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(54) Title: METHODS AND KITS FOR PREDICTING THE RISK OF LOSS OF RENAL FUNCTION IN PATIENTS WITH TYPE
2 DIABETES

(57) Abstract: The present invention relates to methods and kits for predicting the risk of loss of renal function in patients with type 2 di-
abetes. The inventors explored the prognostic value of 3 circulating candidate biomarkers in patients: mid-regional-pro-adrenomedullin
(MR- proADM), soluble tumor necrosis factor receptor 1 (sTNFR1), and N-terminal prohormone brain natriuretic peptide (NT-proB-
NP) for change in renal function in patients with type 2 diabetes. The combination of all 3 biomarkers yielded the highest discrimination.
In particular the present invention relates to a method of predicting the risk of loss of renal function in a patient with type 2 diabetes
comprising determining the levels of MR-proADM, sTNFR1 and NT-proBNP in a blood sample from the patient.

METHODS AND KITS FOR PREDICTING THE RISK OF LOSS OF RENAL FUNCTION IN PATIENTS WITH TYPE 2 DIABETES

FIELD OF THE INVENTION:

The present invention relates to methods and kits for predicting the risk of loss of renal function in patients with type 2 diabetes.

BACKGROUND OF THE INVENTION:

Diabetes mellitus is the leading cause of end-stage renal disease (ESRD) in the United States, Europe and worldwide (1). Recent data have confirmed that patients with diabetes have approximately double the risk of ESRD compared with individuals without diabetes (2). However only 20-40 % of patients with type 2 diabetes will develop diabetic kidney disease (DKD) in their lifespan (3).

Estimated glomerular filtration rate (eGFR) and albuminuria are used to stage chronic kidney disease. Nevertheless, these markers have some limitations, since eGFR may decline in the absence of elevated albuminuria (4) and the structural lesions of DKD may be present in normoalbuminuric patients, including those with normal eGFR (5). Identifying these patients with more sensitive and specific predictive markers of DKD could lead to treatment before irreversible structural injuries occur. This approach could also contribute to more efficient use of medical resources by targeting the patients who could benefit the most from therapeutic intervention.

Mid-regional-pro-adrenomedullin (MR-proADM) concentrations are associated with the doubling of serum creatinine and progression to ESRD (6), and with mortality (7) in type 2 diabetes patients. Circulating soluble tumor necrosis factor receptors (sTNFR) is associated in numerous epidemiological studies cross-sectionally with GFR and DKD (8-10) and prospectively with DKD progression and ESRD occurrence (11-15) in both type 1 diabetes and type 2 diabetes patients. Moreover, sTNFR2 has been associated with GFR variation in type 2 diabetes patients (16) as well as in type 1 diabetes patients (17). sTNFR2 and TNFR1 have also been associated with DKD structural lesions and especially with early glomerular lesions in type 2 diabetes (18). However, even if TNFR2 and TNFR1 show partially overlapping biological effects, we only had access to sTNFR1 assay for the present study. N-terminal prohormone brain natriuretic peptide (NT-proBNP) has been reported to be associated with rapid kidney decline in elderly adults (19) and with ESRD in the general population (20). A

post-hoc analysis of a clinical trial also reported an association of NT-proBNP with ESRD in type 2 diabetes patients (21). Interestingly, all 3 of these biomarkers have been associated with rapid progression of eGFR in type 2 diabetes in the final step of a recent biomarker-panel approach (22).

SUMMARY OF THE INVENTION:

The present invention relates to methods and kits for predicting the risk of loss of renal function in patients with type 2 diabetes. In particular, the present invention is defined by the claims.

DETAILED DESCRIPTION OF THE INVENTION:

The inventors explored the prognostic value of 3 circulating candidate biomarkers (mid-regional-pro-adrenomedullin [MR-proADM], soluble tumor necrosis factor receptor 1 [sTNFR1], and N-terminal prohormone brain natriuretic peptide [NT-proBNP]) for change in renal function in patients with type 2 diabetes. Each biomarker predicted RFL and RRFD. When combined MR-proADM, sTNFR1 and NT-proBNP predicted RFL independently from the established risk factors (Adjusted HR 1.59 [95%CI 1.34-1.89]; $P<0.0001$; 1.33 [1.14-1.55]; $P=0.0003$ and 1.22 [1.07-1.40]; $P=0.004$, respectively) and RRFD (Adjusted OR 1.56 [95%CI 1.7-2.09]; $P=0.003$; 1.72 [1.33-2.22]; $P<0.0001$ and 1.28 [1.03-1.59]; $P=0.02$, respectively). The combination of all 3 biomarkers yielded the highest discrimination (difference in c-statistic=0.054, $P<0.0001$; 0.067, $P<0.0001$ and 0.027, $P<0.0001$; for RFL and RRFD, respectively). In conclusion, in addition to established risk factors, MR-proADM, sTNFR1 and NT-proBNP improve risk prediction of loss of renal function in patients with type 2 diabetes.

Accordingly the first object of the present invention relates to a method of predicting the risk of loss of renal function in a patient with type 2 diabetes comprising determining the levels of MR-proADM, sTNFR1 and NT-proBNP in a blood sample obtained from the patient.

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 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.

As used herein, the expression "loss of renal function" or "RFL" indicates a decline of estimated glomerular filtration rate (eGFR) from baseline superior to 40%, rapid renal function decline (RRFD), and absolute annual eGFR slope < -5ml/min/year.

As used herein, the term "Risk" in the context of the present invention, relates to the probability that an event will occur over a specific time period, as in the conversion to a loss of renal function, 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.

