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Regular Article

# Tissue plasminogen activator (tPA) activity is a novel and early marker of asymptomatic LEAD in type 2 diabetes

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ABSTRACT

*Introduction:* Lower extremity arterial disease (LEAD) is often one of the first signs of a generalized atherosclerotic disease in type 1 and type 2 diabetic subjects.
*Materials and methods:* We studied 143 diabetic subjects at 30-70 years of age, M/F 69/74, 74 with type 1 and 69 with type 2 diabetes, without previously known or suspected lower extremity arterial disease. The relationship between early asymptomatic lower extremity arterial disease and blood levels of HbA1c, lipids and fibrinolysis markers (tPA-activity, tPA mass, PAI-1 activity, tPA-PAI-1 complex) was assessed. In parallel, a group with non-diabetic subjects ($n$=80) was studied.
*Results:* 35 (24%) diabetic subjects were classified as having sign(s) of LEAD, defined as having at least one reduced peripheral blood pressure measurement, 28% in type 1 vs 20% in type 2 diabetic subjects (p=NS). In univariate logistic regression analyses age, glycemic level (HbA1c), male gender (only in type 1 diabetic subjects), hypertension and tPA activity (only in type 2 diabetic subjects) were positively associated with LEAD. When markers of fibrinolysis were entered into a multivariate model adjusting for age, hypertension, and HbA1c, only tPA activity remained independently associated with LEAD (p=0.01) and this was also found in type 2 diabetic subjects (p=0.05). In type 1 diabetic subjects the increase in odds ratio was non-significant.
*Conclusions:* Tissue plasminogen activator (tPA) activity may be an independent and early marker for asymptomatic lower extremity arterial disease in diabetic subjects, particularly in type 2 diabetes. Thus an altered fibrinolytic activity could be an early marker of atherosclerosis development in the lower extremities but the cause-effect relationship remains unclear.



## Introduction

Lower extremity arterial disease (LEAD) is often one of the first signs of a generalized atherosclerotic disease and is characterised by reduced blood pressure in the lower extremities. LEAD can be both asymptomatic or cause symptoms such as intermittent claudicatio. In more advanced stages of disease rest pain and diminished wound healing capacity evolve. The prevalence of LEAD varies between different studies due to differences in classification of LEAD and clinical characteristics of populations studied. In the large population-based Edinburgh Artery Study 1519 men and women between 55 to 74 years were screened and approximately 10% were found to have LEAD, when defined as an ankle brachial index (ABI)<0.9 [1]. LEAD is more severe and generalised in men than in women and the prevalence of LEAD increases with age [2–4].

Diabetes increases the risk of developing LEAD 2- to 5-fold [5,6]. Diabetic subjects develop LEAD at an earlier age compared to non-diabetic subjects and LEAD is also more generalised and extends further into the peripheral vasculature than in age-matched non-diabetic subjects[8]. In diabetic subjects, a foot ulcer may be the first sign of LEAD due to neuropathy and loss of sensation. In addition, neuropathy, impaired wound healing capacity and infections are factors that contribute to the increased risk of leg amputation found in diabetic subjects [7–9].

The aetiology of atherosclerosis, including LEAD, is multifactorial. Major risk factors are hyperglycemia, smoking and hypertension [10,11] but age, duration of diabetes, obesity, and dyslipidemia [12–15] also contributes to the risk. In addition to these well-known risk

*Abbreviations:* T1D, type 1 diabetes; T2D, type 2 diabetes; LEAD, lower extremity arterial disease; ABP, ankle blood pressure; TBP, toe blood pressure; ABI, ankle blood pressure index; TBI, toe blood pressure index; ATA, a. tibialis anterior; ATP, a. tibialis posterior; tPA, tissue plasminogen activator; PAI-1, plasminogen activator inhibitor-1.

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factors, other studies have suggested that alterations in fibrinolysis [16–19] and also in other trombophilic risk factors such as lipoprotein (a), homocystein and antiphospholipid antibodies may be of importance for the development of LEAD [20].

