and damage; elevated levels may reflect the presence of underlying endothelial damage **(Steins *et al.*, 2000).**

The objective of the present study was to evaluate the plasma level of tissue plasminogen activator (t-PA) as an early predictor marker of asymptomatic lower extremity arterial disease (LEAD) in patients with diabetes mellitus. This study was carried out on 57 subjects classified into 3 groups: Group I: - the control group included 13 subjects, Group II: - 22 insulin –dependent diabetic patients, they were sub-classified into NON LEAD and LEAD and Group III: - 22 non-insulin– dependent diabetic patients, they were sub-classified into NON LEAD and LEAD.

In this study BMI was highly significantly increased in group III compared to group I and group II but no significant difference was found between group II and group I. These results coincide with the results reported by **Andreas *et al.*, (2002)** who found a significant increase in BMI in diabetic patients compared to control group, furthermore the results of the study reported by **Ali *et al.* (1995)** are also in agreement with our results. On other hand **Morishita *et al.* (1996)** found no significant difference in BMI between control group and diabetic patients.

The results of this study revealed that there was no significant difference as regard diastolic blood pressure among all groups. These results coincide with the results in the study reported by **Ali *et al.* (1995)** who found no significant difference in DBP between control group and diabetic patients. SBP was significantly increase in group II and group III compared to group I with no significant difference was found between group II and group III, while the study by **Ali *et al.*(1995)** found no significant difference in SBP between control group and diabetic patients.

Moreover, Ankle Brachial Index (ABI) was significantly lower in group II and group III compared to group I with no significant difference was found between group II and group III. These results coincide with the results reported by (**Premanath and Raghunath, 2008)** who found significant decrease in ABI in diabetic patients with and without PAD compared to control group.

In the present study HbA1c was significantly higher in group II and group III compared to group I with no significant difference was found between group II and group III. These results coincide with the results of study reported by **Elizabeth *et al.* (2006)** who found significant increase in levels of HbA1c in type II DM with PAD compared to control group due to chronic elevation of the blood glucose level.

These results suggested that these patients with diabetes were exposed to hyperglycemia over long periods, with increased and accelerated glycosylation of hemoglobin A within the red blood cell throughout its 120 days life span in the circulation.

The results of this study, revealed that t-PA was significantly higher in group II compared to group I, but significantly lower in group III compared to group I and group II. These results coincide with the results reported by **David *et al.*(2009)** who showed that type 2 diabetes had significantly lower levels of t-PA and type 1 diabetes had significantly higher levels of t-PA than non-diabetic subjects**.** These results are explained by detection of elevated levels of the fibrinolytic inhibitor, PAI-1 which is generated from fat-laden

insulin-resistant adipocyte and lead to suppression of the fibrinolysis in diabetic patients due to negative feedback on tissue plasminogen activator (t-PA) **(Lange *et al.*, 2003)**.

In accord with our results, **Bastard *et al.* (2000)** reported that increased PAI-1 secretion due to interaction of a number of metabolic and inflammatory factors in type 2 diabetes can lead to decrease secretion of the tissue plasminogen activator (t-PA).

Our results are also in agreement with **Stegenga *et al.* (2006)** who found that t-PA was significantly lower in type II diabetes because fluctuating hyperglycemia lead to protein glycation that induce the oxidative stress, endothelial cell dysfunction, extracellular matrix formation and apoptosis. This lead to vascular damage that increase thrombotic formation, stimulation the fibrinolysis system, increase of (PAI-1) and decrease t-PA in the late stages.

Our study revealed a significant increase of fibrinogen in group II and group III compared to group I with no significant difference was found between group II and group III. These results coincide with the results reported by **Andreas *et al.* (2002)** who reported that fibrinogen level in diabetic patients were higher than those in the control group due to increased synthesis and turnover of fibrinogen in diabetes that is related to insulin deficiency. These results explained by **Meigs *et al.*(2000)** suggested that in diabetic patients complicated with vascular disease, there are multiple vascular damages which are responsible for the high fibrinogen level. On the other hand, the study of **Pandolfi *et al.*(2001)** found no significant difference in fibrinogen between control group and diabetic patients.

In the present study there was a significant increase of CRP in group II and group III compared to group I, also CRP was significantly higher in group III compared to group II. These results agree with the results of study reported by **Andreas *et al.* (2002)** who reported that CRP levels in diabetic patients were higher than those in the control non-diabetic group, suggesting that the hyperglycemia affects the CRP levels, the study of **Ridker *et al.* (2000)** found that in prolonged exposure to hyperglycemia there is an inflammatory process which lead to elevation of CRP level that leads to vascular damage. This stimulate endothelial production of procoagulant tissue factor, leukocyte adhesion molecules, and chemotactic substances and inhibits endothelial cell nitric oxide (NO) synthase, resulting in increased local production of compounds impairing fibrinolysis, such as plasminogen activator inhibitor (PAI-1) which inhibit the secretion of tissue plasminogen activator .

The results of this study also revealed that there was no significant difference between NON LEAD and LEAD in group II as regards t-PA. These results coincide with the results reported by **David *et al.* (2009)** who found that t-PA was not significantly different in type 1 diabetes with LEAD compared to NON LEAD.

Also, there was no significant difference between NON LEAD and LEAD in group II in the levels of HDL and triglyceride. These results coincide with the results reported by **Tzoulaki *et al.* (2006)** who found that there were no significantly differences in HDL and triglyceride in type 1 diabetes with or without PAD.

Otherwise, cholesterol was significantly increased in group II LEAD compared to NON LEAD. These results not matched with the results reported by **Tzoulaki *et al.* (2006)** who found that there was no significant difference in cholesterol in type 1 diabetes with or without PAD.