As used herein, the term "blood sample" refers to a whole blood sample, serum sample and plasma sample. A blood sample may be obtained by methods known in the art including venipuncture or a finger stick. Serum and plasma samples may be obtained by centrifugation methods known in the art. The sample may be diluted with a suitable buffer before conducting the assay.

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.

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 "sTNFR1" 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.

The measurement of the level of a biomarker (e.g. MR-proADM, NT-proBNP or sTNFR1) 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. MR-proADM, NT-proBNP or sTNFR1) 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. MR-proADM, NT-proBNP or sTNFR1). A sample containing or suspected of containing

the biomarker (e.g. MR-proADM, NT-proBNP or sTNFR1) 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. MR-proADM, NT-proBNP or sTNFR1) (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. MR-proADM, NT-proBNP or sTNFR1) 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. MR-proADM, NT-proBNP or sTNFR1) (Cayman Chemical Company, Ann Arbor, Michigan). According to said embodiment, said means for measuring a biomarker (e.g. MR-proADM, NT-proBNP or sTNFR1) level are for example i) the biomarker (e.g. MR-proADM, NT-proBNP or sTNFR1) buffer, ii) a monoclonal antibody that interacts specifically with the biomarker (e.g. MR-proADM, NT-proBNP or sTNFR1), iii) an enzyme-conjugated antibody specific for the biomarker (e.g. MR-proADM, NT-proBNP or sTNFR1) and a predetermined reference value of the biomarker (e.g. MR-proADM, NT-proBNP or sTNFR1).

In some embodiments, a score which is a composite of the levels of MR-proADM, sTNFR1 and NT-proBNP is calculated and compared to a predetermined reference value wherein when the score is higher to its predetermined reference value, it is concluded that the patient is at risk of having loss or renal function.

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 (e.g. MR-proADM, NT-proBNP or sTNFR1) 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. MR-proADM, NT-proBNP or sTNFR1) 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.

In some embodiments, the score is calculated according to the formula:

score = $3 \times (\beta_1 \times \log[\text{MR-proADM}] + \beta_2 \times \log[\text{sTNFR1}] + \beta_3 \times \log[\text{NT-proBNP}]) / (\beta_1 + \beta_2 + \beta_3)$ wherein beta coefficients were derived from the model 3 multivariable Cox regression model and correspond to the log (HR) of the biomarker ($\beta_1 = 4.892$; $\beta_2 = 4.279$; $\beta_3 = 0.7470$).

When it is determined that the patient is at risk of having loss of renal function, the patient is subsequently administered with a therapeutically effective amount of a drug suitable for the treatment and prevention of loss of renal function. 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 a binding partner specific for each biomarker (i.e. MR-proADM, sTNFR1 and NT-proBNP). In some embodiments, 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.

A further object of the present invention relates to a system for performing the method of the present invention comprising a computer system configured to i) receive a data set for the plurality of biomarkers (i.e. MR-proADM, sTNFR1 and NT-proBNP), ii) calculate the composite score of the present invention and iii) generate the risk status. The software required for receiving, processing, and analyzing biomarker information may be implemented in a single device or implemented in a plurality of devices. The software may be accessible via a network such that storage and processing of information takes place remotely with respect to users. The biomarker analysis system according to various aspects of the present invention and its various

elements provide functions and operations to facilitate biomarker analysis, such as data gathering, processing, analysis, reporting and/or diagnosis. The present biomarker analysis system maintains information relating to biomarkers and facilitates the analysis and/or diagnosis. For example, in the present embodiment, the computer system executes the computer program, which may receive, store, search, analyze, and report information relating to biomarkers. The computer program may comprise multiple modules performing various functions or operations, such as a processing module for processing raw data and generating supplemental data and an analysis module for analyzing raw data and supplemental data to generate a disease status model and/or diagnosis information.

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.

FIGURES:

Figure 1–Cumulative percentage of renal function loss ($\geq 40\%$ GFR drop) according to biomarker quartiles

Panel A for MR-proADM, panel B for sTNFR1 and panel C for NT-proBNP.

Solid line, first quartile (Q1); dashed line, second quartile (Q2), dotted line, third quartile (Q3); short dashed/dotted line, fourth quartile (Q4).

P-values were calculated according to log-rank test.

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

Figure 2 - Receiver operator characteristic (ROC) curves for 5-year Renal Function Loss (panel A) and Rapid Renal Function Decline (panel B). Area under the curve ROC are computed for prediction of risk with the use of traditional risk factors without biomarkers (reference model) and traditional risk factor with biomarkers. Reference model = age, sex, diabetes duration, systolic blood pressure, HbA1c, eGFR and uACR

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 (24). 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 renal function decline (RFL) defined by a decline in eGFR during follow-up of $\geq 40\%$ compared with the baseline value. This endpoint was recently recommended as an alternative endpoint for chronic kidney disease (CKD) progression (25). The secondary endpoint was rapid renal function decline (RRFD), defined by an eGFR annual slope ≤ -5 ml/min/1.73m² per year, according to The Kidney Disease: Improving Global Outcomes (KDIGO) guidelines. Finally, we analyzed determinants of annual eGFR trajectories. The vital status of all study participants was confirmed through December 31, 2013.

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 analyser (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 concentrations of MR-proADM and NT-proBNP were measured in stored plasma-EDTA samples while sTNFR1 was measured in stored serum.