In the present study we assessed the relationship between asymptomatic impairment of peripheral circulation in the lower extremities, i.e. signs of early LEAD, and markers of fibrinolysis.

## Materials and methods

### *Subjects*

143 diabetic subjects (M/F 69/74), diagnosed according to the WHO criteria [21], were included in the study, 74 with type 1 diabetes (T1D), 69 with type 2 diabetes (T2D). They were enrolled consecutively from the outpatient clinic at the Diabetes Unit, Umeå University Hospital. In parallel, a group with 80 non-diabetic subjects (M/F 42/38) was studied. They were recruited among hospital staff and by advertisements in local newspapers. The inclusion criteria were age 30-70 years and no symptoms compatible with LEAD according to a standardised interview and medical records. All the participants gave their informed consent. The study was approved by the Ethics Committee of Umeå University and conducted according to the declaration of Helsinki. The study participants are part of a larger two-centre long-term prospective study addressing the cumulative incidence and natural history of asymptomatic LEAD [22].

Clinical characteristics of the study participants are given in Table 1. Tobacco use was defined as regular use of tobacco (smoking or snuff) within three months prior to the study. Information on antihypertensive medication and the presence of any cardiovascular disease, i.e. ischemic heart disease, previous myocardial infarction or stroke, was collected from the participants and later confirmed from medical records. Hypertension was defined as having a clinical diagnosis of hypertension in medical records, ongoing antihypertensive medication or having a systolic or diastolic blood pressure above 140 mmHg or 90 mmHg, respectively, at study entry.

### *Blood pressure measurements*

A validated non-invasive screening method was used to measure lower extremity blood pressures [22–24]. In brief, blood pressure measurements were performed in the supine position after five minutes rest. Systolic (SBP) and diastolic (DBP) arm blood pressures were assessed according to standard routine at the right arm. Systolic ankle blood pressures (ABP) in the anterior and posterior arteries were assessed using a cuff placed above the ankle. For pulse registration at the dorsalis pedis/tibialis anterior (ATA) and the tibialis posterior (ATP) arteries a Doppler pen (Hadeco MiniDoppler ES-100X and a 5 MHz probe; Arima, Miyamae-Ku, Kawasaki-Shi, Japan) was used. To determine toe blood pressure (TBP) a 2.5×13 cm blood pressure cuff (Criticon™, Rium Medical KB, Sollentuna, Sweden) and a pulse oximetry sensor (BOIX 3740; BOC Ohmeda, Göteborg, Sweden) for detection of pulsatile blood flow were put on the proximal and distal parts of the great toe, respectively. To avoid inter-observer variation the same examiner measured all blood pressures. At all the locations, e.g. arm, ankle and toe, the mean of two blood pressure readings was calculated. Ankle/arm (ATAI, ATPI) and toe/arm (TI) blood pressure indices were then calculated by dividing ABP and TBP, respectively, by the SBP in the right arm.

Reduced peripheral blood pressure, i.e. signs of LEAD, was defined as at least one ankle (ABP) or toe blood pressure (TBP) or one ankle- to-brachial (ABI) or toe-to-brachial blood pressure index (TBI) below the normal range, defined as ankle<98 mmHg, toe<79 mmHg or ABI (ATA)<0.90, ABI (ATP)<0.95, TBI<0.74. The normal ranges used in this study are the means±2SD in 134 non-diabetic subjects without previously known LEAD as reported by us in a previous larger study were we used the same method to assess lower extremity blood pressures [22]. The ranges of normal blood pressures in that study were; ankle blood pressure 98-193 mmHg (a. tibialis anterior; ATA) or 101-198 mmHg (a. tibialis posterior; ATP), toe blood pressure 79-163 mmHg, ankle/brachial index 0.90-1.34 (ATA) or 0.95-1.35 (ATP) and toe/brachial index 0.74-1.12. In the present study 108 subjects (76%) were defined as having a normal lower extremity blood pressure and 35 (24%) as having reduced peripheral blood pressure measurement(s), i.e. sign(s) of LEAD. However, among these 35 subjects 13 (37%) also had at least one measurement above the normal range.

