(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
25 April 2019 (25.04.2019)

WIPO | PCT

(10) International Publication Number
WO 2019/077143 A1

(51) International Patent Classification:
G01N 33/68 (2006.01)

(21) International Application Number:
PCT/EP2018/078795

(22) International Filing Date:
19 October 2018 (19.10.2018)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
17306454.4 20 October 2017 (20.10.2017) EP

(71) Applicants: **INSERM (INSTITUT NATIONAL DE LA SANTÉ ET DE LA RECHERCHE MÉDICALE)** [FR/FR]; 101, rue de Tolbiac, 75013 Paris (FR). **SORBONNE UNIVERSITÉ,** [FR/FR]; 21 rue de l'Ecole de Médecine, 75006 Paris (FR). **ASSISTANCE PUBLIQUE-HÔPITAUX DE PARIS (APHP)** [FR/FR]; 3, avenue Victoria, 75004 Paris (FR).

(72) Inventors: **XU-DUBOIS, Yi-Chun**; INSERM U1155, HOPITAL TENON, 4 rue de la Chine, Bâtiment recherche, 75970 PARIS (FR). **HERTIG, Alexandre**; INSERM U1155, HOPITAL TENON, 4 rue de la Chine, Bâtiment recherche, 75970 PARIS (FR). **RONDEAU, Eric**; INSERM U1155, HOPITAL TENON, 4 rue de la Chine, Bâtiment recherche, 75970 PARIS (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))*

WO 2019/077143 A1

(54) Title: METHODS AND COMPOSITIONS FOR PREDICTING AND EARLY DIAGNOSING CHRONIC ACTIVE FORM OF ABMR WITH TRANSPLANT GLOMERULOPATHY AND RENAL GRAFT LOSS

(57) Abstract: By immunohistochemistry, an easily applicable method in a routine lab, inventors analyzed the pattern and the expression level of fibronectin, collagen IV and fascin in glomeruli in renal grafts. They observed a modified expression pattern with a linear and upregulated expression of fibronectin and collagen IV along the glomerular basement membrane (GBM) and diffused cytoplasmic expressin of fascin in the glomerular endothelial cells in the renal grafts with ABMR, notably, in an early stage of caABMR, in which TG lesion is not detectable by the conventional method with light microscope. Accordingly, inventors have found a new tool to diagnose early stage of glomerulopathy, in a subject having the anti-donor specific antibodies (DSA) and/or suffering from an acute form of antibody-mediated rejection (ABMR). This diagnostic tool can be also used to help the early diagnosis of thrombotic microangiopathy or hepatitis C infection related renal glomerulopathies.

METHODS AND COMPOSITIONS FOR PREDICTING AND EARLY DIAGNOSING CHRONIC ACTIVE FORM OF ABMR WITH TRANSPLANT GLOMERULOPATHY AND RENAL GRAFT LOSS

FIELD OF THE INVENTION:

The invention is in the field of transplantation. More particularly, the invention provides methods to detect whether a renal transplanted subject having the anti-donor specific antibodies (DSA) and/or suffering from an acute form of antibody-mediated rejection (ABMR) is at risk of having or developing a chronic form of ABMR with transplant glomerulopathy (TG).

BACKGROUND OF THE INVENTION:

In spite of a tremendous progress in the transplant field with excellent drugs to prevent the graft rejection in the early transplant course, a major improvement in one year renal graft survival did not translate into similar improvement of graft outcome in the long term. Chronic active form of ABMR with Transplant glomerulopathy (TG) remains a major cause of renal graft loss. In a large series of renal transplanted recipients, graft loss was observed at 5 years post transplant course was 5% in the group of patients without TG. But in those with TG, the rate of graft loss rose to 38% [1,2]. TG is known mostly as a consequence of persist donor-specific alloantibodies (DSA) positivity with proteinuria, and a rapidly decline of graft function and failure which must patients return to dialysis. This form of ABMR can develop silently without acute episodes. When the patients were found to have a reduced graft function and an important proteinuria, a TG has already settled typically with double contour of the glomerular basement membrane (GBM) seen by light microscopy, named as chronic active ABMR (caABMR). This lesion results from a chronic activation and/or injury of glomerular endothelial cells trigged by DSA and an continual accumulation of extracellular matrix in the GBM, which is defined precisely by recent Banff classification (2013, 2015 and 2017) as chronic active ABMR (caABMR) [3-5].