MR-proADM concentration was measured using a commercially available automated immunofluorescent sandwich immunoassay (BRAHMS MR-proADM, BRAHMS GmbH, Hennigsdorf, Germany). The limit of detection (LOD) was assessed as 0.05 nmol/L, intra-assay coefficient of variations (CV) was 3.5-10% and the inter-assay CV was $\leq 20\%$ for 0.2 to 0.5 nmol/L concentrations and $\leq 11\%$ for 0.5 to 6 nmol/L concentrations. Serum sTNFR1 concentrations were measured using Human sTNFR1 ELISA (EKF Diagnostics, Product #BIO94TNFR1, Dublin, Ireland) according to the manufacturers' instructions. The LOD was

1.7 pg/mL, the intra-assay CV 1.8-5.3 % and inter-assay CV 3.6-6.8 %. NT-proBNP plasmatic concentration was measured in a COBAS system (Roche Diagnostics GmbH, Mannheim, Germany) by an automated electrochemiluminescence immunoassay. According to the manufacturer's information, the LOD was 5 ng/l, the intra-assay CV 1.2-1.9% and the inter-assay CV 1.7-3.1%.

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.

The hazard ratio (HR) of RFL 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.

To determine whether patients had achieved RRFD, absolute eGFR slopes were individually determined by linear regression in patients with at least 3 eGFR determinations and 6 months of follow-up between the first and last eGFR. The odds ratio (OR) of RRFD was determined by logistic regression. Three sets of models were used for individual biomarkers: univariate models (model 1), models adjusted for age, sex, diabetes duration, HbA_{1c}, and systolic blood pressure (SBP) (model 2) and models adjusted for the same variables as model 2 plus eGFR and uACR (model 3) as they represent established key markers associated with renal outcomes (26). The outcome risk associated with the biomarker (HR or OR) was expressed for a 1-SD increase in the distribution of the logarithm of the biomarker concentration. Interactions between sex or antidiabetic drugs and biomarkers for the association between biomarkers and RFL or RRFD were evaluated by the addition of interaction terms into the corresponding regression model. Generalized c-statistics were calculated for model 3 accounting for variable follow-up times (27). Comparisons of model adequacy were assessed using the likelihood ratio Chi2 tests. 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 (age, sex, diabetes duration, HbA_{1c}, SBP, eGFR and uACR) (28) (27). Five-year risk was selected because it approximates the median follow-up time for RFL or death. The 95% CIs for the changes in the c-statistic and the relative IDI were computed based on 10,000 bootstrap samples. Receiver-

operating-characteristic (ROC) curves were also generated for models with traditional risk factors (age, sex, diabetes duration, HbA_{1c}, SBP, eGFR and uACR) and traditional risk factors plus biomarkers.

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. MR-proADM, sTNFR1 and NT-proBNP were used to compute a weighted biomarker risk score which was derived by the following equation:

$$\text{score} = \frac{3 \times (\beta_1 \times \log[\text{MR-proADM}] + \beta_2 \times \log[\text{sTNFR1}] + \beta_3 \times \log[\text{NT-proBNP}])}{(\beta_1 + \beta_2 + \beta_3)}.$$

Beta coefficients were derived from the model 3 Cox regression model and correspond to the log (HR) of the biomarker ($\beta_1 = 4.892$; $\beta_2 = 4.279$; $\beta_3 = 0.7470$).

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 also used a linear mixed-effects model to take repeated longitudinal eGFR data into account to test the association between biomarkers and the global pattern of absolute annual eGFR decline (29). The random errors of the linear mixed-effects analyses were defined as a random intercept and slope, assuming that variability between individuals was not identical at baseline and during follow-up. The fixed effects coefficients were presented with their standard error. The validity of the model was demonstrated by the normal distribution of the marginal residuals.

We conducted a series of sensitivity analyses. (1) First, we assessed RFL risk in different subgroup by stratifying the study sample by age (<75 vs ≥ 75 years), sex, diabetes duration (<12.5 vs ≥ 12.5 years), HbA_{1c} (<7% vs $\geq 7\%$), uACR (<30 vs ≥ 30 mg/mmol), diuretic use, SBP (<140 vs ≥ 140 mmHg) and history of cardiovascular disease. To examine whether the associations between biomarkers and renal outcome were independent of cardiovascular history, we successively (2) excluded from the analyses patients with prevalent cardiovascular history (defined as prior myocardial infarction or stroke) at baseline (3) and then patients with incident major cardiovascular event (defined as cardiovascular death or non-fatal myocardial or non-fatal stroke) (4) Additionally, among patients without prevalent cardiovascular history, we examined data by treating non-fatal cardiovascular outcomes that occurred during follow-up as time-varying covariates. (5) To account for individual changes in HbA_{1c} over time, we used a time-dependent Cox regression analyses, including yearly mean of HbA_{1c} as a time-dependent variables. Finally, (6) we then used the competing risk model of Fine and Gray to

estimate the subdistribution hazard ratios for RFL, while accounting for the competing risk of all-cause deaths (30)

P values < 0.05 were considered statistically significant. Statistical analyses were performed with SAS version 9.3 (SAS Institute, Cary, NC).

Results:

Baseline characteristics

The study population included 1,135 patients with available samples and follow-up data. The clinical and biological characteristics of the patients are presented in Table 1. Among the participants, 61 (5%) had a history of stroke at baseline, 171 (15%) a history of myocardial infarction, and 16 (1.4%) had both. We found a significant correlation between baseline eGFR and all 3 biomarkers (Rho = -0.61 to -0.30, all $P < 0.0001$; MR-proADM, NT-proBNP and sTNFR1 were significantly inter-correlated (Rho = 0.39 to 0.72; all $P < 0.0001$). Compared to women, men had significantly lower median concentrations of MR-proADM (0.7 [0.6-0.9] nmol/L vs 0.8[0.6-0.9] nmol/L, $P = 0.0004$), and similar concentrations of sTNFR1 (1,796 [1,524-2,226] pg/mL vs 1,840 [1,566-2,233] pg/mL, $P = 0.22$) and NT-ProBNP (103 [44-276] pg/mL vs 100 [49-252] pg/mL, $P = 0.69$). We observed no significant statistical interaction between sex or antidiabetic drugs and biomarkers for the association of biomarkers with outcomes.

