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(54) Title: METHODS OF PREDICTING THE RISK OF HAVING LOWER-EXTREMITY ARTERY DISEASE IN PATIENTS SUFFERING FROM TYPE 2 DIABETES

(57) Abstract: Inflammation and oxidative stress play an important role in the pathogenesis of lower- extremity artery disease (LEAD). The inventors assessed the prognostic values of inflammatory and redox status biomarkers on the risk of LEAD in individuals with type 2 diabetes. In particular, plasma concentrations of TNF receptor-1 (TNFR1) and ischemia-modified albumin (IMA) were measured at baseline in the SURDIAGENE (SURvie, DIAbete de type 2 et GENétique) cohort. High plasma concentrations of TNFR1 (HR [95%CI] for second versus first tertile and IMA (2.42 [1.38–4.23], p=0.002; 2.04 [1.17–3.57], p=0.01) were independently associated with increased risk of major LEAD. Plasma concentrations of TNFR1, but not IMA, yielded incremental information, over traditional risk factors, for the risk of major LEAD: c-statistic change (0.036 [0.013–0.059], p=0.002), IDI (0.012 [0.005–0.022], p<0.001), continuous NRI (0.583 [0.294–0.847], p<0.001), and categorical NRI (0.171 [0.027–0.317], p=0.02). In conclusion, this is the first report of independent associations between high plasma TNFR1 and/or IMA concentrations and increased 5.6-year risk of major LEAD in people with type 2 diabetes.



METHODS OF PREDICTING THE RISK OF HAVING LOWER-EXTREMITY ARTERY DISEASE IN PATIENTS SUFFERING FROM TYPE 2 DIABETES

FIELD OF THE INVENTION:

The present invention relates to methods of predicting the risk of having lower-extremity artery disease in patients suffering from type 2 diabetes.

BACKGROUND OF THE INVENTION:

Lower-extremity artery disease (LEAD) is one of the major clinical manifestations of atherosclerosis across the world (1). Its prevalence is 2 – 3 fold higher in individuals with type 2 diabetes compared with people without diabetes (2; 3). LEAD is a leading cause of limb loss, and is associated with worse cardiovascular outcomes in subjects with type 2 diabetes (4-7). It is also responsible for a quality of life worsening and a high economic burden (8; 9).

It is well established that low-grade inflammation and oxidative stress play an important role in the development of atherosclerosis and its presentations in different arterial beds, including the arteries of lower limbs (10-13). Several studies have evaluated association between circulating inflammatory or redox biomarkers with chronic kidney disease (CKD) and major adverse cardiovascular events (MACE), but few have been done to test reliably these candidates on the risk of LEAD in subjects with type 2 diabetes. Our team has assessed a broad spectrum set of inflammatory and redox biomarkers to test their interest in the prediction of kidney and vascular complications in the SURDIAGENE (SURvie, DIAbete de type 2 et GENEtique) type 2 diabetes cohort (14-17). Hence, plasma concentrations of tumor necrosis factor- α receptor 1 (TNFR1) and angiopoietin-like 2 (ANGPTL2), two proinflammatory factors, have been associated with increased risk of MACE, CKD, or death (14-16). However, the prediction of MACE has not been enhanced with circulating levels of redox status surrogates, including ischemia-modified albumin (IMA), fluorescent advanced glycation end products (F-AGE), protein carbonyls, and total reductive capacity of plasma (TRCP) (17).

SUMMARY OF THE INVENTION:

The present invention relates to methods of predicting the risk of having lower-extremity artery disease in patients suffering from type 2 diabetes. In particular, the present invention is defined by the claims.

DETAILED DESCRIPTION OF THE INVENTION:

Inflammation and oxidative stress play an important role in the pathogenesis of lower-extremity artery disease (LEAD). The inventors assessed the prognostic values of inflammatory

and redox status biomarkers on the risk of LEAD in individuals with type 2 diabetes. Accordingly, plasma concentrations of TNF receptor-1 (TNFR1), angiopoietin like-2, ischemia-modified albumin (IMA), fluorescent advanced glycation end-products, carbonyls, and total reductive capacity of plasma were measured at baseline in the SURDIAGENE (SURvie, DIAbete de type 2 et GENEtique) cohort. Major LEAD was defined as the occurrence during follow-up of peripheral revascularization or lower-limb amputation. Among 1412 participants at baseline (men: 58.2%, mean (SD) age: 64.7 (10.6) years), 112 (7.9%) subjects developed a major LEAD during 5.6 years of follow-up. High plasma concentrations of TNFR1 (HR [95%CI] for second versus first tertile: 1.12 [0.62–2.03], $p=0.71$; third versus first tertile: 2.16 [1.19–3.92], $p=0.01$) and IMA (2.42 [1.38–4.23], $p=0.002$; 2.04 [1.17–3.57], $p=0.01$) were independently associated with increased risk of major LEAD. Plasma concentrations of TNFR1, but not IMA, yielded incremental information, over traditional risk factors, for the risk of major LEAD: c-statistic change (0.036 [0.013–0.059], $p=0.002$), IDI (0.012 [0.005–0.022], $p<0.001$), continuous NRI (0.583 [0.294–0.847], $p<0.001$), and categorical NRI (0.171 [0.027–0.317], $p=0.02$). In conclusion, this is the first report of independent associations between high plasma TNFR1 and/or IMA concentrations and increased 5.6-year risk of major LEAD in people with type 2 diabetes. TNFR1 allowed incremental prognostic information suggesting its use as a biomarker for LEAD.

Accordingly, the first object of the present invention relates to a method of determining whether a patient suffering from type 2 diabetes is at risk of having lower-extremity artery disease comprising i) measuring the concentration of TNFR1 and/or IMA in a plasma sample obtained from the patient, ii) comparing the concentration measured at step i) with its corresponding predetermined reference value wherein detecting differential between the concentration measured at step i) and its corresponding predetermined reference value indicates whether the patient is or is not at risk of having lower-extremity artery disease.