Despite the increasingly recognized importance of caABMR with TG as a major cause of renal graft loss, there is no consensus regarding its management. One reason is due to uncertainty about its diagnosis, exactly lacking the tool for diagnosing an active and/or ongoing state of disease which needs the clinic interventions. The diagnosis of caABMR according to the recent Banff meeting requires 3 criteria: (1) Morphological evidence of chronic tissue injury including translpant glomerulopathy (TG)(cg score>0) seen by light

microscopy or severe peritubular capillary basement membrane multilayering seen by electron microscopy; (2) evidence of current/recent antibody interaction with vascular endothelium, including linear c4d staining in peritubular capillaries or at least moderate level of microvascular inflammation, or increased expression of gene transcripts in the biopsy tissue indicative of endothelial injury; (3) serologic evidence of circulating DSAs which can be replaced by c4d or ABMR related gene expression.

With regard to DSA, their presence is a pre-requisite for the diagnosis of ABMR. However it does not necessarily indicate an ABMR. Indeed, some antibodies may be present in the context of accommodation. In contrast, absence of detectable of DSA in some ABMR patients can be due to absorption of DSA by the rejected allograft, thus DSA becomes undetectable in the plasma, or, due to the presence only the anti endothelial cell antibodies which are not included in the present panel of DSA detection.

Evidence of current/recent antibody interaction with vascular endothelium should be a most strong proof of disease for the DSA induced graft injury. Currently important peri-tubular capillaritis and glomerulitis are recognized as strong indicators of ABMR in clinic, because endothelial cells are the main target of DSA, and the presence of important inflammatory cells within the capillaries obviously reflects ongoing injury. There are however some limitations to use capillaritis as a diagnostic tool for ABMR: although the presence of inflammatory cells within the capillaries can be the consequence of endothelial cell activation during ABMR, a recent paper has report that the capillaritis in early biopsies was often allo-antibody independent. And as an indirect evidence of endothelial cell activation, it may present in a relative late stage of disease; last, a small amount of inflammatory cells in the capillaries may be difficult to be assessed by routine morphology analysis or to be evaluated as the evidence for the diagnosis of ABMR. Consequently, the proportion of patients diagnosed for ABMR using the capillaritis as criterion could be like the tip of iceberg.

Last, the presence of C4d on the peri-tubular capillaries is clearly suggestive of ABMR, yet it is poorly sensitive as a diagnostic tool because in the most recent series at least 40% of patients with ABMR and 65% in cABMR were C4d negative.

Another reason for the no consensus of management of chronic active ABMR is due to lacking a consistent improvement on the renal graft outcome with current acute ABMR treatment protocols, such as using IGIV, plasmapheresis, antibody against B lymphocytes surface antigen CD20 rituximab, DSA producing plasma cell depletion bortezomib to reduce DSA; complement 5 inhibitor eculizumab to reduce the graft injury. It is mostly due to too

advanced glomerular lesion and too important graft fibrosis once TG was diagnosed by light microscopy which is used in most transplant centers.

Hopefully, some recent studies showed a benefit in the rate of development of TG in patients treated to reduced DSA when the glomerular endothelial lesions were detected during early phase of disease by the electron microscopy [6]. These early observed lesions are endothelial swelling, or hypertrophied endothelium and vacuolation, serration of capillary loop with basement membrane multilayering. However, this medical equipment is not available in many transplant centers and the sample used for the observation with the electron microscopy needs a particular tissue treatment.

So it becomes an urgent need for clinic to develop some sensitive and easily applicable markers in a routine lab, to detect this DSA mediated TG in its early course, especially to assess an active or ongoing state of disease, because in this stage of disease, an adequate treatment for the patients at risk, can be efficient to stop the progression of disease in order to preserve a functional graft in the long term.

Based on the pathogenesis of TG, direct assessment of glomerular endothelial cell activation by 1) its phenotype changes, which was induced by the engagement and aggression of DSA, 2) detection of early ECM deposition in GBM which can be a consequence of endothelial cell activation could meet this need.

In a previous work, using immunohistochemistry, an easily applicable technique in a routine lab, we detected the expression of an endothelial to mesenchymal transition marker fascin, an actin-bundling protein involved in cell motility, in the peri-tubular capillary endothelial cells in the renal grafts with ABMR. The expression of fascin in the endothelial cells reflects an active state of these cells, which was shown to be helpful for an early ABMR diagnosis[7]. This endothelial activation marker can also be used to detect the glomerular endothelial activation.

