



(51) International Patent Classification:

G01N 33/68 (2006.01)

(21) International Application Number:

PCT/EP2019/076155

(22) International Filing Date:

27 September 2019 (27.09.2019)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

18306273.6

28 September 2018 (28.09.2018) EP

(71) Applicants: INSERM (INSTITUT NATIONAL DE LA SANTÉ ET DE LA RECHERCHE MÉDICALE)

[FR/FR]; 101, rue de Tolbiac, 75013 Paris (FR).

UNIVERSITÉ DE POITIERS [FR/FR]; 15 rue de l'Hôtel-Dieu, 86000 Poitiers (FR).

CENTRE HOSPITALIER UNIVERSITAIRE DE POITIERS [FR/FR]; 2 rue de la

Milétrie, 86000 POITIERS (FR).

(72) Inventor: SAULNIER, Pierre Jean; Centre

d'Investigation Clinique CIC - Inserm 1402, CHU, 2 rue de

la Milétrie, 86021 Poitiers cedex (FR).

(74) Agent: INSERM TRANSFERT; 7 rue Watt, 75013 Paris

(FR).

(81) Designated States (unless otherwise indicated, for every

kind of national protection available): AE, AG, AL, AM,

AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ,

CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO,

DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN,

HR, HU, ID, IL, IN, IR, IS, JO, JP, KE, KG, KH, KN, KP,

KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME,

MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ,

OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA,

SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN,

TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every

kind of regional protection available): ARIPO (BW, GH,

GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ,

UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ,

TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK,

EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV,

MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM,

TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW,

KM, ML, MR, NE, SN, TD, TG).

Published:

— with international search report (Art. 21(3))

(54) Title: USE OF BIOMARKERS REPRESENTING CARDIAC, VASCULAR AND INFLAMMATORY PATHWAYS FOR THE PREDICTION OF ACUTE KIDNEY INJURY IN PATIENTS WITH TYPE 2 DIABETES

(57) Abstract: Acute kidney injury (AKI) is a related to chronic kidney disease and death in patients from the general population, with or without type 2 diabetes. Nevertheless AKI biomarkers are rarely validated in diabetes population. The inventors aimed to explore the individual and combined prognostic value of 7 circulating candidate markers for AKI. This include markers of cardiac and endothelial dysfunction (mid-regional-pro-adrenomedullin [MRproADM], angiotensin-like-2 [ANGPTL2], N-terminal prohormone brain natriuretic peptide [NTproBNP]) oxidative stress (fluorescent advanced glycation endproducts [AGE], carbonyls), cardio-renal pathways (copeptin [CTproAVP]), and inflammation (soluble TNF receptor 1 [TNFR1]). They prospectively followed-up 1345 (565 women/780 men) type 2 diabetes participants of a French single-centre hospital-based cohort (SURDIAGENE). In univariate analysis, each biomarker was significantly associated with AKI, and 6 remained associated after multivariable adjustment. The addition of a multimarker score summing standardized and weighted values of these 6 markers to the model including usual risk factors significantly improved C-statistics (0.724 to 0.759, $P < 0.0001$), and 5-year risk-predictive performance (relative integrated discrimination improvement index=0.435, $P < 0.0001$). Thus the panel of 6 biomarkers representing cardiac, vascular and inflammatory pathways improves the prediction of AKI over usual risk factors in patients with type 2 diabetes.

**USE OF BIOMARKERS REPRESENTING CARDIAC, VASCULAR AND
INFLAMMATORY PATHWAYS FOR THE PREDICTION OF ACUTE KIDNEY
INJURY IN PATIENTS WITH TYPE 2 DIABETES**

5

FIELD OF THE INVENTION:

The present invention relates to use of biomarkers representing cardiac, vascular and inflammatory pathways for the prediction of acute kidney injury in patients with type 2 diabetes.

BACKGROUND OF THE INVENTION:

10 Acute kidney injury (AKI) is related to chronic kidney disease and death in patients from the general population, with or without type 2 diabetes. Nevertheless AKI biomarkers are rarely validated in diabetes population.

MR-proADM concentrations are associated with the doubling of serum creatinine and progression to ESRD [2], and with mortality [3] in type 2 diabetes patients. Circulating sTNFR
15 is associated in numerous epidemiological studies cross-sectionally with GFR and DKD [4-6] and prospectively with DKD progression and ESRD occurrence [7-12] in both type 1 diabetes and type 2 diabetes patients. Moreover, sTNFR2 has been associated with GFR variation in type 2 diabetes patients [13] as well as in type 1 diabetes patients. [14]. sTNFR2 and TNFR1 have also been associated with DKD structural lesions and especially with early glomerular
20 lesions in type 2 diabetes [15]. However, even TNFR2 and TNFR1 shows partially overlapping biological effects, we only had access to sTNFR1 assay for the present study. NT-proBNP has been reported to be associated with rapid kidney decline in elderly adults [16] and with ESRD in the general population [17]. A post-hoc analysis of a clinical trial also reported an association of NT-proBNP with ESRD in type 2 diabetes patients [18]. Interestingly, all 3 of these
25 biomarkers were associated with rapid progression of eGFR in type 2 diabetes in a recent biomarker-panel study, and they were included in our study to further evaluate their combined value [19]. However the panel of said 6 biomarkers representing cardiac, vascular and inflammatory pathways has never been investigated for the prediction of AKI over usual risk factors in patients with type 2 diabetes.

SUMMARY OF THE INVENTION:

The present invention relates to use of biomarkers representing cardiac, vascular and inflammatory pathways for the prediction of acute kidney injury in patients with type 2 diabetes. In particular, the present invention is defined by the claims.

DETAILED DESCRIPTION OF THE INVENTION:

The inventors aimed to explore the individual and combined prognostic value of 7 circulating candidate markers for AKI. This include markers of cardiac and endothelial dysfunction (mid-regional-pro-adrenomedullin [MRproADM], angiotensin-like-2 [ANGPTL2], N-terminal prohormone brain natriuretic peptide [NTproBNP]) oxidative stress (fluorescent advanced glycation endproducts [AGE], carbonyls), cardio-renal pathways (copeptin [CTproAVP]), and inflammation (soluble TNF receptor 1 [TNFR1]).