As used herein, the term "type 2 diabetes" or "non-insulin dependent diabetes mellitus (NIDDM)" has its general meaning in the art. Type 2 diabetes often occurs when levels of insulin are normal or even elevated and appears to result from the inability of tissues to respond appropriately to insulin. Most of the type 2 diabetics are obese. As used herein the term "obesity" refers to a condition characterized by an excess of body fat. The operational definition of obesity is based on the Body Mass Index (BMI), which is calculated as body weight per height in meter squared (kg/m^2). Obesity refers to a condition whereby an otherwise healthy subject has a BMI greater than or equal to 30 kg/m^2 , or a condition whereby a subject with at least one co-morbidity has a BMI greater than or equal to 27 kg/m^2 . An "obese subject" is an

otherwise healthy subject with a BMI greater than or equal to 30 kg/m² or a subject with at least one co-morbidity with a BMI greater than or equal to 27 kg/m². A "subject at risk of obesity" is an otherwise healthy subject with a BMI of 25 kg/m² to less than 30 kg/m² or a subject with at least one co-morbidity with a BMI of 25 kg/m² to less than 27 kg/m². The increased risks associated with obesity may occur at a lower BMI in people of Asian descent. In Asian and Asian-Pacific countries, including Japan, "obesity" refers to a condition whereby a subject with at least one obesity-induced or obesity-related co-morbidity that requires weight reduction or that would be improved by weight reduction, has a BMI greater than or equal to 25 kg/m². An "obese subject" in these countries refers to a subject with at least one obesity-induced or obesity-related co-morbidity that requires weight reduction or that would be improved by weight reduction, with a BMI greater than or equal to 25 kg/m². In these countries, a "subject at risk of obesity" is a person with a BMI of greater than 23 kg/m² to less than 25 kg/m².

In some embodiments, the patient is an elderly patient. As used herein, the term "elderly patient" refers to an adult patient sixty-five years of age or older.

As used herein, the expression "lower extremity artery disease" or "LEAD" has its general meaning in the art and refers to a common manifestation of atherosclerosis in lower limbs. LEAD is associated with an increased risk not only for major adverse limb events, but also for all-cause and cardiovascular death as well as for non-fatal myocardial infarction and ischaemic stroke. In some embodiments, the method of the present invention is particularly suitable for determining the risk of major LEAD. As used herein, the term "major LEAD" is defined as the first occurrence of lower-limb amputation (minor, toes or mediotarse; or major, transtibial or transfemoral) or requirement of peripheral revascularization procedure (angioplasty or surgery).

As used herein, the term "Risk" in the context of the present invention, relates to the probability (i.e. at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99% of risk) that an event will occur over a specific time period (e.g. 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 years), as in the conversion to LEAD, and can mean a subject's "absolute" risk or "relative" risk. Absolute risk can be measured with reference to either actual observation post-measurement for the relevant time cohort, or with reference to index values developed from statistically valid historical cohorts that have been followed for the relevant time period. Relative risk refers to the ratio of absolute risks of a subject compared either to the absolute risks of low risk cohorts or an average population risk, which can vary by how clinical risk factors are assessed. Odds ratios, the proportion of positive events to negative events for a given test result, are also commonly used (odds are according to the formula $p/(1-p)$ where p is the

probability of event and $(1 - p)$ is the probability of no event) to no- conversion. "Risk evaluation," or "evaluation of risk" in the context of the present invention encompasses making a prediction of the probability, odds, or likelihood that an event or disease state may occur, the rate of occurrence of the event or conversion from one disease state to another, i.e., from a normal condition to loss of renal function or to one at risk of developing loss of renal function. Risk evaluation can also comprise prediction of future clinical parameters, traditional laboratory risk factor values, or other indices of loss of renal function, either in absolute or relative terms in reference to a previously measured population. The methods of the present invention may be used to make continuous or categorical measurements of the risk of conversion to loss of renal function, thus diagnosing and defining the risk spectrum of a category of subjects defined as being at risk of having loss of renal function. In the categorical scenario, the invention can be used to discriminate between normal and other subject cohorts at higher risk of having loss of renal function. Thus, the terms "high risk", "intermediate risk" and "low risk" refers to differences in the individual predisposition for developing a disease, disorder, complication or susceptibility therefor, preferably after a subject has been treated by one of the therapies referred to below, such as high-dose chemotherapy. Said high, intermediate or low risk can be statistically analyzed. Preferably, the differences between a subject or a group of subjects having a high, intermediate or low risk are statistically significant. This can be evaluated by well-known statistic techniques including Student's t-Test, Chi2-Test, Wilcoxon-Mann-Whitney Test, Kurskal-Wallis Test or Fisher's exact Test, log-rank test, logistic regression analysis, or Cox models. Most preferably, the risk groups are analyzed as described in the accompanied Examples whereby explorative data analysis is carried out and the risk groups are formed with respect to the median, the 25% and the 75% percentiles. Differences in continuous variables of the groups are tested by Wilcoxon-Mann-Whitney Test or Kurskal-Wallis Test depending on the number of groups to be compared. For nominal or ordered categories, Fisher's exact or Chi2-Test for trend are applied. Without further ado, the person skilled in the art can carry out multivariant analysis with stratified versions of the aforementioned tests or Cox models in order to examine the independent impact of predictive factors and to establish the different risk groups.

Typically the plasma sample may be obtained using methods well known in the art. Plasma may then be obtained from the plasma sample following standard procedures of the field including, but not limited to, centrifuging the plasma sample, followed by pipetting of the plasma layer. Platelet-free plasma (PFP) can be obtained following appropriate centrifugation. More preferably, the plasma sample obtained from the patient is a platelet free plasma sample.

As used herein, the term "TNFR1" has its general meaning in the art and is used herein to denote the human soluble tumour necrosis factor receptor type 1. Typically sTNFR1 comprises the extracellular domain of the intact receptor and exhibits an approximate molecular weight of 30KDa.

As used herein, the term "IMA" has its general meaning in the art and refers to ischemia modified albumin (IMA), which is an altered albumin induced by ischemia. Ischemia produces modifications to the N-terminus (Bar-Or, D. et al. (2000) J. Emerg. Med. 19:311-315), and possibly other sites, on the albumin molecule. The N-terminus of albumin has been well characterized as being the primary binding site for several transition metals such as cobalt, nickel and copper (Sadler, P. et al. (1994) Eur. J. Biochem. 220:193-200; Lakusta, H. et al. (1979) J. Inorg. Biochem. 11:303-315; Gasmi, G. et al. (1997) J. Peptide Res. 49:500-509; Predki, P. et al. (1992) Biochem. J. 287:211-215; Lussac, J. et al. (1984) Biochem. 23:2832-38; Matsuoka, J. et al. (1993) J. Biol. Chem. 268:21533-37). Once the N-terminus and possibly other sequestering binding sites have been modified by exposure to ischemic tissue, they are rendered unable to bind metals. This altered albumin is referred to herein as Ischemia Modified Albumin (IMA).