Among the numerous ECM proteins, fibronectin (FN) is an important extracellular matrix (ECM) protein and essential for vascular morphogenesis. During embryonic development, FN is incorporated between endothelial and perivascular cells. It plays important role in cell adhesion, migration, growth and differentiation. Its expression is highly up-regulated during embryogenesis around newly developing vasculature. In some pathological conditions such as atherosclerosis and tumorigenesis, a large amount of FN deposition under endothelium was found around the vasculature. Because FN is produced at time of dynamic tissue remodeling, formation or repair [8,9], the over-expression of FN could be a sensitive marker reflecting a pathological condition or an activation of endothelial cells

during ABMR. In addition, ECM is known to have many effects beyond providing structure support. By close contact with cells, abnormal accumulation of ECM can be account to give inputs into cells and modify cellular behaviors through adhesion receptors such as integrins and DDR tyrosine kinase receptor or indirectly through regulating the activity of growth factors attached or stocked on the surface of ECM. Thus abnormal deposition of FN under endothelial cells is not only a sensitive marker of endothelial cell activation, but also can play a role of activator for these cells.

Type IV collage (coll4) is a major constituent of GBM. Its expression was up-regulated during diabetic glomerulopathy. The thickening of GBM in the diabetic nephropathy is due to accumulation of coll4 and alteration in its structure and composition.

We made hypothesis that during the early course of ABMR, the activated glomerular endothelial cells will stimulate its FN and coll4 synthesis which can deposit and/or incorporate into the GBM. The latter changes GBM structure and results in double contours of GBM which can be observed in the late stage by light microscope.

SUMMARY OF THE INVENTION:

The invention relates to a method for predicting whether a renal transplant subject having the anti-donor specific antibodies (DSA) and/or suffering from an acute active form of antibody-mediated rejection (ABMR), , is at risk of having or developing late a chronic active form of ABMR (caABMR) with transplant glomerulopathy (TG), comprising the steps of: i) measuring the expression of fibronectin and/or type IV collagen in a biological sample obtained from said subject; ii) comparing the expression pattern and level measured at step i with its predetermined reference value, and iii) concluding that the subject is at risk of having or developing a chronic active form of ABMR with transplant glomerulopathy (TG) when a linear expression of fibronectin and/or type IV collagen is observed along the GBM and the level of expression is higher than its predetermined reference value or concluding that the subject is not at risk of developing a chronic form of ABMR with transplant glomerulopathy (TG) when the expression pattern and level of fibronectin and/or type IV collagen is the same as its predetermined reference value. In particular, the invention is defined by claims.

DETAILED DESCRIPTION OF THE INVENTION:

By immunohistochemistry, inventors have analyzed that the fibronectin and collagen type IV (coll4) expression pattern in the glomeruli and the importance of expression in the glomerular basement membrane (GBM) was semi quantified in all biopsies. The pattern of expression of fibronectin and coll4 in the glomeruli was changed from a focal, irregular expression in the mesangial area of glomeruli in the normal kidneys to an up-regulated and

linear expression along the GBM in glomeruli in the renal graft with antibody mediated rejection (ABMR), especially higher in the chronic form of ABMR (cABMR) with transplant glomerulopathy (TG). Moreover, the expression level of fibronectin and coll4 was significantly correlated with the level of donor specific antibodies (DSA) detected in the recipients at time of biopsy. They also observed a modified expression pattern of fascin in the glomeruli (from a weak and limited peri-nuclear localization in normal kidney to an up-regulated and diffused cytoplasmic expression in the glomerular endothelial cells in the renal grafts with ABMR). The level of glomerular fascin expression was correlated with those of FN and coll4 in GBM. Notably, the expression of these markers can predict the risk of developing late chronic form of ABMR with transplant glomerulopathy (TG). These sensitive markers can also predict strongly late graft loss. Particularly in the recipients in whom the diagnosis of caABMT cannot be made because of lacking of TG determined with the conventional method by light microscope. TG lesion is undetectable by the conventional method with light microscopy.

For the first time, inventors have shown by immunohistochemistry that the expression of fibronectin and collagen type IV (coll4) in the subject suffering from glomerulopathy of the allograft is linear along the GBM in glomeruli. Whereas in the control subject, they do not observe such linear expression of fibronectin and collagen type IV (coll4). Accordingly, inventors have found a new tool to diagnose early stage and active state of glomerulopathy, thrombotic microangiopathy or hepatitis C infection related renal pathologies.