They prospectively followed-up 1345 (565 women/780 men) type 2 diabetes participants of a French single-centre hospital-based cohort (SURDIAGENE) with baseline $\text{GFR} \geq 30$ ml/min/1.73m² and no renal replacement to onset of AKI, death, or December 31, 2015, whichever came first. Intrahospital AKI was diagnosed and staged using the KDIGO criteria (increase in serum creatinine concentration by 0.3 mg/dL or increase in serum creatinine to ≥ 1.5 times baseline). Cox models were used to estimate the risk of AKI for each biomarker at baseline after adjustment for usual risk factors: sex, diabetes duration, HbA1c, systolic blood pressure, GFR, ACR, use of antihypertensive, and history of cardiovascular disease. Hazard ratios were reported per 1 SD increment of the logarithm of the biomarker concentration.

At baseline, mean $\pm$ SD age was 64 $\pm$ 11 years, diabetes duration 14 $\pm$ 10 years, HbA1c 7.8 $\pm$ 1.6 %, and eGFR 77 $\pm$ 21 ml/min/1.73m², and median (IQR) ACR 3 (1-10) mg/mmol. During a median follow-up of 4.7 years, 449 (33%) patients developed an AKI. In univariate analysis, each biomarker was significantly associated with AKI, and 6 remained associated after multivariable adjustment. The addition of a multimarker score summing standardized and weighted values of these 6 markers to the model including usual risk factors significantly improved C-statistics (0.724 to 0.759, $P < 0.0001$), and 5-year risk-predictive performance (relative integrated discrimination improvement index=0.435, $P < 0.0001$).

Thus a panel of 6 biomarkers representing cardiac, vascular and inflammatory pathways improved the prediction of AKI over usual risk factors in patients with type 2 diabetes.

Accordingly, the present invention relates to a method of determining whether a patient suffering from type 2 diabetes is at risk of having acute kidney injury (AKI) comprising i) measuring the level of at least one biomarker representing cardiac, vascular or inflammatory pathways in a plasma sample obtained from the patient, ii) comparing the level measured at step i) with its corresponding predetermined reference value wherein detecting differential between the level measured at step i) and its corresponding predetermined reference value indicates whether the patient is or is not at risk of having acute kidney injury.

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/m^2 or a subject with at least one co-morbidity with a BMI greater than or equal to 27 kg/m^2 . A "subject at risk of obesity" is an otherwise healthy subject with a BMI of 25 kg/m^2 to less than 30 kg/m^2 or a subject with at least one co-morbidity with a BMI of 25 kg/m^2 to less than 27 kg/m^2 . 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/m^2 . 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/m^2 . In these countries, a "subject at risk of obesity" is a person with a BMI of greater than 23 kg/m^2 to less than 25 kg/m^2 .

As used herein, the term "acute kidney injury" or "acute kidney failure" is typically identified by a rapid deterioration in renal function sufficient to result in the accumulation of nitrogenous wastes in the body (see, e.g., Anderson and Schrier (1994), in Harrison's Principles of Internal Medicine, 13th edition, Isselbacher et al, eds., McGraw Hill Text, New York). Rates of increase in BUN of at least 4 to 8 mmol/L/day (10 to 20 mg/dL/day), and rates of increase of serum creatinine of at least 40 to 80 $\mu\text{mol/L/day}$ (0.5 to 1.0 mg/dL/day), are typical in acute renal failure.

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 AKI, 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 and 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.

According to the present invention, the biomarkers are selected from markers of cardiac and endothelial dysfunction (mid-regional-pro-adrenomedullin [MRproADM], angiopoietinlike-2 [ANGPTL2], N-terminal prohormone brain natriuretic peptide [NTproBNP]) oxidative stress (fluorescent advanced glycation endproducts [AGE], carbonyls), cardio-renal pathways (copeptin [CTproAVP]), and inflammation (soluble TNF receptor 1 [TNFR1]).

As used herein, the term “Mid-regional-pro-adrenomedullin” or “MR-proADM” has its general meaning in the art and refers to a fragment of adrenomedullin of unknown function and with high ex vivo stability (Struck et al. (2004), Peptides 25(8):1369-72). More particularly, mid-regional proANP comprises at least amino acid residues 53-90 of proadrenomedullin.

As used herein, the term “ANGPTL2” has its general meaning in the art and refers to the angiopoietin-related protein 2 also known as angiopoietin-like protein 2 is a protein that in humans is encoded by the ANGPTL2 gene.

As used herein, the term “NT-proBNP” has its general meaning in the art and relates to a polypeptide comprising, preferably, 76 amino acids in length corresponding to the N-terminal portion of the human NT-proBNP molecule. The structure of the human BNP and NT-proBNP has been described already in detail in the prior art, e.g., WO 02/089657, WO 02/083913, Bonow 1996, New Insights into the cardiac natriuretic peptides. Circulation 93: 1946-1950. Human NT-proBNP as disclosed in EP 0 648 228 B1 or under GeneBank accession number NP-002512.1; GI:4505433.

As used herein, the term “AGE” refers to the compound which it modifies as the reaction product of either an advanced glycosylation endproduct or a compound which forms AGEs and the compound so modified, such as the bovine serum albumin (BSA). Thus, AGEs include, but are not limited to, AGE-proteins (such as BSA-AGE), AGE-lipids, AGE-peptides, and AGE-DNA.

As used herein, the term “CTproAVP” has its general meaning in the art and refers to a 39-amino acid-long peptide derived from the C-terminus of pre-pro-hormone of arginine vasopressin, neurophysin II and copeptin. The term is also known as copeptin.

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.