The measurement of the level of a biomarker (e.g. TNFR1) in the blood sample is typically carried out using standard protocols known in the art. For example, the method may comprise contacting the blood sample with a binding partner capable of selectively interacting with the biomarker (e.g. TNFR1) in the sample. In some embodiments, the binding partners are antibodies, such as, for example, monoclonal antibodies or even aptamers. For example the binding may be detected through use of a competitive immunoassay, a non-competitive assay system using techniques such as western blots, a radioimmunoassay, an ELISA (enzyme linked immunosorbent assay), a "sandwich" immunoassay, an immunoprecipitation assay, a precipitin reaction, a gel diffusion precipitin reaction, an immunodiffusion assay, an agglutination assay, a complement fixation assay, an immunoradiometric assay, a fluorescent immunoassay, a protein A immunoassay, an immunoprecipitation assay, an immunohistochemical assay, a competition or sandwich ELISA, a radioimmunoassay, a Western blot assay, an immunohistological assay, an immunocytochemical assay, a dot blot assay, a fluorescence polarization assay, a scintillation proximity assay, a homogeneous time resolved fluorescence assay, a IAsys analysis, and a BIAcore analysis. The aforementioned assays generally involve the binding of the partner (ie. antibody or aptamer) to a solid support. Solid supports which can be used in the practice of the invention include substrates such as nitrocellulose (e.g., in membrane or microtiter well form); polyvinylchloride (e.g., sheets or microtiter wells);

polystyrene latex (e.g., beads or microtiter plates); polyvinylidene fluoride; diazotized paper; nylon membranes; activated beads, magnetically responsive beads, and the like. An exemplary biochemical test for identifying specific proteins employs a standardized test format, such as ELISA test, although the information provided herein may apply to the development of other biochemical or diagnostic tests and is not limited to the development of an ELISA test (see, e.g., *Molecular Immunology: A Textbook*, edited by Atassi et al. Marcel Dekker Inc., New York and Basel 1984, for a description of ELISA tests). Therefore ELISA method can be used, wherein the wells of a microtiter plate are coated with a set of antibodies which recognize the biomarker (e.g. TNFR1). A sample containing or suspected of containing the biomarker (e.g. TNFR1) is then added to the coated wells. After a period of incubation sufficient to allow the formation of antibody-antigen complexes, the plate(s) can be washed to remove unbound moieties and a detectably labelled secondary binding molecule added. The secondary binding molecule is allowed to react with any captured sample marker protein, the plate washed and the presence of the secondary binding molecule detected using methods well known in the art. Measuring the level of a biomarker (e.g. TNFR1) (with or without immunoassay-based methods) may also include separation of the compounds: centrifugation based on the compound's molecular weight; electrophoresis based on mass and charge; HPLC based on hydrophobicity; size exclusion chromatography based on size; and solid-phase affinity based on the compound's affinity for the particular solid-phase that is used. Once separated, said one or two biomarkers proteins may be identified based on the known "separation profile" e.g., retention time, for that compound and measured using standard techniques. Alternatively, the separated compounds may be detected and measured by, for example, a mass spectrometer. Typically, levels of immunoreactive biomarker (e.g. TNFR1) in a sample may be measured by an immunometric assay on the basis of a double-antibody "sandwich" technique, with a monoclonal antibody specific for a biomarker (e.g. TNFR1) (Cayman Chemical Company, Ann Arbor, Michigan). According to said embodiment, said means for measuring a biomarker (e.g. TNFR1) level are for example i) the biomarker (e.g. TNFR1) buffer, ii) a monoclonal antibody that interacts specifically with the biomarker (e.g. TNFR1), iii) an enzyme-conjugated antibody specific for the biomarker (e.g. TNFR1) and a predetermined reference value of the biomarker (e.g. TNFR1).

IMA may be measured by several methods including but not limited to mass spectrometry, HPLC, immunoassay, electrochemical and colorimetric techniques. The detection of IMA is described for example by Wu et al. (*Journal: MLO Medical Laboratory Observer* 35 (2003), 36-38; 40). the assay for ischemia modified albumin can be, for example,

the Albumin Cobalt Binding (ACB®) Test or an immunoassay specific for ischemia modified albumin, i.e., using antibodies directed to the altered N-terminus of albumin, a metal affinity assay for IMA, or an electrochemical or optical test for IMA. Some of these methods are described in U.S. Pat. Nos. 5,290,519, 5,227,307, 6,492,179, and 6,461,875, and co-pending
5 patent applications which are hereby incorporated by reference: U.S. Ser. No. 10/304,610, U.S. Ser. No. 09/849,956, U.S. Ser. No. 10/319,263, U.S. Ser. No. 09/846,411 and WO2008095358.

A predetermined reference value can be relative to a number or value derived from population studies, including without limitation, such subjects having similar body mass index, total cholesterol levels, LDL/HDL levels, systolic or diastolic blood pressure, subjects of the
10 same or similar age range, subjects in the same or similar ethnic group, and subjects having the same severity of type 2 diabetes. Such predetermined reference values can be derived from statistical analyses and/or risk prediction data of populations obtained from mathematical algorithms and computed indices of metabolic syndrome. In some embodiments, the predetermined reference values are derived from the level of a biomarker (e.g. TNFR1 and/or
15 IMA) in a control sample derived from one or more subjects who were not subjected to the event. Furthermore, retrospective measurement of the level of a biomarker (e.g. TNFR1 and/or IMA) in properly banked historical subject samples may be used in establishing these predetermined reference values. The predetermined reference value has to be determined in order to obtain the optimal sensitivity and specificity according to the function of the test and
20 the benefit/risk balance (clinical consequences of false positive and false negative). Typically, the optimal sensitivity and specificity (and so the predetermined reference value) can be determined using a Receiver Operating Characteristic (ROC) curve based on experimental data. For example, after determining the level of the marker in a group of reference, one can use algorithmic analysis for the statistic treatment of the measured levels of the marker in samples
25 to be tested, and thus obtain a classification standard having significance for sample classification. The full name of ROC curve is receiver operator characteristic curve, which is also known as receiver operation characteristic curve. It is mainly used for clinical biochemical diagnostic tests. ROC curve is a comprehensive indicator that reflects the continuous variables of true positive rate (sensitivity) and false positive rate (1-specificity). It reveals the relationship
30 between sensitivity and specificity with the image composition method. A series of different cut-off values (thresholds or critical values, boundary values between normal and abnormal results of diagnostic test) are set as continuous variables to calculate a series of sensitivity and specificity values. Then sensitivity is used as the vertical coordinate and specificity is used as the horizontal coordinate to draw a curve. The higher the area under the curve (AUC), the higher

the accuracy of diagnosis. On the ROC curve, the point closest to the far upper left of the coordinate diagram is a critical point having both high sensitivity and high specificity values. The AUC value of the ROC curve is between 1.0 and 0.5. When $AUC > 0.5$, the diagnostic result gets better and better as AUC approaches 1. When AUC is between 0.5 and 0.7, the accuracy is low. When AUC is between 0.7 and 0.9, the accuracy is moderate. When AUC is higher than 0.9, the accuracy is quite high. This algorithmic method is preferably done with a computer. Existing software or systems in the art may be used for the drawing of the ROC curve, such as: MedCalc 9.2.0.1 medical statistical software, SPSS 9.0, ROCPOWER.SAS, DESIGNROC.FOR, MULTIREADER POWER.SAS, CREATE-ROC.SAS, GB STAT VI0.0 (Dynamic Microsystems, Inc. Silver Spring, Md., USA), etc.