Method for predicting and/or early diagnosing the risk of having a chronic active form of ABMR with transplant glomerulopathy (TG)

Accordingly, in a first aspect, the invention relates to a method for predicting whether a transplant subject having the anti-donor specific antibodies (DSA) and/or suffering from an acute form of antibody-mediated rejection (ABMR) is at risk of having or developing a chronic form of ABMR with transplant glomerulopathy (TG), comprising the steps of: i) measuring the expression of fibronectin and/or type IV collagen in a biological sample obtained from said subject; ii) comparing the expression pattern and level measured at step i with its predetermined reference value, and iii) concluding that the subject is at risk of having or developing chronic active form of ABMR with transplant glomerulopathy (TG) when the expression pattern is different and level of fibronectin and/or type IV collagen is higher than its predetermined reference value or concluding that the subject is not at risk of developing a chronic form of ABMR with transplant glomerulopathy (TG) when the expression pattern and level of fibronectin and/or type IV collagen is the same as its predetermined reference value.

In a particular embodiment, the invention relates to a method for predicting whether a transplant subject having the anti-donor specific antibodies (DSA) and/or suffering from an acute form of antibody-mediated rejection (ABMR) is at risk of having or developing a chronic active form of ABMR with transplant glomerulopathy (TG), comprising the steps of: i) measuring the expression of fibronectin and/or type IV collagen in a biological sample obtained from said subject; ii) comparing the expression pattern and level measured at step i with its predetermined reference value, and iii) concluding that the subject is at risk of having or developing chronic form of ABMR with transplant glomerulopathy (TG) when a linear expression of fibronectin and/or type IV collagen is observed along the GBM and the level of expression of fibronectin and/or type IV collagen is higher than its predetermined reference value or concluding that the subject is not at risk of developing a chronic form of ABMR with transplant glomerulopathy (TG) when the expression pattern and level of fibronectin and/or type IV collagen is the same as its predetermined reference value.

In a second aspect, the invention relates to a method for early diagnosing whether a transplant subject having the anti-donor specific antibodies (DSA) and/or suffering from an acute form of antibody-mediated rejection (ABMR) is having a chronic form of ABMR with transplant glomerulopathy (TG), comprising the steps of: i) measuring the expression of fibronectin and/or type IV collagen in a biological sample obtained from said subject; ii) comparing the expression pattern and level measured at step i) with its predetermined reference value, and iii) concluding that the subject is having chronic active form of ABMR with transplant glomerulopathy (TG) when the expression pattern is different and level of fibronectin and/or type IV collagen is higher than its predetermined reference value or concluding that the subject is not having a chronic form of ABMR with transplant glomerulopathy (TG) when the expression pattern and level of fibronectin and/or type IV collagen is the same as its predetermined reference value.

As used herein, the term “predicting” means that the subject to be analyzed by the method of the invention is allocated either into the group of subjects who will have a chronic form of ABMR with transplant glomerulopathy, or into a group of subjects who will not have chronic form of ABMR with transplant glomerulopathy. Typically, said risk is elevated as compared to the average risk in a cohort of transplanted subjects. In the context of the invention, the risk of having a chronic form of ABMR with transplant glomerulopathy in a subject shall be predicted. The term "predicting the risk", as used herein, refers to assessing the probability according to which the patient as referred to herein will have a chronic form of ABMR with transplant glomerulopathy. As will be understood by those skilled in the art, such

an assessment is usually not intended to be correct for 100% of the subjects to be investigated. The term, however, requires that prediction can be made for a statistically significant portion of subjects in a proper and correct manner. Whether a portion is statistically significant can be determined without further ado by the person skilled in the art using various well known statistic evaluation tools, e.g., determination of confidence intervals, p-value determination, Student's t-test, Mann- Whitney test etc. Details are found in Dowdy and Wearden, Statistics for Research, John Wiley & Sons, New York 1983. Preferred confidence intervals are at least 90%, at least 95%, at least 97%, at least 98% or at least 99 %. The p-values are, preferably, 0.1, 0.05, 0.01, 0.005, or 0.0001. Preferably, the probability envisaged by the invention allows that the prediction of an increased risk will be correct for at least 60%, at least 70%, at least 80%), or at least 90% of the subjects of a given cohort or population. The term, preferably, relates to predicting whether or not there is an increased risk of having a chronic form of ABMR with transplant glomerulopathy in a population of subjects rather than giving a precise probability for the said risk.