Biomarkers and renal function loss (i.e. $\geq 40\%$ eGFR drop)

Patients were followed for RFL for a median of 4.3 years (2.4–7.3 years), during which 397 cases of RFL occurred (incidence rate 73 per 1,000 person-years, 95% confidence interval, 66 to 80) and 292 deaths occurred (incidence rate 40 per 1,000 person-years, 95%CI 35 to 44). The cumulative incidence of RFL across quartiles of biomarkers and biomarker risk score is shown in Figure 1. All 3 biomarkers predicted RFL independently in univariate and multivariate models (Table 2). Additionally when considering the combination of the 3 biomarkers in the adjusted model 3, MR-proADM, and NT-proBNP remained independently associated with an increase of RFL risk: adjusted HR per 1-SD 1.59 (95%CI 1.34, 1.89); $P < 0.0001$; 1.33, (1.14, 1.55); $P = 0.0003$ and 1.22 (1.07, 1.40); $P = 0.004$, respectively. This analysis was repeated with diabetes duration and ACR categorized (diabetes duration at its median ≤ 12.5 years or > 12.5 years) and uACR < 30 mg/mmol or ≥ 30 mg/mmol) after including patients with a baseline eGFR < 30 ml/min without requirement of a renal replacement therapy and the findings were unchanged.

Biomarkers and rapid renal function decline (i.e. annualized GFR < -5 ml/min/1.73m²/year.)

The prevalence of RRFD was 20% ($n = 233$) in the 1,109 patients with at least 3 determinations and 6 months between first and last eGFR. In models 1 to 3, all 3 biomarkers were individually associated with the risk of RRFD (Table 2). As in the prospective approach for RFL, MR-proADM, sTNFR1 and NT-proBNP remained independently associated with increased risk of RRFD, when considering all 3 biomarkers with model 3 covariates: adjusted OR for a 1-SD increase 1.56 (95%CI 1.7, 2.09); $P = 0.003$; 1.72 (1.33, 2.22); $P < 0.0001$ and 1.28 (1.03, 1.59); $P = 0.02$, respectively.

Biomarkers and annual renal function decline (eGFR slope)

Median annual variation of eGFR was -1.6 (-4.1 to 0.03) ml/min/1.73m²/year. We registered 24,776 eGFR determinations (corresponding to a median of 14 determinations (7-26) per person) and found a significant negative correlation between baseline concentrations of each biomarker and annual eGFR slope ($r = -0.16$; -0.19 and -0.19 for MR-proADM, sTNFR1 and NT-proBNP, respectively; all $P < 0.0001$).

Considering a mixed-effect model, a significant association was observed between annual eGFR slope and sex, age, and baseline HbA1c, uACR, MR-proADM, sTNFR1 and NT-proBNP.

Discrimination

We assessed improvement in risk discrimination for each candidate biomarkers compared with the model with traditional risk factors (model 3). We observed a significant improvement in RFL risk prediction, when including separate biomarkers or any of their combinations in the model (Table 3). Inclusion of all 3 biomarkers in the model yielded the highest discrimination (difference in c-statistic=0.060, $P < 0.0001$; rIDI=52.4%, $P < 0.0001$) and also a better overall fit in predicting RFL (smallest AIC).

For RRFD, we found that a combination of MR-proADM, sTNFR1 and NT-proBNP yielded the highest discrimination (difference in c-statistic=0.068, $P < 0.0001$; rIDI=70.2%, $P < 0.0001$) as well as the best fit (Table 3).

Figure 2 shows 5-year RFL and RRFD ROC curves for models with traditional risk factor alone and for model with traditional risk factor plus biomarkers.

The biomarker risk score yielded similar results (difference in c-statistic=0.060, $P < 0.0001$; rIDI=56.4%, $P < 0.0001$) for RFL and for RRFD (difference in c-statistic=0.068, $P < 0.0001$; rIDI=68.9 %, $P < 0.0001$).

Sensitivity analyses

Each biomarker was associated significantly with incident RFL in all subgroups tested, except for NT-proBNP in the subgroup of patients with SBP>140 mm Hg where it conferred a

borderline non-significant increased risk. In the second sensitivity analysis, following exclusion of 216 participants with baseline cardiovascular history results were unchanged as MR-proADM, sTNFR1 and NT-proBNP remained associated with RFL and RRFD risk. Accounting for incident cardiovascular disease did not modify the associations. In the subset 216 participants with baseline cardiovascular history, the 3 biomarkers together yielded the highest discrimination as well as the best overall fit in predicting RFL and RRFD. The time-dependent analysis of yearly mean HbA1c values did not change our conclusions. After accounting for the competing risk of all-cause mortality in a Fine and Gray analysis (Table 2), MR-proADM, sTNFR1 and NT-proBNP remained independently associated with an increased risk of RFL.

Conclusions :

In this study, we investigated 3 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. MR-proADM, sTNFR1 and NT-proBNP independently predicted renal outcomes, even when we excluded participants with prior history of cardiovascular disease. We also showed that the combination of these 3 biomarkers improved prediction of renal complications going beyond traditional risk factors.