In some embodiments, the level of 1, 2, 3, 4, 5, or 6 biomarkers is determined in the plasma sample. In some embodiments, the level of MR-proADM, sTNFR1 and NT-proBNP is determined in the plasma sample.

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).

Typically, the level of biomarkers are determined in the plasma sample by any method well known in the art and more preferably as described in the EXAMPLE.

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 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 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 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 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 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 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 level of the biomarker is higher than its corresponding predetermined reference value, it is concluded that the patient is a risk of having AKI. On the contrary, when the level of the biomarker is lower than its corresponding predetermined reference value, it is concluded that the patient is a risk of having AKI.

Once it is concluded that the patient is at risk of having AKI, treatment options may be prescribed. Typical treatment position include weight management, physical activity, smoking cessation and medications. Medicines for preventing typically includes Examples of drug suitable for the prevention of loss of renal function include but is not limited to inhibitors of the renin-angiotensin system (RAS), including angiotensin-converting enzyme (ACE) inhibitors,

angiotensin II receptor blockers (ARBs) or antidiabetic drugs such as insulin or Sodium-glucose co-transporter 2 (SGLT2) inhibitors among patients with diabetes.

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. 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 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 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 figures and examples. However, these examples and figures should not be interpreted in any way as limiting the scope of the present invention.

EXAMPLE:

Methods

Study patients

The SURDIAGENE study is a French single-center inception cohort of type 2 diabetes patients regularly visiting the diabetes department at Poitiers University Hospital, France [20]. Patients were consecutively enrolled from 2002 to 2012 and outcome updates were performed every 2 years since 2007. Since this is a referral population, some participants may be more complicated than those in the general diabetes population. The Poitiers University Hospital Ethics Committee approved the design (CPP Ouest III). All participants in the study gave their informed written consent.

At baseline, all patients were examined to collect relevant clinical and biological data. A history of cardiovascular disease at baseline was defined as a personal history of myocardial infarction, and/or stroke. Patients with a baseline eGFR <30 ml/min/1.73m² and/or prior renal replacement therapy were excluded from the present analysis.

Definition of outcomes

The primary outcome in the longitudinal analyses was AKI defined according to The Kidney Disease: Improving Global Outcomes (KDIGO) guidelines criteria [21]. Only serum creatinine criteria were used to diagnose and stage AKI, and, therefore, urinary output criteria were omitted. We considered the lowest creatinine value found between the dates of hospital admission and discharge as the reference creatinine value. We identified and classified AKI by comparing the highest creatinine value found during full hospitalization to the reference serum creatinine value. AKI was defined as an increase in serum creatinine by $\geq 0.3\text{mg/dL}$ ($\geq 26.5\text{ }\mu\text{mol/L}$) or ≥ 1.5 baseline versus the reference serum creatinine level. AKI was further classified by stage according to this ratio (stage 1 1.5–1.9%, stage 2 2.0–2.9%, stage 3 $\geq 300\%$). Stage 3 AKI was also defined by a serum creatinine increase of $\geq 4.0\text{ mg/dL}$ ($\geq 353.6\text{ mmol/L}$). For each patient, we considered only the first episode of AKI.

The vital status of all study participants was confirmed through December 31, 2015.

Biomarker selection process

Biomarker selection was based on existing evidence from the literature (manual literature review) and the availability of reliable validated assays to measure biomarker concentrations in small volumes of serum.

Assays

Blood samples and second morning urine samples were obtained in patients after an overnight fast. Serum and urine creatinine and urinary albumin were measured by colorimetry and immunoturbidimetry tests, respectively, on a COBAS System analyzer (Roche Diagnostics GmbH, Mannheim, Germany). Glomerular filtration rate was estimated using the Chronic Kidney Disease Epidemiology (2009 CKD-EPI) creatinine equation. Glycated hemoglobin was determined using a high-performance liquid chromatography method with a HA-8160 analyzer (Menarini, Florence, Italy).

Remaining samples were processed under standardized conditions and stored at -80°C in the Poitiers Biological Resource Center (BRC BB-0033-00068) undergoing only one prior freeze-thaw cycle prior to assay. The fluorescence intensity of AGEs and the levels of carbonyls, ANGPTL2, CTproAVP, MR-proADM and NT-proBNP were measured in stored plasma-EDTA samples while sTNFR1 was measured in stored serum. Detailed method of the assays were already published but briefly, AGEs were measured by spectrofluorometer, carbonyls[22], ANGPTL2[23] and sTNFR1 by ELISA, and CTproAVP, MR-proADM and NT-proBNP [24] by immunoassay. Concentrations below the limit of detection were replaced by the $\text{LOD}/\sqrt{2}$ [25].

Choice of covariates in the model

Clinical covariates included in the models for biomarker selection were selected based upon their inclusion in known associations AKI or CKD: age, sex, diabetes duration, HbA_{1c}, systolic blood pressure (SBP), use of antihypertensive, history of cardiovascular disease, eGFR, uACR...

Statistical analysis

Quantitative variables were expressed as means $\pm$ standard deviation (SD) or medians (25th-75th percentile) for skewed distributions; qualitative variables were presented as frequencies and percentages. Because of non-Gaussian distribution, concentrations of biomarkers were log-transformed. Spearman's correlations were used to assess the relationship of biomarkers with each other and with clinical variables. A complete case method was used to handle missing data. Thus, 2 subjects with at least one missing value for biomarkers were omitted in the present study. Patients with follow up less than 1 month were omitted, living 1342 participants included in the complete case study.

The hazard ratio (HR) of AKI for each biomarker measured at baseline was determined by using Cox proportional hazards regression. We tested each model for log-linearity and proportionality assumptions using Schoenfeld residuals. Results were given with HR and 95% confidence intervals and expressed for a 1-SD increase in the distribution of the logarithm of the biomarker concentration.