As used herein, the term “early diagnosing” means that the subject to be analyzed by the method of the invention is allocated either into the group of subjects who have a chronic but active form of ABMR with transplant glomerulopathy, or into a group of subjects who do not have chronic active form of ABMR with transplant glomerulopathy.

As used herein, the term “DSA” refers to antibodies which are anti-HLA antibodies, specifically generated against donor cells. The term “antibody-mediated rejection, (ABMR)” refers to a type of rejection of transplant tissue or organ by the recipient’s immune system. The rejection of a transplanted tissue or organ is triggered by the action of anti-donor's antibodies developed in the recipients against antigens found on the endothelial surface of blood vessels of graft. There are two types of ABMR: acute active ABMR and chronic active ABMR. In the context of the invention, the ABMR is an acute ABMR. As used herein, the term “acute rejection” refers to an acute episode of tissue or transplanted organ injury. In a particular embodiment, it can begin in early stage such as shortly after implantation or at any time during the course of transplant. Acute rejection is often identified clinically by decreased function of the transplanted organ. Lesions of acute active ABMR at the site of the renal transplant characteristically are infiltrated with large numbers of neutrophils, lymphocytes and macrophages in the microvasculature of glomeruli or of peri-tubular capillaries that cause tissue or organ damage. In the context of the invention, the method is suitable to predict the risk and diagnose early caABMR in a subject having DSA and/or suffering from acute active form of ABMR. As used herein, the term “chronic active ABMR” refers to a long-term loss of

function in transplanted tissues or organs via fibrosis of the transplanted tissue because of ongoing chronic vascular lesions and subsequently chronic organ ischemia. In a particular embodiment, the chronic active ABMR is associated with a transplant glomerulopathy (TG). The term "transplant glomerulopathy" refers to a disease of the glomeruli in transplanted kidneys. Typically, TG is characterized by duplication of the glomerular basement membrane assessed by light or electronic microscopy, this pattern of injury may result from a number of disease processes affecting the glomerular endothelium. In particular embodiment, the chronic active ABMR is a chronic active antibody mediated rejection (caABMR). As used herein, the term "caABMR" refers to a chronic form of ABMR but in an active state of disease, which destroy progressively and actively the grafted organ and ended by the graft loss. caABMR is identified clinically by progressively decreased function of the transplanted organ and often associated with proteinuria in the case of renal transplantation. Lesions at the site of the transplant characteristically are double contour of GBM with inflammatory cells and c4d deposition in the glomerular and peritubular capillaries in the case of renal transplantation. In a particular embodiment, the invention is to sensitize the early diagnosis and suitable to predict the risk of having or developing late a chronic active form of ABMR with transplant glomerulopathy (TG).

As used herein, the term "transplant subject" also called as grafted subject, refers to a subject who has received an organ transplantation. The term "organ transplantation" refers to the procedure of replacing diseased organs, parts of organs, or tissues by healthy organs or tissues. The transplanted organ or tissue can be obtained either from the subject himself (= autograft), from another human donor (= allograft) or from an animal (= xenograft). Transplanted organs may be artificial or natural, whole (such as kidney, heart and liver) or partial (such as heart valves, skin and bone). In a particular embodiment, the subject is a renal transplanted subject. In particular, said renal transplanted subject may further have been grafted with the pancreas, and optionally a piece of duodenum, of the kidney donor. Said subject is treated with immunosuppressive drugs or another drugs that are currently known in the art or that will be identified in the future. In a particular embodiment, the subject is under immunosuppressive treatment, which means that the subject is administered with one or more immunosuppressive drugs.

As used herein, the term "fibronectin" (FN) refers to a glycoprotein present in a soluble dimeric form in plasma and in a dimeric or multimeric form at the cell surface and in extracellular matrix. FN mediates a wide variety of cellular interactions with the extracellular matrix (ECM) and plays important roles in cell adhesion, migration, growth and

differentiation. FN plays a crucial role in wound healing, FN stops the bleeding by forming a blood clot. The naturally occurring human fibronectin gene has a nucleotide sequence as shown in Genbank Accession numbers: NM_001306129.1, NM_001306130.1, NM_001306131.1, NM_001306132.1, NM_002026.3, NM_054034.2, NM_212474.2, NM_212476.2, NM_212478.2 and NM_212482.2. And the naturally occurring human fibronectin protein has an aminoacid sequence as shown in Genbank Accession numbers: NP_001293058.1, NP_001293059.1, NP_001293060.1, NP_001293061.1, NP_002017.1, NP_473375.2, NP_997639.1, NP_997641.1, NP_997643.1 and NP_997647.1.