MR-proADM (47 amino acids) is a surrogate of adrenomedullin, a short half-life peptide. MR-proADM peptide is formed in equimolar amounts to adrenomedullin during the cleavage of its precursor (31). Adrenomedullin is synthesized by many mammalian tissues including kidney, adrenal medulla, cardiomyocytes endothelial and vascular smooth muscle cells, (32) Adrenomedullin exerts pleiotropic actions such as vasodilation, natriuresis/diuresis, tumor growth and anti- inflammation (33). It also inhibits the proliferation of mesangial cells (34). Some epidemiological data support the association of adrenomedullin with CKD progression in non-diabetic individuals (35) and with cardiorenal syndrome (36) in type 2 diabetes patients. In SURDIAGENE cohort, MR-proADM was associated with the risk of doubling of plasma creatinine concentration and/or progression to ESRD as already shown by Velho et al. (6). That study considered 2 independent type 2 diabetes populations: the DIABHYCAR patients and the CKD stage 1-4 SURDIAGENE patients, i.e. 149 additional subjects compared to the present study population.

TNFR1 is ubiquitously synthesized and participates in the TNF-alpha-signalling inflammatory pathway. Circulating sTNFR1 is either released by proteolytic sheddase-mediated cleavage of the membrane-anchored proteins or via alternative splicing of mRNA transcripts (37). TNFRs are then constitutively released in the circulation where they stabilize

circulating TNF (38) or even modify its effect (39). Our results are in accordance with prior epidemiological work which shows that circulating TNFR concentrations are robust prognostic factors for progression to advanced CKD or ESRD (11-16). Moreover, in women with type 2 diabetes that eGFR variations were associated with TNFR2 levels (40). In the SURDIAGENE we found similar results for both men and women and we found no sex interaction. Pena et al. (41) showed recently that TNFR1 considered in a biomarker panel contributed to improved eGFR decline prediction in type 2 diabetes patients.

BNP and its precursors are secreted from myocytes as a reaction to myocytes stretching. BNP and its complementary inactive peptide NT-proBNP (76 amino acids) are secreted in equimolar amounts. BNP has a multitude of actions including relaxation of vascular smooth muscle cells, natriuresis and diuresis, direct antagonism on the RAAS, and lowering of plasma glucose concentrations (42). NT-proBNP is associated with diagnosis and prognosis of chronic heart failure and left ventricular hypertrophy and dysfunction. High plasma concentrations of NT-pro-BNP are secondary not only to increased myocardial production and myocardial stress, but also to impaired kidney function (43). Additionally, our findings are consistent with previous reports showing the association of NT-proBNP with progression of CKD in the general population (21), or impaired GFR (44). Finally, we confirmed the renal prognostic value of elevated NT-proBNP for ESRD found in the post hoc analysis of type 2 diabetes patients selected for a placebo-controlled trial of darbepoetin alfa for the treatment of anemia (22).

Together, our findings support the hypothesis that increased MR-proADM, sTNFR1 and NT-proBNP are independently associated with renal function decline, and they each enhance prediction beyond traditional risk factors. The relationship between these biomarkers and RFL was consistent across the subgroups we explored, including those without cardiovascular disease at baseline. Nevertheless, control of traditional risk factors remains important irrespective of the levels of the new biomarkers.

Physiopathology

MR-proADM, TNFR and NT-proBNP pathways are not completely independent of each other. First, there is some evidence linking low-grade inflammation and endothelial dysfunction. MR-proADM actions include vasodilatation, natriuresis, inhibition of ACTH release, and delay of insulin secretion (45). TNF-alpha can induce adrenomedullin secretion by vascular smooth muscle cells (46). In a mouse model of type 2 diabetes (*Lepr^{db}*), increased TNF expression induced endothelial dysfunction by overproduction of reactive oxidative

species (47), and in patients with chronic heart failure, anti-TNF therapies improve systemic endothelial vasodilator capacity (48).

Chronic heart failure is a medical condition in which NT-proBNP and MR-proADM concentration are elevated. Chronic heart failure could not only lead to renal impairment throughout chronic activation of the renin angiotensin system, sympathetic activation, increase of inflammation and oxidative stress, but also through impairment of vascular endothelium (49).

Interestingly, MR-proADM, sTNFR1 and NT-proBNP concentrations are significantly inter-correlated in our data set. The fact that they remained independent predictors of renal complications suggests that the deleterious renal effect of one biomarker could express itself directly on the kidney or might be mediated by more sophisticated interactions between these biological pathways.

Study Strengths and Limitations

Our study had a number of potential limitations. French patients from the SURDIAGENE study were consecutively recruited in a hospital-based single center cohort, and this design may have caused selection bias. Since our cohort is not population-based, it might not be fully representative of the type 2 diabetes population of France. Moreover, renal function was estimated with the CKD-EPI formula rather than being measured. This study was not design to assess repeated research uACR measurement and exploration of the association of biomarkers with renal function decline in the absence of albuminuria was therefore beyond our scope. Our study also has a number of strengths, including the large size of the cohort, the standardized procedures for sample collection, processing and storage, and the storage at -80°C. Furthermore, the results of the study were consistent across several different definitions of declining renal function.

Assays for these biomarkers are commercially available and have good analytical performance. NT-proBNP is currently in routine use for diagnosis of congestive heart failure. From a clinical perspective, we documented that these biomarkers improve renal risk prediction in addition to traditional risk factors, including eGFR and albuminuria. In addition, the biomarker risk score provides a simple and practical tool to improve the predictive ability of these markers for renal function decline. Nevertheless external validation and cost-benefit studies are needed.

In conclusion, we found, in a prospective cohort study, that beyond traditional risk factors, an increased circulating level of MR-proADM, sTNFR1 and NT-proBNP improves

risk prediction of renal function alterations in a type 2 diabetes population. Their value in clinical practice remains to be determined.