Two sets of models were used for individual biomarkers: univariate models (model 1), models adjusted for age, sex, diabetes duration, HbA_{1c}, systolic blood pressure (SBP), use of antihypertensive, history of cardiovascular disease, eGFR and uACR (model 2) as they represent established key markers associated with renal outcomes [26]. Interactions between sex and biomarkers for the association between biomarkers and AKI were evaluated by the addition of interaction terms into the corresponding regression model. The Akaike's information criterion (AIC) was used to compare global fit among models (nested or not nested), the model with the smallest AIC was considered as the best model. Comparisons of model adequacy were assessed using the likelihood ratio Chi2 tests.

Generalized c-statistics were calculated for model 2 accounting for variable follow-up times [27]. The relative integrated discrimination improvement (IDI) index was calculated to assess the improvement in 5-year risk prediction of each biomarker in addition to traditional risk factors (model 2) [28]. Five-year risk was selected because of its clinical relevance. The 95% CIs for the changes in the c-statistic and the relative IDI were computed based on 10,000 bootstrap samples. Areas under the curve of Five-year receiver-operating-characteristic (ROC)

curves were also generated for models with traditional risk factors (model 2) and traditional risk factors plus biomarkers.

To examine the combined effects of biomarkers we considered biomarkers significantly associated with AKI after model 2 adjustment to compute a weighted AKI biomarker risk score

(AKI-BRS). The AKI-BRS which derived from the following equation:

$$AKI\ BRS = \frac{k \sum_{k=1}^{k=n} \beta^k X^k}{\sum_{k=1}^{k=n} \beta^k}$$

where X^k are log of biomarker concentration and β^k are beta coefficients were derived from the model 2 Cox regression model and correspond to the log (HR) of the biomarker.

We also computed a score (RFL-BRS) for the combination of MR-proADM, NT-proBNP and sTNFR1 since we published its prognostic value for renal function loss in the SURDIAGENE cohort [24]. The time to event was plotted as Kaplan-Meier cumulative incidence curves according to quartiles of biomarkers and biomarker risk score, and comparison was made using the log-rank test.

We conducted a series of sensitivity analyses.

(1) We used the competing risk model of Fine and Gray to estimate the subdistribution hazard ratios for AKI, while accounting for the competing risk of all-cause deaths [29].

(2) In order to verify that intrahospital AKI was independent from hospitalization risk we tested for an interaction between number of all-cause hospital admissions over time and risk of AKI when considering first hospitalization as a time-dependent variable.

(3) Finally, we modeled the risk of first intrahospital AKI grade 1 and AKI grade2+ using the model 2 Cox regression model.

P values < 0.05 were considered statistically significant. Statistical analyses were performed with SAS version 9.4 (SAS Institute, Cary, NC).

Results

Baseline characteristics

The study population included 1,343 patients with available samples and follow-up data. The clinical and biological characteristics of the patients are presented in Table 1.

All the biomarkers did not have any significant correlation with PAS or HbA1c. However, all biomarkers showed a negative correlation with baseline eGFR (Rho = -0.63 to -0.06, all $P < 0.0001$, a positive correlation with ACR eGFR (Rho = -0.25 to -0.06, all $P < 0.005$). All biomarkers showed a positive correlation with age (Rho = -0.52 to -0.06, all $P < 0.005$) and all biomarkers - except AGEs and carbonyls - with diabetes duration (Rho = -0.32 to -0.08, all $P < 0.005$).

Compared to women, men had significantly higher concentrations of CTproAVP (7.4 [4.6-12.2] vs 4.8 [2.8-8.5] pmol/L; $P < 0.0001$) and significantly lower concentrations of MR-proADM (0.70 [0.56-0.90] nmol/L vs 0.75 [0.62-0.92] nmol/L, $P = 0.0008$). Men had no significance difference in AGEs (111294 [92585-130403] vs 108919 [92585-130403], $P = 0.449$), carbonyls (28 [25-30] vs 28 [26-31], $P = 0.959$), ANGPTL2 (14.5 [11.1-19.8] vs 15.2 [11.2-19.1], $P = 0.458$), NT-ProBNP (107 [45-286] pg/mL vs 99 [49-240] pg/mL, $P = 0.500$), and sTNFR1 (1,804 [1,520-2,257] pg/mL vs 1,822 [1,564-2,200] pg/mL, $P = 0.458$)

We observed no significant statistical interaction between sex and biomarkers for the association of biomarkers with AKI incidence so we reported findings for men and women together.

Biomarkers and acute kidney injury

Patients were followed for AKI for a median of 4.7 years (2.4-8.1 years), during which 451 cases of AKI occurred (incidence rate 65 per 1,000 person-years, 95% confidence interval, 59 to 71) and 361 deaths occurred (incidence rate 45 per 1,000 person-years, 95%CI 40 to 49) including 155 before AKI.

Each biomarker except carbonyls predicted AKI in univariate and multivariate models (Table 2). RFL-BRS and AKI-BRS scores were also associated with a significant increased risk of AKI: adjusted HR per 1-SD 2.24 (1.96-2.55); $P < 0.0001$ and 2.36 (2.06-2.71); $P < 0.0001$ for RFL-BRS and AKI-BRS, respectively.

Discrimination

We assessed goodness of fit and improvement in risk discrimination for each biomarker score compared with the model with traditional risk factors (model 2). The inclusion of AKI-RFL and RFL-BRS increased c-statistic of 0.032, $P < 0.0001$ and of 0.034, $P < 0.0001$, respectively; whereas the rIDI was 0.324, $P < 0.0001$ and 0.436, $P < 0.0001$, respectively. (Table 3).

Sensitivity analyses

After accounting for the competing risk of all-cause mortality in a Fine and Gray analysis, each BRS remained independently associated with an increased risk of AKI ; both $P < 0.0001$.

When considering first non-AKI hospitalization as a time-dependent variable in multiaadjusted model 2, we confirmed that each BRS remained independently associated with an increased risk of AKI both $P < 0.0001$.