As used herein, the term "collagen IV" refers to Type IV collagen, also known as ColIV or Col4. Collagen IV is the major structural component of basement membranes, is a multimeric protein composed of 3 alpha subunits. These subunits are encoded by 6 different genes, alpha 1 through alpha 6, each of which can form a triple helix structure with 2 other subunits to form type IV collagen. The collagen IV C4 domain at the C-terminus is not removed in post-translational processing, and the fibers link head-to-head, rather than in parallel. The naturally occurring human fibronectin gene has a nucleotide sequence as shown in Genbank Accession numbers: NM_001303110.1, NM_001845.5, NM_000495.4, NM_033380.2, NM_000091.4, NM_001846.3 and NM_000092.4. And the naturally occurring human fibronectin protein has an aminoacid sequence as shown in Genbank Accession numbers: NP_001290039.1, NP_001836.3, NP_000486.1, NP_203699.1, NP_000082.2, NP_001837.2 and NP_000083.3.

As used herein, the term "expression pattern and level" refers to both qualitative as well as quantitative differences in the temporal and tissue expression patterns of a gene or a protein in transplant tissue or organ versus normal adjacent tissue or organ. A differential in the expression pattern, observed and defined by morphological studies, and level of the fibronectin and/or collagen IV between the biological sample and the reference value is indicative that said subject suffers from an active state of ABMR. The expression pattern is particularly observed in the glomeruli. Typically, a healthy subject has a focal and irregular expression of FN and/or coll4 in the mesangial area in the glomeruli, whereas the subject having a chronic active form of ABMR with transplant glomerulopathy (TG) has an up-regulated and linear expression of these extra cellular matrix along the GBM in the glomeruli.

Particularly, the method is suitable for the patients who has not developed or cannot be diagnosed as chronic active ABMR in the early stage of disease because of lacking the detectable TG (has not double contour of GBM) examined with the conventional method by light microscope. Determining the expression pattern and level of fibronectin and/or collagen

IV level is mainly defined by the immune-staining on the tissue with specific antibodies and observed by light microscopy. But the increased expression level of these extracellular matrix during disease may be assessed also by any of a wide variety of well-known methods for detecting expression of a transcribed nucleic acid or translated protein. In one embodiment, the fibronectin and/or collagen IV expression level is assessed by analyzing the expression of the protein translated from said gene. Said analysis can be assessed using an antibody (e.g., a radio-labeled, chromophore- labeled, fluorophore-labeled, or enzyme-labeled antibody), an antibody derivative (e.g., an antibody conjugate with a substrate or with the protein or ligand of a protein of a protein/ligand pair (e.g., biotin-streptavidin)), or an antibody fragment (e.g., a single-chain antibody, an isolated antibody hypervariable domain, etc.) which binds specifically to the protein translated from the gene encoding for the biomarker. Methods for measuring the expression level of a biomarker in a sample may be assessed by any of a wide variety of well-known methods from one of skill in the art for detecting expression of a protein including, but not limited to quantification methods such as IHC.

As used herein, the term "biological sample" refers to any sample obtained from a transplanted subject, such as a serum sample, a plasma sample, a urine sample, a blood sample, a lymph sample, or a tissue biopsy. In the context of the invention, the biological sample is a biopsy. Typically, the biopsy refers to an extraction of tissues from the transplant or no transplant tissue or organ for examination to determine expression pattern and level of fibrinogen and collagen IV. In a particular embodiment, the biological sample is glomeruli which is obtained by biopsy. The expression pattern and level of fibronectin and/or collagen IV are measured in the glomeruli.

As used herein, the term "predetermined reference value" refers to a threshold value or a cut-off value, which was defined after analysing the normal tissue from the normal kidney samples obtained from the healthy part of kidney removed because of a renal cancer and also a great quantity of normal renal graft samples from protocol biopsy.