TABLES:

TABLES

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

Variables	All n=1,135
Male	651 (57%)
Age (years)	64 ± 11
Body mass index (kg/m ²)	31 ± 6
Active smoking	125 (11%)
Known diabetes duration (years)	14 ± 10
History of cardiovascular disease	216 (19%)
History of stroke	61 (5%)
History of myocardial infarction	171 (15%)
Systolic blood pressure (mmHg)	132 ± 18
Diastolic blood pressure (mmHg)	72 ± 11
Resting heart rate (beats per min)	71 ± 13
Therapeutics	
Any antihypertensive drug	950 (84%)
Diuretics	504 (44%)
RAAS blockers	711 (63%)
Beta blockers	388 (34%)
Calcium antagonists	418 (37%)
Any antidiabetic drug	1091 (96%)
Insulin	683 (60%)
OAD agents	740 (65%)
Biological determinations	
HbA1c (%)	7.8 ± 1.5
HbA1c (mmol/mol)	62 ± 16.4
eGFR (ml/min/1.73m ²)	76 ± 21
uACR (mg/mmol)	2.6 (1.0-10.4)
Normoalbuminuria, microalbuminuria, macroalbuminuria*	455 (45%), 368 (36%), 187 (19%)
MR-proADM (nmol/L)	0.72 (0.59-0.90)
sTNFR1 (pg/mL)	1,818 (1,544-2,231)
NT-pro BNP (pg/mL)	101 (47-262)
Biomarker risk score	4.51 ± 0.45

Data are mean ± 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

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 $\text{uACR} < 30 \text{ mg/g}$, microalbuminuria as $\text{uACR} 30\text{--}299 \text{ mg/g}$ and macroalbuminuria as $\text{uACR} \geq 300 \text{ mg/g}$

Table 2– Risk of renal function loss ($\geq 40\%$ GFR drop) and rapid renal function decline ($< -5\text{ml/min/year}$) according to biomarkers in patients of the SURDIAGENE

Variables	Model 1	Model 2	Model 3
Hazard ratio for renal function loss ($\geq 40\%$ GFR drop)*			
MR-proADM (per 1 SD)	2.05 (1.86-2.25)	2.01 (1.81-2.24)	2.06 (1.81-2.34)
sTNFR1 (per 1 SD)	1.97 (1.78-2.17)	1.81 (1.63-2.01)	1.78 (1.57-2.02)
NT-proBNP (per 1 SD)	1.90 (1.71-2.10)	1.73 (1.54-1.94)	1.52 (1.34-1.72)
Sub Hazard Ratio of renal function loss ($\geq 40\%$ GFR drop)†			
MR-proADM (per 1 SD)	1.85 (1.68-2.05)	1.80 (1.6-2.02)	1.84 (1.6-2.12)
sTNFR1 (per 1 SD)	1.84 (1.65-2.04)	1.70 (1.52-1.91)	1.69 (1.47-1.95)
NT-proBNP (per 1 SD)	1.64 (1.47-1.81)	1.51 (1.34-1.71)	1.32 (1.15-1.52)
Odds ratio of rapid renal function decline ($< -5\text{ml/min/year}$)‡			
MR-proADM (per 1 SD)	1.74 (1.50-2.02)	1.87 (1.58-2.22)	2.44 (1.94-3.07)
sTNFR1 (per 1 SD)	1.83 (1.57-2.13)	1.83 (1.55-2.15)	2.31 (1.86-2.87)
NT-proBNP (per 1 SD)	1.65 (1.43-1.92)	1.77 (1.48-2.11)	1.64 (1.36-1.99)

Ratios are presented with 95% confidence interval and P Value.

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

Model 1 = univariate

Model 2 = Age, Sex, Diabetes duration, Systolic blood pressure, HbA1c

Model 3 = model 2 + 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	C-statistics with biomarker	Difference in C-statistics (95% CI)	Likelihood ratio P value	Relative IDI (95% CI)	P value
Dependent variable = renal function loss ($\geq 40\%$ GFR drop)					
MR-proADM	0.751	0.050 (0.031-0.069)	<0.0001	0.462 (0.234-0.745)	<0.0001
sTNFR1	0.739	0.038 (0.020-0.057)	<0.0001	0.331 (0.148-0.562)	<0.0001
NT-proBNP	0.731	0.029 (0.014-0.047)	<0.0001	0.159 (0.034-0.332)	0.001
MR-proADM + NT-proBNP	0.757	0.056 (0.036-0.077)	<0.0001	0.486 (0.249-0.774)	<0.0001
MR-proADM + sTNFR1	0.755	0.054 (0.035-0.073)	<0.0001	0.509 (0.200-0.693)	<0.0001
NT-proBNP + sTNFR1	0.753	0.051 (0.032-0.072)	<0.0001	0.428 (0.269-0.812)	<0.0001
MR-proADM + sTNFR1 + NT-proBNP	0.761	0.060 (0.040-0.081)	<0.0001	0.524 (0.281-0.838)	<0.0001
Dependent variable = rapid renal function decline ($<5\text{ ml/min/year}$)					
MR-proADM	0.778	0.052 (0.027-0.078)	<0.0001	0.522 (0.232-0.954)	<0.0001
sTNFR1	0.780	0.053 (0.029-0.079)	<0.0001	0.529 (0.244-0.953)	<0.0001
NT-proBNP	0.755	0.028 (0.009-0.053)	<0.0001	0.186 (0.037-0.451)	0.0003
MR-proADM + NT-proBNP	0.781	0.055 (0.030-0.083)	<0.0001	0.548 (0.247-0.999)	<0.0001
MR-proADM + sTNFR1	0.791	0.065 (0.039-0.091)	<0.0001	0.669 (0.338-1.000)	<0.0001
NT-proBNP + sTNFR1	0.789	0.062 (0.036-0.092)	<0.0001	0.626 (0.295-1.000)	<0.0001
MR-proADM + sTNFR1 + NT-proBNP	0.794	0.068 (0.042-0.097)	<0.0001	0.702 (0.349-1.000)	<0.0001

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Reference Model = age, sex, Diabetes duration, Systolic blood pressure, HbA1c, eGFR, uACR.