Moreover, there was a significant decrease in ankle brachial index in LEAD compared to NON LEAD in group II. These results coincide with the result showed by (**Premanath and Raghunath, 2008)** who found significant decrease in ABI in diabetic patients with PAD compared to diabetic patients without PAD.

On the other hand, there were no significant difference in age, body mass index, systolic blood pressure, diastolic blood pressure, glycosylated hemoglobin, fibrinogen, fasting serum glucose, post prandial serum glucose and diabetic duration between LEAD and NON LEAD of group II.

In the group III, tPA was significantly increased in LEAD compared to NON LEAD .These results coincide with the results reported by **David *et al.* (2009)** who found that tPA was significantly elevated in type 2 diabetes with LEAD than NON LEAD.

These results were explained by **(Kooistra *et al.*, 1994)** who said that peripheral ischemia stimulate the compensatory mechanism to sustain circulation in the vessels in the early stage of the disease and during this stage the endothelium still contact and secretory moreover, it is the major source for secretion of tPA so, the tPA secretion increase after vascular insufficient caused by peripheral vascular disease in type 2 diabetes.

Also there was no significant difference between NON LEAD and LEAD in group III as regards cholesterol, HDL-cholesterol and triglyceride. These results coincide with the results reported by **Tzoulaki *et al.* (2006)** who found that cholesterol, HDL-cholesterol and triglyceride not significantly different in type 2 diabetes with or without PAD.

Moreover, there was a significant decrease in ankle brachial index in LEAD compare to NON

LEAD in the group III. These results coincide with the result showed by **(Premanath and Raghunath, 2008)** who found significant decrease of ABI in diabetic patients with PAD compare to diabetic patients without PAD. This study found that atherosclerosis stimulate formation of the atheromatic plaque which affect the blood flow in the blood vessels simultaneously the change in the endothelial wall of the blood vessels which loss its elasticity and thickened leading to decrease the sound wave that transmitted through it as it become more flattened and rounded and lead to decrease the ankle systolic pressure.

Otherwise, there was a significant increase in glycosylated hemoglobin in LEAD compare to NON LEAD in the group III. These results coincide with the results of study reported by **Elizabeth *et al.* (2006)** who found significant increase in levels of HbA1c in type II DM with PAD compared to type II DM without PAD suggested that these patients with uncontrolled diabetes were exposed to hyperglycemia over long periods, with increased and accelerated glycosylation of hemoglobin A within the red blood cell throughout its 120 days life span in the circulation

On the other hand, there were no significant difference in body mass index, diastolic blood pressure, fasting serum sugar, post prandial serum sugar and diabetic duration between LEAD and NON LEAD of group III.

In the present study, tissue plasmingen activator was positively correlated in group II with age and duration of diabetes.

Furthermore, in our study tissue plasminogen activator was positively correlated in group III with age, duration of diabetes and with HbA1c in (total).

In our study, there was no correlation between tPA and body mass index in group II and group III, While another study by **Ali Keskin *et al.* (1995)** show positive correlation of t-PA with BMI, this observation further supports documented decreased fibrinolytic activity in obesity. These results were explained as an elevation in BMI predisposes to insulin resistance and hyperinsulinism rather than to insulin deficiency.

In our study we found a significant correlation between tissue plasminogen activator and glycosylated hemoglobin in group III (total). These results were agree with the result shown by **Thomas *et al.* (2009)** who found that there was a significant correlation between t-PA and HbA1c, on the other hand the study reported by **Ali Keskin *et al.* (1995)** who found no correlation between t-PA and HbA1c was against our study. So, the concentration of the tissue plasmingen activator according to our study seems to be dependent on diabetic control.

Otherwise, our study in group II found no correlation among t-PA and glycosylated hemoglobin, systolic blood pressure, diastolic blood pressure, fibrinogen, C-reactive protein, ankle brachial index, fasting serum sugar,post prandial serum sugar, HDL- cholesterol, cholesterol and triglyceride .

our study in group III found no correlation among t-PA and systolic blood pressure, diastolic blood pressure, fibrinogen, C-reactive protein, ankle brachial index, fasting serum sugar,post prandial serum sugar, HDL- cholesterol, cholesterol and triglyceride.

In **conclusion** Tissue plasminogen activator (tPA) present in the early stage after vascular damage so, measurement of t-PA is a reflection for increased activity of the fibrinolytic system and can potentially be useful as an early predicator marker for the measure of activated coagulation in diabetic patients with asymptomatic lower extremity arterial disease (LEAD) . In our study there was no significant difference between NON LEAD and LEAD in type 1DM (groupII) in levels of t-PA. Otherwise, in type 2DM (group III) levels of t-PA were significantly increase in LEAD compared to NON LEAD These results were explained by a compensatory mechanism to sustain circulation that present in the early progression of the disease when the endothelium still is a major source of t-PA so, the t-PA secretion increase in the early stage of vascular insufficient in type 2 diabetes. Although this mechanism present in type1DM but the difference in the results can be explained by the hypothesis that provide the deterioration of this mechanisms by the long duration.

There is correlation between tissue plasminogen activator and glycosylated hemoglobin in type 2 DM; these results reveal that in uncontrolled type 2 DM there was an increase in incidence of lower extremity arterial disease.

In conclusion, measurement of tissue plasminogen activator can potentially be useful as an early marker for prevention of vascular complications before they become clinically evident.

We recommended that tissue plasminogen activator (t-PA) can be used as an early marker for diagnosis and follow up in diabetic patients. More studies on large numbers of patients are needed to confirm these results. Also, further studies should be done as PAI-1 and factor VII in diabetic patients.

**Corresponding author**
**Mahmoud A. Ashour**
Department of Internal Medicine, Faculty of Medicine, Zagazig University, Egypt.
kamel_ashour2050@hotmail.com

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