A total 92 cases of AKI grade 2+ occurred during follow up (incidence rate 13 per 1,000 person-years, 95% confidence interval, 11 to 16). Modeling the risk of first intrahospital AKI

grade2+ using the model 2 Cox regression model retrieved similar findings for RFL-BRS and AKI-BRS (both $P < 0.0001$).

Conclusion:

In this study, we investigated 7 biomarkers in a hospital-based sample of 1,135 type 2 diabetes patients with normal to mildly impaired renal function ($\text{eGFR} > 30 \text{ ml/min/1.73m}^2$ and no history of RRT) who were followed for up to 11.8 years for AKI. In conclusion, we found, in a prospective cohort study, that beyond traditional risk factors, an increased circulating level of a combination of MR-proADM, sTNFR1 and NT-proBNP improves risk prediction of AKI as it was for RFL in a type 2 diabetes population.

TABLES

Table 1 – Clinical and biological characteristics in the SURDIAGENE study.

Variables	All n=1,343
Male	778 (58)
Age (years)	64 ± 11
Body mass index (kg/m^2)	31 ± 6
Active smoking	148 (11)
Known diabetes duration (years)	14 ± 10
History of cardiovascular disease	255 (19)
Systolic blood pressure (mmHg)	132 ± 17
Diastolic blood pressure (mmHg)	72 ± 11
Resting heart rate (beats per min)	71 ± 14
Therapeutics	
Any antihypertensive drug	1112 (83)
Diuretics	597 (44)
RAAS blockers	847 (63)
Beta blockers	231 (17)
Calcium antagonists	400 (30)
Insulin	790 (59)
OAD agents	893 (66)
Biological determinations	
HbA1c (%)	7.8 ± 1.6
HbA1c (mmol/mol)	62 ± 16.4
eGFR (ml/min/1.73m^2)	77 ± 21
uACR (mg/mmol)	2.6 (1.0-10.4)
Normoalbuminuria, microalbuminuria, macroalbuminuria*	544 (45)/429 (36)/226 (19)
Fluorescent AGE (AU)	110132 (91635-130196)
ELISA carbonyls (mmol/mg)	28 (26-31)
ANGPTL2 (ng/ml)	15 (11-20)
MR-proADM (nmol/L)	6 (4-11)
CT-proAVP (pmol/L)	0.7 (0.6-0.9)

NT-pro BNP (pg/mL)

103 (47-262)

sTNFR1 (pg/mL)

1816 (1544-2236)

Data are mean $\pm$ standard deviation, median (25th-75th percentile) or n (%)

History of cardiovascular disease was defined as history of stroke and/or myocardial infarction prior to baseline

AU, arbitrary unit; RAAS blocker, Renin Angiotensin aldosterone system blocker (Angiotensin receptor blocker and/or ACE inhibitor); OAD agent, oral antidiabetic agent; eGFR, estimated glomerular filtration rate by CKD EPI equation; uACR, urinary albumin-to-creatinine ratio; MR-proADM, Mid-regional-pro-adrenomedullin; NT-proBNP; sTNFR1, soluble Tumor Necrosis Factor receptor 1; N-terminal of the prohormone brain natriuretic peptide

*Normoalbuminuria was defined as uACR < 30mg/g, microalbuminuria as uACR 30–299 mg/g and macroalbuminuria as uACR $\geq$ 300 mg/g

Missing values: diabetes duration, 2; PAS/PAD, 7; HR, 6; HbA1c, 1; uACR, 4; smoking status, 18; albuminuria status, 145.

Table 2– Risk of acute kidney injury according to biomarkers in patients of the SURDIAGENE cohort

Variables	univariate		Adjusted	
Hazard ratio for AKI *				
Fluorescent AGE	1.30 (1.18-1.43)	<0.0001	1.12 (1.02-1.23)	0.024
ELISA carbonyls	1.09 (0.99-1.19)	0.072	1.02 (0.94-1.11)	0.590
ANGPTL2	1.61 (1.47-1.75)	<0.0001	1.29 (1.16-1.43)	<0.0001
CT-proAVP	1.62 (1.48-1.78)	<0.0001	1.33 (1.19-1.48)	<0.0001
MR-proADM	2.13 (1.95-2.33)	<0.0001	1.93 (1.71-2.18)	<0.0001
NT-pro BNP	1.86 (1.70-2.04)	<0.0001	1.52 (1.36-1.70)	<0.0001
sTNFR1	2.12 (1.93-2.33)	<0.0001	1.79 (1.58-2.03)	<0.0001
RFL-BRS	2.32	<0.0001	2.08 (1.83-2.35)	<0.0001
AKI-BRS	2.39	<0.0001	2.36 (2.06-2.71)	<0.0001

Ratios are presented for 1 SD increment with 95% confidence interval and P Value.

* Cox model, † Fine and Gray model (competing risk = all-cause death)

Adjustment for age, sex, diabetes duration, systolic blood pressure, hba1c, eGFR, UACR

MR-proADM, Mid-regional-pro-adrenomedullin; sTNFR1, soluble Tumor Necrosis Factor receptor 1; NT-proBNP; N-terminal prohormone brain natriuretic peptide

Table 3-C-statistics, relative integrated discrimination improvement index (rIDI) using individual biomarkers or their combination for the prediction of renal function loss ($\geq 40\%$ GFR drop) and of rapid renal function decline ($<5\text{ml/min/year}$)

Variables	AIC	Likelihood ratio P value	Difference in C-statistics	Likelihood ratio P value	Relative IDI (95% CI)	P value
Reference model	5687					
+ AKI-BRS	5555	<0.0001	0.032	<0.0001	0.324	<0.0001
+ RFL-BRS	5546	<0.0001	0.034	<0.0001	0.436	0.001

Reference Model = age, sex, diabetes duration, systolic blood pressure, hba1c, eGFR, UACR

C-statistics reference = 0.725

AIC, Akaike information criterion; Relative IDI, relative integrated discrimination improvement index; MR-proADM, Mid-regional-pro-adrenomedullin; sTNFR1, soluble Tumor Necrosis Factor receptor 1; NT-proBNP; N-terminal prohormone brain natriuretic peptide

REFERENCES:

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.