Typically, when the concentration of TNFR1 and/or IMA is higher than its corresponding predetermined reference value, it is concluded that the patient is a risk of having LEAD. On the contrary, when the concentration of TNFR1 and/or IMA is lower than its corresponding predetermined reference value, it is concluded that the patient is a risk of having LEAD.

Once it is concluded that the patient is at risk of having LEAD, treatment options may be prescribed. Typical treatment position include weight management, physical activity, smoking cessation and medications. Medicines for preventing LEAD include lipid-lowering drugs, anti-platelet drugs as well as anti-hypertensive drug. For instance lipid-lowering drugs include statin. As used herein, the term "statin" refers to an inhibitor of the 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCR) enzyme, which catalyzes the limiting step of cholesterol biosynthesis and includes any natural, synthetic or semi-synthetic statin. In some embodiments, the statin is atorvastatin, cerivastatin, fluvastatin, lovastatin, mevastatin, pitavastatin, pravastatin, rosuvastatin, or simvastatin. Non-limiting examples of antiplatelet drugs include: irreversible cyclooxygenase inhibitors (e.g., aspirin or triflusal), adenosine diphosphate receptor inhibitors (e.g., clopidogrel, prasugrel, ticagrelor, or ticlopidine), phosphodiesterase inhibitors (e.g., cilostazol), glycoprotein IIB/IIIA inhibitors (e.g., abciximab, eptifibatide, or tirofiban), adenosine reuptake inhibitors (e.g., dipyridamole), and thromboxane inhibitors (e.g., thromboxane synthase inhibitors or thromboxane receptor antagonists). Anti-hypertensive drugs such as Diuretics, beta-blockers, calcium antagonists, angiotensin-converting enzyme inhibitors (ACEIs) and angiotensin receptor blockers (ARBs).

A further object relates to a kit suitable for performing the method of the present invention which comprises means for measuring the biomarker of the present invention (i.e. TNFR1 and/or IMA). In some embodiments, the kit comprises binding partner specific for the

biomarker(s). Said binding partners are antibodies as described above. In some embodiments, these antibodies are labelled as described above. Typically, the kits described above will also comprise one or more other containers, containing for example, wash reagents, and/or other reagents capable of quantitatively detecting the presence of bound antibodies. Typically
5 compartmentalised kit includes any kit in which reagents are contained in separate containers, and may include small glass containers, plastic containers or strips of plastic or paper. Such containers may allow the efficient transfer of reagents from one compartment to another compartment whilst avoiding cross-contamination of the samples and reagents, and the addition of agents or solutions of each container from one compartment to another in a quantitative
10 fashion. Such kits may also include a container which will accept the tumor tissue sample, a container which contains the antibody(s) used in the assay, containers which contain wash reagents (such as phosphate buffered saline, Tris-buffers, and like), and containers which contain the detection reagent.

The invention will be further illustrated by the following examples. However, these
15 examples should not be interpreted in any way as limiting the scope of the present invention.

EXAMPLE:

Methods

Participants

SURDIAGENE is a French single center prospective cohort designed to identify genetic
20 and biochemical determinants of vascular complications in individuals with type 2 diabetes (18). Adults with an established diagnosis of type 2 diabetes for at least 2 years were recruited in 2002 – 2012, and followed every 2 years since 2007 until December 31, 2015. Non-diabetic kidney disease and short follow-up duration (<1 month) were the main exclusion criteria. The SURDIAGENE study protocol was approved by the Poitiers University Hospital Ethics
25 Committee (CPP Ouest 3) and all participants gave written informed consent before enrolment.

Definition of clinical parameters at baseline

The history of macrovascular disease was defined as the presence at baseline of at least one of the following: myocardial infarction, stable angina, stroke, transient ischaemic attack, coronary or carotid artery revascularization. Glomerular filtration rate (eGFR) was estimated
30 using the Chronic Kidney Disease (CKD)–Epidemiology Collaboration equation. CKD was defined at baseline as eGFR <60 ml/min/1.73 m². Diabetic retinopathy was staged as absent, non-proliferative, pre-proliferative, or proliferative.

Definition of endpoints

The primary endpoint, major LEAD, was defined as the first occurrence during follow-up of lower-limb amputation (minor, toes or mediotarse; or major, transtibial or transfemoral) or requirement of peripheral revascularization procedure (angioplasty or surgery), whichever came first. Requirement of peripheral revascularization procedure and lower-limb amputation were considered separately as secondary endpoints. An independent adjudication committee adjudicated each endpoint.

Laboratory procedures

Blood samples and second morning urines were obtained after an overnight fast and stored at -80°C until use in the CHU Poitiers biobanking facility (CRB0033 - 00068). HbA1c was assessed using a high performance liquid chromatography method (ADAMS A1C HA - 8160 analyzer; Menarini, Florence, Italy). Serum and urine creatinine and urinary albumin were measured by nephelometry on a Modular System P (Roche Diagnostics GmbH, Mannheim, Germany). Plasma concentrations of triglycerides, total- and HDL-cholesterol were measured with enzymatic methods.

Plasma concentrations of TNFR1 (EKF Diagnostics, Dublin, Ireland) and human ANGPTL2 (Cloud-Clone Corp, Houston, TX, USA) were measured using ELISA kits. Samples were tested in duplicate, and the mean of the two measurements was considered. The intra- and inter-assay coefficient of variations (CV) were 1.8–5.3% and 3.6–6.8% for TNFR1; and <10% and <15% for ANGPTL2, respectively. The results of both biomarkers are expressed as ng/ml. Plasma C-reactive protein (CRP) was measured at baseline using immunoturbidimetric assay (Roche/Hitachi cobas c systems, Mannheim, Germany). CV was 2.07% and 2.85% for CRP concentrations at 8.01 and 36.9 mg/l, respectively.

The comprehensive biological process used to measure the redox biomarkers were recently reported (17). Briefly, plasma IMA index, an early marker of ischemia, was assessed by spectrophotometry. The measurement was based on the decreased capacity of IMA to bind cobalt, and the results are expressed as arbitrary units (AU). Plasma F-AGE concentrations were assessed using a spectrofluorometer (Fluostar Omega, BM6 Labtech) and the results are expressed as 10^{-3}AU . Plasma concentrations of protein carbonyls, reflecting the degree of carbonylation in plasma, were determined by ELISA kit (OxiselectTM, Protein Carbonyl ELISA Kit, Cell Biolabs, Inc), and the results are expressed as mmol/mg. TRCP, a marker of anti-oxidant capacity of plasma, was measured using the Folin-Ciocalteu method. The gallic acid (Sigma) was used as a standard, and the results are expressed as gallic acid equivalents.