In a particular embodiment, the invention relates method for predicting and early diagnosing whether a transplant subject having the anti-donor specific antibodies (DSA) and/or suffering from an acute form of antibody-mediated rejection (ABMR) is at risk of having or developing a late a chronic active form of ABMR with transplant glomerulopathy (TG), comprising the steps of: i) measuring the expression pattern and level of fibronectin, collagen IV and/or fascin in a biological sample obtained from said subject; ii) comparing the expression pattern and level measured at step i with its predetermined reference value, and iii) concluding that the subject is of having or developing a chronic form of ABMR with

transplant glomerulopathy (TG), when a linear expression of fibronectin and/or type IV collagen is observed along the GBM and/or a diffused cytoplasmic expression of fascin in the glomerular endothelial cells, and the level of expression ishigher than its predetermined reference value or concluding that the subject is not at risk of having or developing a chronic form of ABMR with transplant glomerulopathy (TG) when the expression pattern and level of fibronectin, collagen IV and/or fascin is not different from its predetermined reference value.

As used herein, the term "fascin" refers to an endothelial to mesenchymal transition marker, an actin-bundling protein involved in cell motility, in the peri-tubular capillary endothelial cells in the renal grafts with ABMR. Fascin proteins organize F-actin into parallel bundles, and are required for the formation of actin-based cellular protrusions. The naturally occurring human fascin gene has a nucleotide sequence as shown in Genbank Accession number NM_003088.3 and the naturally occurring human fascin protein has an aminoacid sequence as shown in Genbank Accession number NP_003079.1.

Method for predicting of having a chronic form glomerular sclerosis and/or glomerular obsolescence

The method of the invention as described above is also suitable to predict whether a subject suffering from thrombotic microangiopathy is at risk of having or developing a glomerular sclerosis and/or obsolescence.

In a third aspect, the invention relates to a method for predicting whether a subject suffering from thrombotic microangiopathy has the glomerular involvement and/or is at risk of having or developing a glomerular sclerosis and/or glomerular obsolescence, comprising the steps of: i) measuring the expression of fibronectin and/or type IV collagen in the glomeruli in a biological sample obtained from said subject; ii) comparing the expression pattern and level measured at step i) with its predetermined reference value, and iii) concluding that the subject is at risk of having or developing glomerular sclerosis and/or glomerular obsolescence when the expression pattern is different and level of fibronectin and/or type IV collagen is higher than its predetermined reference value or concluding that the subject is not at risk of developing glomerular sclerosis and/or glomerular obsolescence when the expression pattern and level of fibronectin and/or type IV collagen is the same as its predetermined reference value.

In a particular embodiment, the invention relates to a method for predicting whether a subject suffering from thrombotic microangiopathy is at risk of having or developing a glomerular sclerosis and/or glomerular obsolescence, comprising the steps of: i) measuring the expression of fibronectin and/or type IV collagen in the glomeruli in a biological sample

obtained from said subject; ii) comparing the expression pattern and level measured at step i) with its predetermined reference value, and iii) concluding that the subject is at risk of having or developing glomerular sclerosis and/or glomerular obsolescence when a linear expression of fibronectin and/or type IV collagen is observed along the GBM and the level of expression is higher than its predetermined reference value or concluding that the subject is not at risk of developing glomerular sclerosis and/or glomerular obsolescence when the expression pattern and level of fibronectin and/or type IV collagen is the same as its predetermined reference value.

As used herein, the term "thrombotic microangiopathy" refers to a pattern of damage that can occur in the smallest blood vessels inside many of vital organs. In the context of the invention, the endothelial cells of capillaries become damaged, blood flow through the kidney slows.

As used herein, the term "glomerular sclerosis and/or glomerular obsolescence" refers to a morphological term caused by long term or serious glomerular injuries which result of destruction of normal glomerular architecture and then replaced by the fibrotic tissue or fibrosclerosis or hyalinosis and finally resulting of glomerular function loss.

In a further aspect, the invention is suitable to predict whether a subject suffering from hepatitis C infection has the glomerular involvement and/or is at risk of having or developing a glomerular sclerosis and/or glomerular obsolescence. Accordingly, the invention relates to a method for predicting whether a subject suffering from hepatitis C infection is at risk of having or developing a glomerular sclerosis and/or glomerular obsolescence, comprising the steps of: i) measuring the expression of fibronectin and/or type IV collagen in the glomeruli in a biological sample obtained from said subject; ii) comparing the expression pattern and level measured at step i) with its predetermined reference value, and iii) concluding that the subject is at risk of having or developing glomerular sclerosis and/or glomerular obsolescence when a linear expression of fibronectin and/or type IV collagen is observed along the GBM and the level of expression is higher than its predetermined reference value or concluding that the subject is not at risk of developing a glomerular sclerosis and/or glomerular obsolescence when the expression pattern and level of fibronectin and/or type IV collagen is the same as its predetermined reference value.