1. Lassalle M, Ayav C, Frimat L, Jacquelinet C, Couchoud C, au nom du registre R. The essential of 2012 results from the French Renal Epidemiology and Information Network (REIN) ESRD registry. *Nephrol Ther* 2015; 11:78-87.

2. Velho G, Ragot S, Mohammedi K, Gand E, Fraty M, Fumeron F, et al. Plasma Adrenomedullin and Allelic Variation in the ADM Gene and Kidney Disease in People With Type 2 Diabetes. *Diabetes* 2015; 64:3262-3272.

3. Landman GW, van Dijk PR, Drion I, van Hateren KJ, Struck J, Groenier KH, et al. Midregional fragment of proadrenomedullin, new-onset albuminuria, and cardiovascular and all-cause mortality in patients with type 2 diabetes (ZODIAC-30). *Diabetes care* 2014; 37:839-845.

4. Gomez-Banoy N, Cuevas V, Higueta A, Aranzalez LH, Mockus I. Soluble tumor necrosis factor receptor 1 is associated with diminished estimated glomerular filtration rate in colombian patients with type 2 diabetes. *J Diabetes Complications* 2016.

5. Ng DP, Fukushima M, Tai BC, Koh D, Leong H, Imura H, et al. Reduced GFR and albuminuria in Chinese type 2 diabetes mellitus patients are both independently associated with activation of the TNF-alpha system. *Diabetologia* 2008; 51:2318-2324.
6. Niewczas MA, Ficociello LH, Johnson AC, Walker W, Rosolowsky ET, Roshan
5 B, et al. Serum concentrations of markers of TNFalpha and Fas-mediated pathways and renal function in nonproteinuric patients with type 1 diabetes. *Clin J Am Soc Nephrol* 2009; 4:62-70.
7. Carlsson AC, Ostgren CJ, Nystrom FH, Lanne T, Jennersjo P, Larsson A, et al. Association of soluble tumor necrosis factor receptors 1 and 2 with nephropathy, cardiovascular events, and total mortality in type 2 diabetes. *Cardiovasc Diabetol* 2016; 15:40.
- 10 8. Forsblom C, Moran J, Harjutsalo V, Loughman T, Waden J, Tolonen N, et al. Added value of soluble tumor necrosis factor-alpha receptor 1 as a biomarker of ESRD risk in patients with type 1 diabetes. *Diabetes care* 2014; 37:2334-2342.
9. Lopes-Virella MF, Baker NL, Hunt KJ, Cleary PA, Klein R, Virella G. Baseline markers of inflammation are associated with progression to macroalbuminuria in type 1 diabetic
15 subjects. *Diabetes care* 2013; 36:2317-2323.
10. Niewczas MA, Gohda T, Skupien J, Smiles AM, Walker WH, Rosetti F, et al. Circulating TNF receptors 1 and 2 predict ESRD in type 2 diabetes. *J Am Soc Nephrol* 2012; 23:507-515.
11. Saulnier PJ, Gand E, Ragot S, Ducrocq G, Halimi JM, Hulin-Delmotte C, et al.
20 Association of serum concentration of TNFR1 with all-cause mortality in patients with type 2 diabetes and chronic kidney disease: follow-up of the SURDIAGENE Cohort. *Diabetes care* 2014; 37:1425-1431.
12. Pavkov ME, Nelson RG, Knowler WC, Cheng Y, Krolewski AS, Niewczas MA. Elevation of circulating TNF receptors 1 and 2 increases the risk of end-stage renal disease in
25 American Indians with type 2 diabetes. *Kidney Int* 2015; 87:812-819.
13. Miyazawa I, Araki S, Obata T, Yoshizaki T, Morino K, Kadota A, et al. Association between serum soluble TNFalpha receptors and renal dysfunction in type 2 diabetic patients without proteinuria. *Diabetes Res Clin Pract* 2011; 92:174-180.
14. Skupien J, Warram JH, Niewczas MA, Gohda T, Malecki M, Mychaleckyj JC,
30 et al. Synergism between circulating tumor necrosis factor receptor 2 and HbA1c in determining renal decline during 5-18 years of follow-up in patients with type 1 diabetes and proteinuria. *Diabetes care* 2014; 37:2601-2608.