Analyses and statistical methods

Continuous variables are expressed as mean (SD), or as median (25th, 75th percentiles) for those with skewed distribution. Categorical variables are expressed as the number of participants with corresponding percentage. Participants were categorized into three equally sized groups corresponding to increasing tertiles (T1, T2 and T3) of each biomarker.

5 Characteristics of participants at baseline by the incidence of major LEAD during follow-up were compared using chi-squared, ANOVA, or Wilcoxon tests.

A complete case method was used to handle missing data. Thus, 56 subjects with at least one missing value were omitted in the present study, and 1412 participants were included in the complete case study.

10 Restrictive cubic splines regression analyses were performed (using quantiles as knots, and medians as reference values) to assess the nonlinearity in the relationship between each biomarker and the primary endpoint. Kaplan-Meier curves were plotted to evaluate the primary endpoint free-survival rates by biomarker tertiles at baseline, and compared using the log-rank test. Cox proportional hazards regression models were fitted to estimate hazard ratios (HR),
15 with associated 95% CI, for endpoints during follow-up for the second and the third tertiles of each biomarker compared with the first one. Analyses were adjusted for sex and age (model 1); and for all potential confounding covariates at baseline: model 1 plus BMI, duration of diabetes, HbA1c, systolic and diastolic blood pressure, urinary albumin to creatinine ratio (ACR), eGFR, diabetic retinopathy stages, plasma concentrations of total-, HDL-cholesterol, and triglycerides,
20 history of macrovascular disease, current smoking, and use of insulin therapy, antihypertensive, statin, fibrate, and antiplatelet drugs (model 2). The Schoenfeld residuals method was used to check the proportional hazards assumption for the association between primary endpoint and each biomarker.

Harrell's c-statistic (19), Integrated Discrimination Improvement (IDI), and Net
25 Reclassification Improvement (NRI) were performed, in participants with no major LEAD at baseline, to compare discrimination and classification of primary endpoint, assessed using survival methodology, between two prognostic models: model 2 versus model 2 plus plasma concentrations of relevant (independently associated with major LEAD) biomarkers.

We conducted a series of sensitivity analyses: (1) to use the competing risk model of
30 Fine and Gray to estimate the subdistribution hazard ratios for major LEAD, while accounting for the competing risk of cardiovascular death (20); (2) to evaluate associations between plasma biomarker levels and primary endpoint in individuals free at baseline of history of major LEAD, macrovascular disease, or CKD; and (3) to assess associations between biomarkers and an alternative primary endpoint defined as the first occurrence during follow-up of one of the

following: (i) minor lower-limb amputation with peripheral revascularization; (ii) major lower-limb amputation; or (iii) requirement of peripheral revascularization procedure. Finally, we evaluated the prognostic value of plasma CRP concentrations on the risk of major LEAD in the subset of participants for whom CRP data was available at baseline.

Statistics were performed using SAS software, version 9.4 (SAS Institute, www.sas.com) and Stata software version 13 (StataCorp, www.stata.com). Two-sided P-values less than 0.05 were considered significant.

Results

Characteristics of participants at baseline according to the incidence of major LEAD during follow-up:

We investigated 1412 participants with 58.2 % of men, mean (SD) age at 64.7 (10.6) years, and median [25th, 75th percentiles] duration of diabetes at 13 [6, 21] years at baseline. New cases of major LEAD occurred in 112 (7.9%) subjects during a median duration of follow-up of 5.6 [3.0, 8.6] years. The incidence rate of major LEAD was 1.4 per 100 person-years. Individuals who developed a major LEAD during follow-up, compared with those who did not, were significantly older, more frequently men, had a longer duration of diabetes, higher systolic blood pressure and ACR, lower BMI, eGFR and HDL-cholesterol, were less likely to use fibrates, more likely to use statins, antihypertensives and antiplatelet drugs, and had more prevalent diabetic retinopathy, lower limb amputation, and peripheral revascularization at baseline (Table 1).

Risk of primary endpoint by plasma concentrations of inflammatory and oxidative stress biomarkers at baseline:

Participants who developed a major LEAD during follow-up, compared with those who did not, had higher plasma concentrations of TNFR1, ANGPTL2, IMA, and protein carbonyls (Table 1). The relationship between plasma concentrations of each biomarker at baseline and the primary endpoint were not linear ($p < 0.0001$ for all, data not shown). They were higher in the upper compared with the lowest tertiles of TNFR1 (4.2 %, 4.7 %, and 17.7 % for T1, T2 and T3, respectively, $p < 0.0001$), ANGPTL2 (4.4 %, 6.8 %, and 13.9 %, $p < 0.0001$), IMA (4.3 %, 10.5 %, and 9.7 %, $p = 0.002$), and protein carbonyls (7.4 %, 6.0 %, and 10.4 %, $p = 0.03$). No significant association was observed between major LEAD and F-AGE or TRCP tertiles (Table 2). The Cox regression model 1 confirmed the associations between TNFR1, ANGPTL2, and IMA tertiles and major LEAD (Table 2). However, only TNFR1 and IMA tertiles remained significantly associated with the risk of major LEAD in the fully adjusted model 2. Similar results were observed after including both TNFR1 and IMA tertiles together into model 2

(TNFR1, T2 vs. T1: 1.15 [0.63 – 2.10], $p=0.64$; T3 vs. T1: 2.28 [1.25 – 4.15], $p=0.007$; IMA, T2 vs. T1: 2.52 [1.44 – 4.42], $p=0.001$; T3 vs. T1: 2.09 [1.20 – 3.66], $p=0.009$). No evidence for interaction was observed between plasma concentrations of TNFR1 and IMA on the risk of major LEAD (p for interaction=0.30).

Our findings were reliable after adjusting for cardiovascular death as a competing risk (data not shown), and after considering participants with no baseline history of major LEAD ($n=1290$), CKD ($n=1016$), or macrovascular disease ($n=905$, except for the absence of significant IMA-LEAD association in this subset of participants) (data not shown). The use of the alternative primary outcome did not materially alter the results (data not shown).

Plasma concentrations of CRP were measured at baseline in 291 (20.6) subjects. Participants with available CRP measurements at baseline, compared with others, had slightly higher BMI and HbA1c, lower systolic blood pressure, HDL-cholesterol and total-cholesterol, were less likely to use fibrates, and more likely to use antihypertensive and antiplatelet drugs (data not shown). Among participants for whom CRP measurements were available at baseline, major LEAD occurred during follow-up in 31 (10.6%) subjects. Plasma CRP concentrations were higher in individuals who experienced major LEAD compared to those who did not (8.5 [5.4 – 22.5] vs. 4.7 [2.0 – 12.6] mg/l, $p=0.001$), with a non-linear relationship (p for non-linearity <0.0001 , data not shown). The Kaplan–Meier estimate of the 6-year cumulative incidence of major LEAD during follow-up was higher in the second (15.0%) and third CRP tertiles (18.1%) compared to the first one (3.2%, $p=0.02$, data not shown). Hazard ratios for major LEAD increased with growing CRP tertiles (T2 vs. T1: 5.52 [1.38 – 22.09], $p=0.02$; T3 vs. T1: 7.14 [1.82 – 27.96], $p=0.005$) in the fully adjusted model. A significant interaction was observed between TNFR1 and CRP on the risk of major LEAD (p for interaction=0.03).