### *Blood and urine chemistry*

Blood samples were collected in the morning between 7.00 and 9.00 am after an over-night fast (10 h). Blood glucose was determined using the HemoCue glucose system (HemoCue AB, Ängelholm, Sweden). HbA1c was measured by high-pressure liquid chromatography (Integral 4000, BioRad, Anaheim, CA, USA) (normal range 3.6-5.0%). Analyses of markers of fibrinolysis were made within one year of sampling in stored, frozen samples. Venous blood samples for hemostatic assays were drawn without stasis into Biopool stabilyte™ tubes. Plasma was obtained by centrifugation at 2500 g for 15 minutes, aliquot, and stored frozen at −80 °C until analysis. Mass concentrations of PAI-1 and tPA-PAI-1 complex were determined by ELISA, Imulyse™ PAI-1 for PAI-1 and sTintElize™ tPA/PAI-1 for tPA-PAI-1 complex. tPA mass was determined by Imulyse™ t-PA or TintElize™ tPA. These two assays use the same antibody and internal validation at the manufacturer has shown a high correlation between the two tests (information provided upon request). Intraassay CV for tPA with Imulize™ t-PA was 5.8% and for Tintelize™ t-PA intra- and interassay CV was 5.5% and 3.5% at 6 ng/ml, respectively. Intra- and interassay CV for tPA/PAI-1 complex was 5.7% and 6.8%, all according to the manufacturer. Activity of tPA and PAI-1 were determined by a bio immunoassay (BIA), Chromolize™ t-PA for tPA and Chromolize™ PAI-1 for PAI-1. All reagent kits were purchased from Biopool AB (Umeå, Sweden). Intra- and interassay CV for tPA-activity was 3.9% and 5.2% at 1.25 IU/mL, respectively, and for PAI-1 activity was 2.7% and 4.6% at 22 IU/mL, respectively. Analyses of serum triglycerides and cholesterol were performed by routine methods at the Department of Clinical Chemistry, University Hospital of Umeå.

### *Statistical methods*

Data are presented as mean±SD, or as indicated. The Kolmogorow-Smirnov test was used to assess the distribution of data for each subgroup (T1D and T2D) separately and for all participants taken together. Differences in mean values between groups were analysed using the Student's two-tailed t-test for normally distributed data. For

**Table 1**
Clinical characteristics of study participants (n=223)

| Variables | *Non-diabetics (n=80)* | Type 1 diabetes (n=74) | Type 2 diabetes (n=69) |
|---|---|---|---|
| Age (years) | *48±11* | 48±8[a] | 53±9 |
| BMI (kg/m2) | *25±3.4* | 25±3.0[b] | 30±5.0 |
| Diabetes duration | | | |
| LEAD (Y/N) (years) | *n.a.* | 35±11 [b] /25±11 [b] | 7±5/8±7 |
| Gender, M/F (%) | *42/38 (53%)* | 36/38 (48%) | 33/36 (48%) |
| Tobacco use (n, %) | *19 (24%)* | 14 (19%) | 14 (20%) |
| Antihypertensive | | | |
| medication (n, %) | *3 (4%)* | 27 (36%) | 29 (42%) |
| Hypertension (n, %) | *17 (21%)* | 42 (57%) | 47 (68%) |
| Cardiovascular disease (n, %) | *1 (1%)* | 7 (10%) [a] | 16 (23%) |
| Sign of LEAD (n, %) | *7 (10%)* | 21 (28%) | 14 (20%) |
| HbA1c (%) | *4.2±0.4* | 7.2±1.3[c] | 6.8±1.4 |

Values are means±SD or n, %, n.a., Not applicable, [a]p<0.05, [b]p<0.001 vs T2D, [c]p=0.0053 vs T2D.
LEAD=lower extremity arterial disease.