15. Pavkov ME, Weil EJ, Fufaa GD, Nelson RG, Lemley KV, Knowler WC, et al. Tumor necrosis factor receptors 1 and 2 are associated with early glomerular lesions in type 2 diabetes. *Kidney Int* 2015.
16. Bansal N, Katz R, Dalrymple L, de Boer I, DeFilippi C, Kestenbaum B, et al.
5 NT-proBNP and troponin T and risk of rapid kidney function decline and incident CKD in elderly adults. *Clin J Am Soc Nephrol* 2015; 10:205-214.
17. Kim Y, Matsushita K, Sang Y, Grams ME, Skali H, Shah AM, et al. Association of high-sensitivity cardiac troponin T and natriuretic peptide with incident ESRD: the Atherosclerosis Risk in Communities (ARIC) study. *Am J Kidney Dis* 2015; 65:550-558.
- 10 18. Desai AS, Toto R, Jarolim P, Uno H, Eckardt KU, Kewalramani R, et al. Association between cardiac biomarkers and the development of ESRD in patients with type 2 diabetes mellitus, anemia, and CKD. *Am J Kidney Dis* 2011; 58:717-728.
19. Looker HC, Colombo M, Hess S, Brosnan MJ, Farran B, Dalton RN, et al. Biomarkers of rapid chronic kidney disease progression in type 2 diabetes. *Kidney Int* 2015.
- 15 20. Hadjadj S, Fumeron F, Roussel R, Saulnier PJ, Gallois Y, Ankotche A, et al. Prognostic value of the insertion/deletion polymorphism of the ACE gene in type 2 diabetic subjects: results from the Non-insulin-dependent Diabetes, Hypertension, Microalbuminuria or Proteinuria, Cardiovascular Events, and Ramipril (DIABHYCAR), Diabete de type 2, Nephropathie et Genetique (DIAB2NEPHROGENE), and Survie, Diabete de type 2 et
20 Genetique (SURDIAGENE) studies. *Diabetes care* 2008; 31:1847-1852.
21. Kellum JA, Lameire N, Group KAGW. Diagnosis, evaluation, and management of acute kidney injury: a KDIGO summary (Part 1). *Crit Care* 2013; 17:204.
22. Cournot M, Burillo E, Saulnier PJ, Planesse C, Gand E, Rehman M, et al. Circulating Concentrations of Redox Biomarkers Do Not Improve the Prediction of Adverse
25 Cardiovascular Events in Patients With Type 2 Diabetes Mellitus. *J Am Heart Assoc* 2018; 7.
23. Gellen B, Thorin-Trescases N, Sosner P, Gand E, Saulnier PJ, Ragot S, et al. ANGPTL2 is associated with an increased risk of cardiovascular events and death in diabetic patients. *Diabetologia* 2016; 59:2321-2330.
24. Saulnier PJ, Gand E, Velho G, Mohammedi K, Zaoui P, Fraty M, et al.
30 Association of Circulating Biomarkers (Adrenomedullin, TNFR1, and NT-proBNP) With Renal Function Decline in Patients With Type 2 Diabetes: A French Prospective Cohort. *Diabetes care* 2017; 40:367-374.
25. Hornung RW, Reed LD. Estimation of Average Concentration in the Presence of Nondetectable Values. *Applied Occupational and Environmental Hygiene* 1990; 5:46-51.

26. Ninomiya T, Perkovic V, de Galan BE, Zoungas S, Pillai A, Jardine M, et al. Albuminuria and kidney function independently predict cardiovascular and renal outcomes in diabetes. *J Am Soc Nephrol* 2009; 20:1813-1821.

5 27. Pencina MJ, D'Agostino RB. Overall C as a measure of discrimination in survival analysis: model specific population value and confidence interval estimation. *Statistics in Medicine* 2004; 23:2109-2123.

28. Pencina MJ, D'Agostino RB, Sr., D'Agostino RB, Jr., Vasan RS. Evaluating the added predictive ability of a new marker: from area under the ROC curve to reclassification and beyond. *Stat Med* 2008; 27:157-172; discussion 207-112.

10 29. Fine JP, Gray RJ. A proportional hazards model for the subdistribution of a competing risk. *J Am Stat Assoc* 1999; 94:496-509.

CLAIMS:

1. A method of determining whether a patient suffering from type 2 diabetes is at risk of having acute kidney injury (AKI) comprising i) measuring the level of at least one biomarker representing cardiac, vascular or inflammatory pathways in a plasma sample
5 obtained from the patient, ii) comparing the level measured at step i) with its corresponding predetermined reference value wherein detecting differential between the level measured at step i) and its corresponding predetermined reference value indicates whether the patient is or is not at risk of having acute kidney injury.
- 10 2. The method of claim 1 wherein the biomarker is selected from markers of cardiac and endothelial dysfunction (mid-regional-pro-adrenomedullin [MRproADM], angiotensin-like-2 [ANGPTL2], N-terminal prohormone brain natriuretic peptide [NTproBNP]) oxidative stress (fluorescent advanced glycation endproducts [AGE], carbonyls), cardio-renal pathways (copeptin [CTproAVP]), and inflammation (soluble TNF receptor 1 [TNFR1]).
- 15 3. The method of claim 1 wherein level of 1, 2, 3, 4, 5, or 6 biomarkers is determined in the plasma sample.
4. The method of claim 1 wherein the level of MR-proADM, sTNFR1 and NT-proBNP is determined in the plasma sample.

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2019/076155

A. 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	WO 2018/069487 A1 (INSERM INSTITUT NATIONAL DE LA SANTE ET DE LA RECH MEDICALE [FR]; CENT) 19 April 2018 (2018-04-19) see claims 1-2, 6 and pages 2-8 -----	1-4
Y	WO 2014/197729 A1 (ASTUTE MEDICAL INC [US]) 11 December 2014 (2014-12-11) see claims 1-2, pages 6-11, 14-20 ----- -/-	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

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

17 October 2019

Date of mailing of the international search report

28/10/2019

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2
 NL - 2280 HV Rijswijk
 Tel. (+31-70) 340-2040,
 Fax: (+31-70) 340-3016

Authorized officer

Vix, Olivier

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2019/076155

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	GELLEN BARNABAS ET AL: "ANGPTL2 is associated with an increased risk of cardiovascular events and death in diabetic patients", DIABETOLOGIA, SPRINGER, BERLIN, DE, vol. 59, no. 11, 4 August 2016 (2016-08-04), pages 2321-2330, XP036291240, ISSN: 0012-186X, DOI: 10.1007/S00125-016-4066-5 [retrieved on 2016-08-04] see abstract and pages 2322-2325 -----	1-4
Y	AXEL C. CARLSSON ET AL: "Association of soluble tumor necrosis factor receptors 1 and 2 with nephropathy, cardiovascular events, and total mortality in type 2 diabetes", CARDIOVASCULAR DIABETOLOGY, vol. 15, no. 1, 29 February 2016 (2016-02-29), XP055342896, DOI: 10.1186/s12933-016-0359-8 see abstract and pages 2-5 -----	1-4
Y	N. BANSAL ET AL: "NT-ProBNP and Troponin T and Risk of Rapid Kidney Function Decline and Incident CKD in Elderly Adults", CLINICAL JOURNAL OF THE AMERICAN SOCIETY OF NEPHROLOGY, vol. 10, no. 2, 20 January 2015 (2015-01-20), pages 205-214, XP055342895, ISSN: 1555-9041, DOI: 10.2215/CJN.04910514 see abstract and pages 205-208 -----	1-4
Y	GILBERTO VELHO ET AL: "Plasma Adrenomedullin and Allelic Variation in the ADM Gene and Kidney Disease in People With Type 2 Diabetes", DIABETES, vol. 64, no. 9, 6 May 2015 (2015-05-06), pages 3262-3272, XP055342830, US ISSN: 0012-1797, DOI: 10.2337/db14-1852 see abstract and pages 3263-3266 -----	1-4
Y	WO 2010/068686 A2 (JOSLIN DIABETES CENTER INC [US]; NIEWCZAS MONIKA A [US]; KROLEWSKI AND) 17 June 2010 (2010-06-17) see claims 1-9 and pages 4-11 ----- -/--	1-4