Additive value of plasma concentrations of TNFR1 and/or IMA at baseline in discrimination and classification of major LEAD during follow-up:

The addition of plasma concentrations of TNFR1 to traditional risk factors (as in model 2) improved c-statistic (0.036, 95% CI [0.013 – 0.059], $p=0.002$), IDI (0.012 [0.005 – 0.022], $p<0.001$), continuous NRI (0.583 [0.294 – 0.847], $p<0.001$), and categorical NRI (0.171 [0.027 – 0.317], $p=0.02$) for the 5.6-year risk of major LEAD during follow-up. Plasma concentrations of IMA at baseline did not enhance discrimination or classification of the investigated risk (Table 3). No further improvement was observed by the addition of both plasma concentrations of TNFR1 and IMA together into model 2 (data not shown). Plasma CRP concentrations enhanced c-statistic (0.071 [0.008 – 0.135], $p=0.03$), IDI (1.031 [0.789 – 1.90], $p<0.001$), and continuous NRI (0.291 [0.205 – 0.385], $p<0.001$) for the risk of major LEAD during follow-

up. A greater improvement in c-statistic was observed when both TNFR1 and CRP were introduced together in the final model (0.086 [0.020 - 0.151], $p=0.01$).

Risk of secondary endpoints by plasma concentrations of inflammatory and oxidative stress biomarkers at baseline:

Peripheral revascularization and lower-limb amputation occurred during follow-up in 79 (5.6 %) and 58 (4.1 %) participants, respectively. Their incidence rates were 1.0 and 0.7 per 100 person-years, respectively. The risk of peripheral revascularization was significantly higher in the greatest TNFR1 and IMA tertiles, while the risk of lower-limb amputation was greater in the upper TNFR1 and ANGPTL2 tertiles, compared with the lowest ones (data not shown).

Conclusion:

In the present study, we evaluated the relationship between plasma concentrations of inflammatory and redox status biomarkers and the risk of major LEAD in a prospective cohort of individuals with type 2 diabetes. We have observed associations between plasma concentrations of TNFR1 and IMA at baseline and excess risk of major LEAD during follow-up. Whereas no independent associations were observed between major LEAD and circulating levels of ANGPTL2, F-AGE, protein carbonyls, or TRCP.

Participants in the upper TNFR1 tertile had a 2-fold increased risk of major LEAD compared with those in the lowest one. This finding was derived from analyse of the whole cohort and remained significant in the subset of participants with no history of major LEAD at baseline. This association was independent on potential confounders, including key cardiovascular risk factors. Similar results were observed with either peripheral revascularization or lower-limb amputation, considered individually as secondary endpoints. Furthermore, plasma concentrations of TNFR1 provided additive prognostic information, beyond conventional risk factors, on the risk of major LEAD. They improved C-statistics, IDI and NRI.

As far as we know, we reported here for the first time reliable evidence for the prognostic value of plasma TNFR1 concentrations on the risk of major LEAD in people with type 2 diabetes. Few cross-sectional studies have investigated the association between LEAD and TNF alpha, or its two soluble receptors TNFR1 or TNFR2, in the general population. Two small studies, including an overall of a hundred participants, showed higher circulating TNF- α , TNFR1 or TNFR2 concentrations in individuals with LEAD compared with controls (21; 22). The Framingham Offspring Study, a larger community-based cohort, showed an association between TNFR2 and LEAD, defined as ankle-brachial index <0.9 , intermittent claudication and/or lower extremity revascularization (23). In type 2 diabetes setting, higher circulating

TNFR1 concentrations have been reported to be mainly associated with increased risk of kidney disease, cardiovascular events, or mortality (14; 16; 24), but no investigation has been performed regarding the risk of LEAD. It is unlikely that our findings have been driven by kidney or cardiovascular disease. Yet, TNFR1-LEAD association remained significant after
5 adjustment for renal parameters and cardiovascular risk factors but also in participants free of history of kidney or macrovascular disease at baseline. Furthermore, we did not observe evidence for competing risk of cardiovascular death on this association.

Our findings cannot allow any etiologic conclusion, but they are, however, consistent with previous studies supporting the implication of systemic inflammation in peripheral artery
10 disease (25; 26) (27). TNF pro-inflammatory activities promote atherosclerosis by increasing endothelial cell permeability, inducing the expression of surface leukocyte adhesion molecules, and enhancing the production of cytokines (28; 29). Furthermore, TNF decreases the activity of adipocyte-derived lipoprotein lipase and increases the production of hepatic very low-density lipoproteins in response to acute endotoxin exposure (30; 31). Increased TNF- α activity may
15 also reflect oxidative stress (32), and was correlated with pulse wave velocity, an established surrogate for arterial stiffness (33), which plays an important role in the pathophysiology of LEAD (34).

High plasma CRP concentrations were associated with increased risk of major LEAD, and provided additive prognostic information, over traditional risk factors. Plasma CRP
20 concentrations significantly interacted with TNFR1 levels on their associations with major LEAD, suggesting that these relationships may be related to the inflammatory background. However, these findings are limited by the issue that they were derived from a subset of 291 participants from whom CRP data was available at baseline.

Our study shows also an independent association between plasma IMA concentrations
25 and increased risk of major LEAD, peripheral revascularization, but not lower-limb amputation. The association between circulating IMA levels and major LEAD remained significant in participants free of LEAD or CKD at baseline, but not in those with no history of macrovascular disease. IMA reflects ischemia regardless of vascular bed, and it has been suggested as a biomarker of acute myocardial ischemia, skeletal muscle ischemia, and stroke (35-37). In
30 ischemic condition, structural changes take place in the N-terminus of the human albumin, which reduce its binding capacity (38), possibly as a result of exposure to reactive oxygen species. However, the diagnostic and prognostic values of IMA have not been clearly established yet. In our study, circulating IMA levels did not improve discrimination or classification of major LEAD risk. In the same line, IMA did not provide an incremental

diagnostic information for cardiovascular events in SURDIAGENE type 2 diabetes cohort or in patients with suspected acute coronary syndrome in the IMAGINE multicenter prospective study (17; 39).