C-statistics reference = 0.702 and 0.726 for renal function loss and rapid renal function decline.

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

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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 predicting the risk of loss of renal function in a patient with type 2 diabetes comprising determining the levels of MR-proADM, sTNFR1 and NT-proBNP in a blood sample obtained from the patient.
- 5 2. The method of claim 1 wherein a score which is a composite of the levels of MR-proADM, sTNFR1 and NT-proBNP is calculated and compared to a predetermined reference value wherein when the score is higher to its predetermined reference value, it is concluded that the patient is at risk of having loss of renal function.
- 10 3. The method of claim 2 wherein the score is calculated according to the formula $\text{score} = 3 \times (\beta_1 \times \log[\text{MR-proADM}] + \beta_2 \times \log[\text{sTNFR1}] + \beta_3 \times \log[\text{NT-proBNP}]) / (\beta_1 + \beta_2 + \beta_3)$ wherein $\beta_1 = 4.892$; $\beta_2 = 4.279$; and $\beta_3 = 0.7470$.
4. A kit suitable for performing the method of any of preceding claims which comprises a set of binding partners specific for MR-proADM, sTNFR1 and NT-proBNP.
5. The kit of claim 4 wherein the binding partners are antibodies.
- 15 6. A system for performing the method of claim 1 comprising a computer system configured to i) receive a data set for the plurality of biomarkers (i.e. MR-proADM, sTNFR1 and NT-proBNP), ii) calculate the composite score by using the method of claim 2 and iii) generate the risk status.

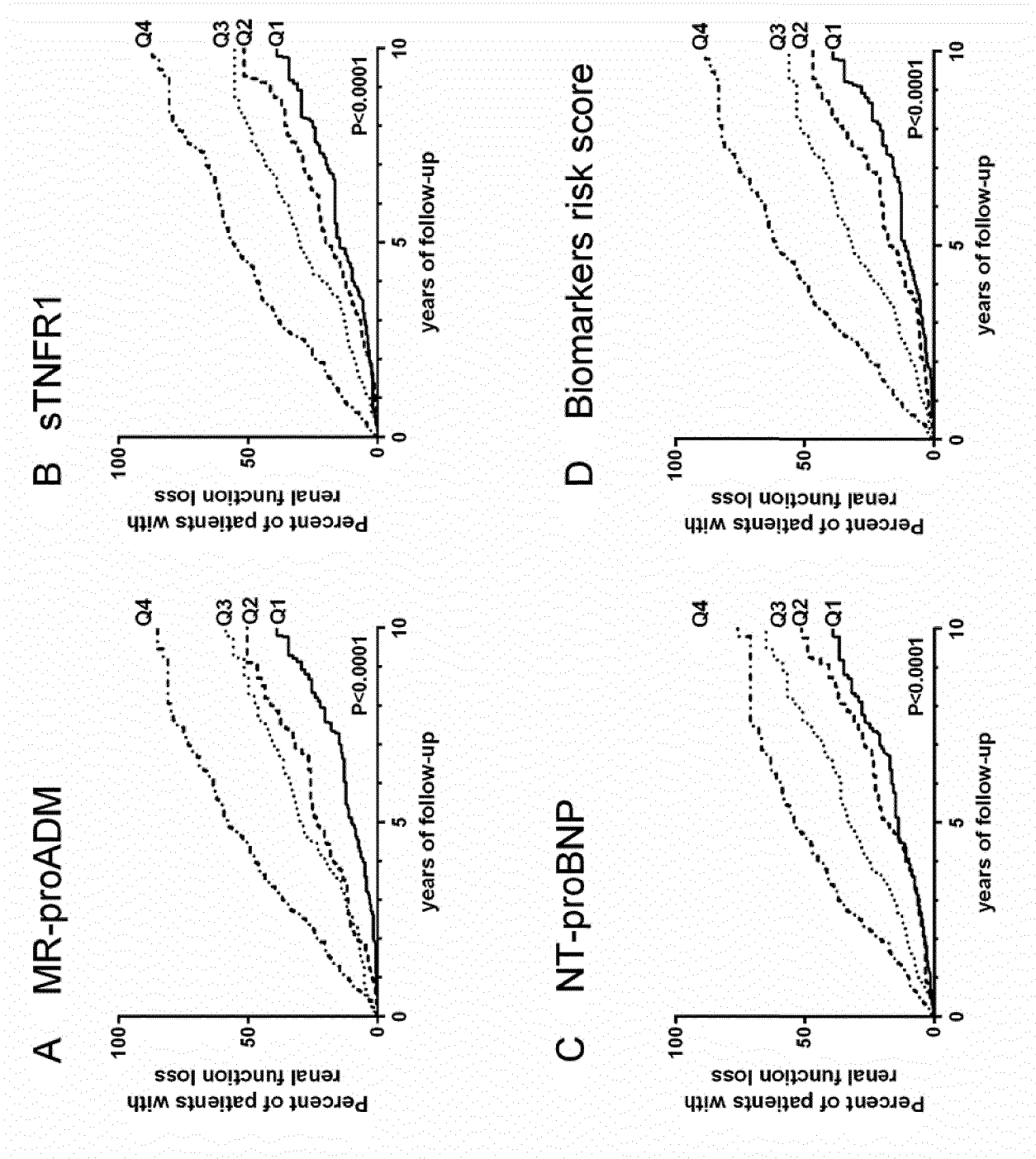


Figure 1

A-Renal function loss (year-5)