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2019/076155

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2014/096110 A1 (NOVARTIS AG [CH]; DIETERLE FRANK [CH]; HOEFLING HOLGER [CH]) 26 June 2014 (2014-06-26) see claims 1-9 and pages 6-15 -----	1-4
Y	WO 2012/146645 A1 (ROCHE DIAGNOSTICS GMBH [DE]; HOFFMANN LA ROCHE [CH]; HESS GEORG [DE];) 1 November 2012 (2012-11-01) see claims -----	1
Y	SIMONA POZZOLI ET AL: "Predicting acute kidney injury: current status and future challenges", JOURNAL OF NEPHROLOGY : JN, vol. 31, no. 2, 17 April 2018 (2018-04-17) , pages 209-223, XP055535728, IT ISSN: 1121-8428, DOI: 10.1007/s40620-017-0416-8 the whole document -----	1-4
Y	Karthikeyan Kandasamy ET AL: "Prediction of drug-induced nephrotoxicity and injury mechanisms with human induced pluripotent stem cell-derived cells and machine learning methods", Scientific reports, 27 July 2015 (2015-07-27), page 12337, XP055535702, England DOI: 10.1038/srep12337 Retrieved from the Internet: URL:https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6080076/pdf/10.1177_2054358118776326.pdf the whole document -----	1-4
A	M. E. HELLEMONS ET AL: "Validity of biomarkers predicting onset or progression of nephropathy in patients with Type2 diabetes: a systematic review", DIABETIC MEDICINE., vol. 29, no. 5, 16 April 2012 (2012-04-16) , pages 567-577, XP055342826, GB ISSN: 0742-3071, DOI: 10.1111/j.1464-5491.2011.03437.x the whole document -----	1-4
A	WO 2010/025434 A1 (ASTUTE MEDICAL INC [US]; ANDERBERG JOSEPH [US]; GRAY JEFF [US]; MCPHER) 4 March 2010 (2010-03-04) the whole document ----- -/--	1-4

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2019/076155

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2009/116023 A1 (BIOTRIN INTELLECTUAL PTY LTD [IE]; KILTY CORMAC GERARD [IE]; KOYNER JA) 24 September 2009 (2009-09-24) the whole document	1-4
A	----- H. R. H. DE GEUS ET AL: "Biomarkers for the prediction of acute kidney injury: a narrative review on current status and future challenges", CLINICAL KIDNEY JOURNAL, vol. 5, no. 2, 26 March 2012 (2012-03-26), pages 102-108, XP055535699, ISSN: 2048-8505, DOI: 10.1093/ckj/sfs008 the whole document -----	1-4

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2019/076155

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2018069487	A1	19-04-2018	NONE
WO 2014197729	A1	11-12-2014	CN 105556305 A 04-05-2016 EP 3004873 A1 13-04-2016 HK 1222455 A1 30-06-2017 US 2016146832 A1 26-05-2016 WO 2014197729 A1 11-12-2014
WO 2010068686	A2	17-06-2010	DK 2406636 T3 18-02-2019 EP 2406636 A2 18-01-2012 EP 3474016 A1 24-04-2019 ES 2706403 T3 28-03-2019 US 2011281758 A1 17-11-2011 US 2018011109 A1 11-01-2018 WO 2010068686 A2 17-06-2010
WO 2014096110	A1	26-06-2014	AR 094118 A1 08-07-2015 AU 2013360685 A1 02-07-2015 AU 2017232081 A1 12-10-2017 BR 112015014232 A2 11-07-2017 CA 2895096 A1 26-06-2014 CL 2015001768 A1 09-10-2015 CN 104871004 A 26-08-2015 EP 2936160 A1 28-10-2015 HK 1209838 A1 08-04-2016 JP 6416778 B2 31-10-2018 JP 2016503162 A 01-02-2016 JP 2019053067 A 04-04-2019 KR 20150096728 A 25-08-2015 MA 38171 A1 30-11-2016 PH 12015501399 A1 07-09-2015 RU 2015129496 A 26-01-2017 SG 11201504329R A 30-07-2015 TN 2015000263 A1 03-10-2016 TW 201430347 A 01-08-2014 US 2015309052 A1 29-10-2015 WO 2014096110 A1 26-06-2014
WO 2012146645	A1	01-11-2012	NONE
WO 2010025434	A1	04-03-2010	CA 2735590 A1 04-03-2010 CN 102187219 A 14-09-2011 CN 105021826 A 04-11-2015 EP 2324354 A1 25-05-2011 EP 2813848 A2 17-12-2014 HK 1153267 A1 06-03-2015 HK 1212776 A1 17-06-2016 JP 5947544 B2 06-07-2016 JP 2012501457 A 19-01-2012 JP 2016053587 A 14-04-2016 US 2011174062 A1 21-07-2011 US 2015285824 A1 08-10-2015 WO 2010025434 A1 04-03-2010
WO 2009116023	A1	24-09-2009	EP 2255206 A1 01-12-2010 US 2009239242 A1 24-09-2009 US 2011136138 A1 09-06-2011 US 2013236908 A1 12-09-2013

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2019/076155

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
W0 2009116023 A1 24-09-2009			