We have also observed an association between greater circulating ANGPTL2 levels and increased risk of lower-limb amputation. However, plasma concentrations of ANGPTL2 did not enhance the discrimination or classification of limb loss (data not shown), and were not independently associated with the risk of the primary endpoint. Although, the absence of strong evidence to support the usefulness of plasma ANGPTL2 as a reliable predictor for major LEAD, our observation is consistent with the role of vascular inflammation in the natural history of lower-limb amputation. Excess ANGPTL2 may accelerate vascular inflammation by activating proinflammatory pathways in endothelial cells and increasing macrophage infiltration, leading to endothelial dysfunction and atherosclerosis progression (40).

The main strength of our study is the use of a contemporary prospective cohort designed to investigate clinical, biochemical, and genetic determinants of vascular complications in people with type 2 diabetes. SURDIAGENE contains a comprehensive data on clinical and biochemical parameters at baseline, as well as adjudicated vascular endpoints during follow-up. We assessed here wide-ranging biomarkers of such major pathways involved in the pathophysiology of lower-extremity atherosclerosis, including inflammation, oxidative stress, and advanced glycation end products. The major limitation of our study is related to the issue that SURDIAGENE cohort was conducted in a single French diabetes department, and may not be representative of all populations with type 2 diabetes. Our findings can only be generalized for Caucasian people with type 2 diabetes, but not other ethnic groups. The present study lacks data related to intermittent claudication and ankle-brachial index, which can lead to underestimate association between candidate biomarkers and early stages of LEAD. SURDIAGENE also lacks data regarding peripheral neuropathy and foot infection, which can have confounding effects, especially in the risk of lower-limb amputation. Nevertheless, similar associations were observed when we considered the alternative endpoint including lower-limb amputation (transmetatarsal with need of revascularization, transtibial, or transfemoral) believed to be due to artery disease. Furthermore, the main association and prognostic value of plasma TNFR1 concentrations were observed not only for the combined LEAD endpoint but also for peripheral revascularization considered individually as a secondary endpoint.

Overall, high plasma concentrations of TNFR1 and IMA were independently associated with increased 6-year risk of major LEAD in people with type 2 diabetes. TNFR1 yielded

incremental prognostic information on the risk of major LEAD, suggesting that it may be a useful biomarker for peripheral arterial disease in this population.

TABLES:

Table 1. Characteristics of participants at baseline according to the incidence of major lower-extremity artery disease during follow-up.

	Major lower-extremity artery disease			
	Overall	No	Yes	P
N	1412	1300	112	
Clinical parameters				
Male sex	822 (58.2)	733 (56.4)	89 (79.5)	< 0.0001
Age (years)	64.7 (10.6)	64.5 (10.8)	66.9 (8.9)	0.02
Duration of diabetes (years)	13 (6, 21)	12 (6, 20)	16 (10, 24)	0.0009
Body mass index (kg/m ²)	31.3 (6.3)	31.4 (6.4)	29.7 (5.0)	0.005
Systolic blood pressure (mmHg)	132 (18)	132 (17)	138 (20)	0.0004
Diastolic blood pressure (mmHg)	72 (11)	72 (11)	73 (12)	0.83
Biological parameters				
HbA1c (%)	7.8 (1.5)	7.8 (1.5)	7.6 (1.5)	0.25
HbA1c (mmol/mol)	62 (17)	62 (17)	60 (16)	
Urinary albumin to creatinine ratio (mg/mmol)	3 (1, 14)	3 (1, 12)	13 (2, 131)	<0.0001
eGFR (ml/min/1.73m ²)	73 (24)	74 (24)	62 (28)	<0.0001

Serum	total	cholesterol	4.79 (1.15)	4.79 (1.14)	4.81 (1.24)	0.89
(mmol/l)						

Serum	HDL	cholesterol	1.21 (0.41)	1.21 (0.42)	1.13 (0.35)	0.03
(mmol/l)						

Serum triglycerides (mmol/l)	1.57 (1.12, 2.30)	1.57 (1.12, 2.30)	1.69 (1.14, 2.27)	0.71
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Medical history

Current smoking	152 (10.8)	135 (10.4)	17 (15.2)	0.15
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Diabetic retinopathy	624 (44)	544 (42)	80 (71)	<0.0001
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Macrovascular disease	507 (36)	459 (35)	48 (43)	0.12
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Major lower-extremity artery disease	122 (9)	83 (6)	39 (35)	<0.0001
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Lower-limb amputation	69 (5)	43 (3)	26 (23)	<0.0001
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Peripheral revascularization	69 (5)	50 (4)	19 (17)	<0.0001
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History of treatments

Use of antihypertensive treatment	1172 (83)	1067 (82)	105 (94)	0.0009
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Use of statin	638 (45)	576 (44)	62 (55)	0.03
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Use of fibrate	160 (11)	154 (12)	6 (5)	0.04
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Use of antiplatelet drugs	593 (42)	532 (41)	61 (54)	0.007
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Use of insulin therapy	846 (60)	771 (59)	75 (67)	0.13
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Plasma concentrations of biomarkers

TNFR1 (ng/ml)	1.8 (1.5, 2.3)	1.8 (1.5, 2.3)	2.3 (1.8, 3.1)	<0.0001
ANGPTL2 (ng/ml)	15 (11, 21)	15 (11, 20)	19 (13, 28)	<0.0001
IMA (AU)	0.51 (0.33, 0.63)	0.51 (0.32, 0.63)	0.58 (0.48, 0.68)	<0.0001
F-AGE (10 ⁻³ AU)	111 (93, 132)	111 (93, 132)	117 (94, 141)	0.24
Protein carbonyls (mmol/mg)	28 (26, 31)	28 (26, 31)	29 (26, 33)	0.02
TRCP (gallic acid equivalents)	120 (103, 145)	120 (103, 145)	122 (103, 159)	0.45

Data presented as numbers (%), mean (SD), or median (25th, 75th percentiles) for variables with skewed distribution: duration of diabetes, urinary albumin to creatinine ratio, triglycerides, tumor necrosis factor receptor 1 (TNFR1), angiopoietin-like 2 protein (ANGPTL2), ischemia-modified albumin (IMA), fluorescent advanced glycation end products (F-AGE), and total reductive capacity of plasma (TRCP).

Comparisons of qualitative and quantitative parameters were performed using Chi-square and ANOVA tests, respectively. Wilcoxon test was used for comparisons of variables with skewed distribution. $p < 0.05$ was significant.

Table 2. Risk for major lower-extremity artery disease during follow-up according to plasma concentrations of inflammatory and oxidative stress biomarkers at baseline.