B-Rapid renal function decline

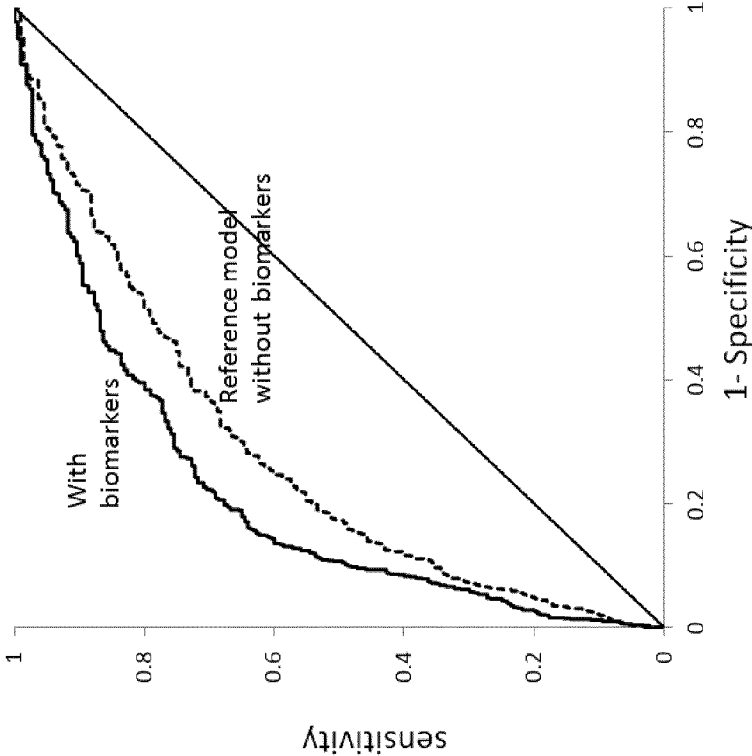
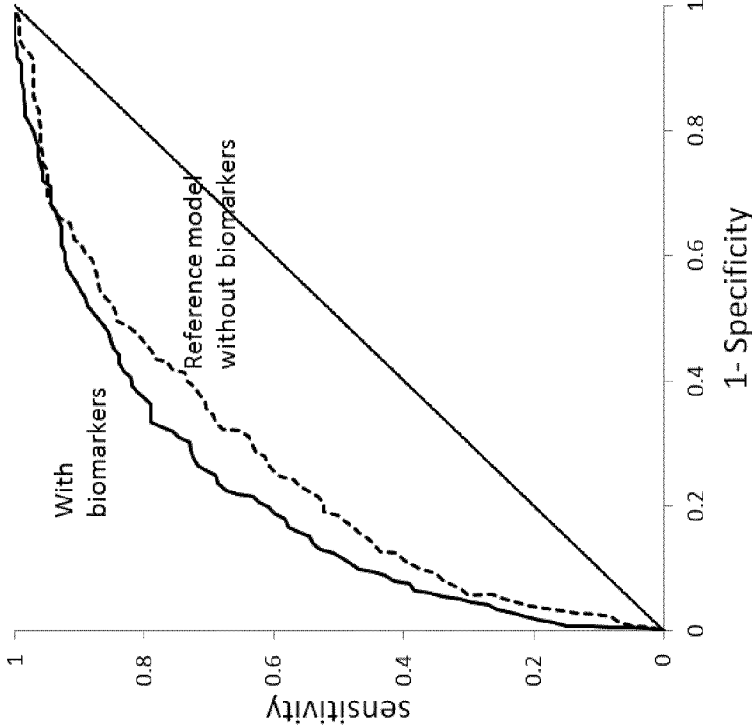


Figure 2

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2017/076157A. 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, COMPENDEX, EMBASE, FSTA, INSPEC, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2015/140551 A1 (UNIV DUNDEE [GB]) 24 September 2015 (2015-09-24) claims 1-28	1-6
A	----- AKSHAY S DESAI ET AL: "Association Between Cardiac Biomarkers and the Development of ESRD in Patients With Type 2 Diabetes Mellitus, Anemia, and CKD", AMERICAN JOURNAL OF KIDNEY DISEASES, W.B. SAUNDERS, PHILADELPHIA, PA, US, vol. 58, no. 5, 28 May 2011 (2011-05-28), pages 717-728, XP028320449, ISSN: 0272-6386, DOI: 10.1053/J.AJKD.2011.05.020 [retrieved on 2011-06-22] page 1, section "results" ----- -/--	1-6



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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"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

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"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

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Date of the actual completion of the international search

20 November 2017

Date of mailing of the international search report

04/12/2017

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INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2017/076157

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	MICHELLE J. PENA ET AL: "A Panel of Novel Biomarkers Representing Different Disease Pathways Improves Prediction of Renal Function Decline in Type 2 Diabetes", PLOS ONE, vol. 10, no. 5, 14 May 2015 (2015-05-14), page e0120995, XP055342809, DOI: 10.1371/journal.pone.0120995 abstract -----	1-6
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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 abstract -----	1-6
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 abstract ----- -/--	1-6

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2017/076157

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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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	1-6
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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2017/076157

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2015140551 A1	24-09-2015	EP 3120150 A1	25-01-2017
		JP 2017516113 A	15-06-2017
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