Major LEAD			Model 1		Model 2	
	No, n	Yes, n (%)	HRs (95% CI)	P	HRs (95% CI)	P
TNFR1	All	1300	112 (7.9)			
	1 st tertile	448	23 (4.9)		Ref.	
	2 nd tertile	447	24 (5.1)	0.45	1.12 (0.62 - 2.03)	0.71
	3 rd tertile	405	65 (13.8)	<0.0001	2.16 (1.19 - 3.92)	0.01
ANGPTL2	1 st tertile	449	22 (4.7)		Ref.	
	2 nd tertile	439	32 (6.8)	0.14	1.31 (0.74 - 2.32)	0.36
	3 rd tertile	412	58 (12.3)	<0.0001	1.59 (0.88 - 2.85)	0.12
IMA	1 st tertile	453	18 (3.8)		Ref.	
	2 nd tertile	428	43 (9.1)	0.001	2.42 (1.38 - 4.23)	0.002
	3 rd tertile	419	51 (10.9)	0.002	2.04 (1.17 - 3.57)	0.01
F-AGE	1 st tertile	437	34 (7.2)		Ref.	
	2 nd tertile	433	38 (8.1)	0.34	1.10 (0.68 - 1.80)	0.70
	3 rd tertile	430	40 (8.5)	0.05	1.15 (0.69 - 1.92)	0.59
Protein carbonyls	1 st tertile	436	35 (7.4)		Ref.	
	2 nd tertile	443	28 (5.9)			
	3 rd tertile	421	49 (10.4)	0.27	0.66 (0.40 - 1.12)	0.12
				0.16	1.16 (0.73 - 1.83)	0.53
TRCP	1 st tertile	433	38 (8.1)		Ref.	
	2 nd tertile	437	34 (7.2)	0.74	0.92 (0.57 - 1.48)	0.73
	3 rd tertile	430	40 (8.5)	0.38	1.08 (0.67 - 1.75)	0.74

Hazard ratios (HRs) and 95% confidence intervals (CI) for the 2nd and 3rd tertiles compared with the 1st one. Analyses adjusted for baseline age and sex (model 1); and for model 1 plus BMI, duration of diabetes, HbA1c, systolic and diastolic blood pressure, urinary albumin-to-creatinine ratio, eGFR, diabetic retinopathy stages, plasma concentrations of total-, HDL-cholesterol, and triglycerides, use of insulin therapy, antihypertensive, statin, fibrate and antiplatelet drugs, and history of current smoking and macrovascular disease (model 2). P<0.05 was significant.

Table 3. Discrimination and classification assessments for risk of major lower-extremity artery disease during follow-up according to traditional risk factors without and with plasma concentrations of TNFR1 and/or IMA at baseline.

Risk of lower extremity artery disease	P
C-statistic (95%CI) for model 2	0.753 (0.688 – 0.817)
Change in C-statistic (95%CI) for model 2 + TNFR1	0.036 (0.013 – 0.059) 0.002
Change in C-statistic (95%CI) for model 2 + IMA	0.007 (-0.009 – 0.022) 0.38
IDI (95% CI) for TNFR1	0.012 (0.005 – 0.022) <0.001
Continuous NRI (95% CI) for TNFR1	0.583 (0.294 – 0.847) <0.001
Categorical NRI (95% CI) for TNFR1	0.171 (0.027 – 0.317) 0.02
IDI (95% CI) for IMA	0.001 (-0.006 – 0.009) 0.63
Continuous NRI (95% CI) for IMA	0.239 (-0.043 – 0.508) 0.11
Categorical NRI (95% CI) for IMA	0.055 (-0.021 – 0.134) 0.18

Integrated discrimination improvement (IDI), continuous and categorical (5 and 10% risk thresholds) net reclassification improvement (NRI) tests performed for model 2 plus baseline plasma concentrations of TNFR1 or IMA, compared with model 2 alone.

Model 2: age, sex, BMI, duration of diabetes, HbA1c, systolic and diastolic blood pressure, urinary albumin-to-creatinine ratio, estimated glomerular filtration rate, diabetic retinopathy stages, plasma concentrations of HDL-cholesterol, total cholesterol and

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Throughout this application, various references describe the state of the art to which this invention pertains. The disclosures of these references are hereby incorporated by reference into the present disclosure.

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CLAIMS:

1. A method of determining whether a patient suffering from type 2 diabetes is at risk of having lower-extremity artery disease (LEAD) comprising i) measuring the concentration of TNFR1 and/or IMA in a plasma sample obtained from the patient, ii)
5 comparing the concentration measured at step i) with its corresponding predetermined reference value wherein detecting differential between the concentration measured at step i) and its corresponding predetermined reference value indicates whether the patient is or is not at risk of having lower-extremity artery disease.
2. The method of claim 1 wherein the patient is obese.
- 10 3. The method of claim 1 wherein the patient is an elderly patient.
4. The method of claim 1 wherein when the concentration of TNFR1 and/or IMA is higher than its corresponding predetermined reference value, it is concluded that the patient is a risk of having LEAD.

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2019/064929A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N33/68
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	FABRICE SCHNEIDER ET AL: "Influence of micro- and macro-vascular disease and Tumor Necrosis Factor Receptor 1 on the level of lower-extremity amputation in patients with type 2 diabetes", CARDIOVASCULAR DIABETOLOGY, vol. 17, no. 1, 8 June 2018 (2018-06-08), XP055499379, DOI: 10.1186/s12933-018-0725-9 abstract, page 2, left-hand column - page 4, right-hand column. ----- -/--	1-4



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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Date of the actual completion of the international search

8 July 2019

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INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2019/064929

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Shao-Gang Ma ET AL: "Ischemia-modified albumin in type 2 diabetic patients with and without peripheral arterial disease", Clinics (Sao Paulo, Brazil), 1 January 2011 (2011-01-01), pages 1677-1680, XP055499383, Brazil DOI: 10.1590/S1807-59322011001000003 Retrieved from the Internet: URL: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3180151/pdf/cln-66-10-1677.pdf [retrieved on 2018-08-13] abstract, pages 1678-1679	1-4
A	----- ANDREW D. ALTHOUSE ET AL: "Risk Factors for Incident Peripheral Arterial Disease in Type 2 Diabetes: Results From the Bypass Angioplasty Revascularization Investigation in Type 2 Diabetes (BARI 2D) Trial", DIABETES CARE, vol. 37, no. 5, 4 March 2014 (2014-03-04), pages 1346-1352, XP055499388, US ISSN: 0149-5992, DOI: 10.2337/dc13-2303 abstract	1-4
A	----- Cameli C. Diaconu ET AL: "Clinical presentation of lower extremity arterial disease (LEAD)", E-Journal of Cardiology Practice, 7 March 2018 (2018-03-07), pages 1-9, XP055499394, Retrieved from the Internet: URL: https://www.escardio.org/Journals/E-Journal-of-Cardiology-Practice/Volume-16/Clinical-presentation-of-lower-extremity-arterial-disease-LEAD [retrieved on 2018-08-13] the whole document -----	1-4