Method for predicting the risk of graft loss

In a fourth aspect, the invention relates to a method for predicting whether a transplanted subject is at risk of graft loss comprising the steps of: i) measuring the expression pattern and level of fibronectin and/or collagen IV in a biological sample obtained from said

subject; ii) comparing the expression pattern and level measured at step i) with its predetermined reference value, and iii) concluding that the subject is at risk of graft loss when a linear expression of fibronectin and/or type IV collagen is observed along the GBM and the level of expression is higher than its predetermined reference value or concluding that the subject is not at risk of graft loss when the expression pattern and level of fibronectin and/or collagen IV is not different from its predetermined reference value.

As used herein, the term “predicting” means that the subject to be analyzed by the method of the invention is allocated either into the group of subjects who will lose his graft, or into a group of subjects who will not lose his graft. Typically, said risk is elevated as compared to the average risk in a cohort of transplanted subjects. In the context of the invention, the risk of graft loss in a subject shall be predicted. The term "predicting the risk", as used herein, refers to assessing the probability according to which the patient as referred to herein will lose graft. As will be understood by those skilled in the art, such an assessment is usually not intended to be correct for 100% of the subjects to be investigated. The term, however, requires that prediction can be made for a statistically significant portion of subjects in a proper and correct manner. Whether a portion is statistically significant can be determined without further ado by the person skilled in the art using various well known statistic evaluation tools, e.g., determination of confidence intervals, p-value determination, Student's t-test, Mann- Whitney test etc. Details are found in Dowdy and Wearden, Statistics for Research, John Wiley & Sons, New York 1983. Preferred confidence intervals are at least 90%, at least 95%, at least 97%, at least 98% or at least 99 %. The p-values are, preferably, 0.1, 0.05, 0.01, 0.005, or 0.0001. Preferably, the probability envisaged by the invention allows that the prediction of an increased risk will be correct for at least 60%, at least 70%, at least 80%), or at least 90% of the subjects of a given cohort or population. The term, preferably, relates to predicting whether or not there is an increased risk of having chronic ABMR compared to the average risk of chronic ABMR in a population of subjects rather than giving a precise probability for the said risk.

As used herein, the term “graft loss” also known as graft failure or transplant loss, refers to loss of function in a transplanted organ or tissue.

In a particular embodiment, the invention relates to a method for predicting whether a transplanted subject is at risk of graft loss comprising the steps of: i) measuring the expression pattern and level of fibronectin, collagen IV and/or fascin in a biological sample obtained from said subject; ii) comparing the expression pattern and level measured at step i) with its predetermined reference value, and iii) concluding that the subject is at risk of graft loss

when the expression pattern and level of fibronectin, collagen IV and/or fascin is different and higher than its predetermined reference value or concluding that the subject is not at risk of graft loss when the expression pattern and level of fibronectin and/or collagen IV is not different from its predetermined reference value.

More particularly, the invention relates to a method for predicting whether a transplanted subject is at risk of graft loss comprising the steps of: i) measuring the expression pattern and level of fibronectin, collagen IV and/or fascin in a biological sample obtained from said subject; ii) comparing the expression pattern and level measured at step i) with its predetermined reference value, and iii) concluding that the subject is at risk of graft loss when a linear expression of fibronectin and/or type IV collagen is observed along the GBM and/or a diffused cytoplasmic expression of fascin in the glomerular endothelial cells, and the level of expression is higher than its predetermined reference value or concluding that the subject is not at risk of graft loss when the expression pattern and level of fibronectin and/or collagen IV is not different from its predetermined reference value.

A differential in the expression pattern and level of the fibronectin, collagen IV and/or fascin between the biological sample and the reference value is indicative that said subject is at risk of having a graft loss. The expression pattern and level of fibronectin and/or collagen IV and fascin is determined by the methods as described above.

The methods as described above, wherein, the subject can be also no renal transplanted recipients and thrombotic microangiopathy or hepatitis C infection related renal pathologies can developed on their own kidneys.

Method for treating early and/or preventing late chronic form of ABMR with transplant glomerulopathy (TG) and subsequently graft loss

In a fifth aspect, the invention relates to a method of treating the transplant recipients to prevent the occurrence of chronic active form of ABMR with transplant glomerulopathy (TG) in a subject comprising a step of administering to said subject a therapeutically effective amount of immunosuppressive drugs.

In particular embodiment, the invention is suitable to treat early and to prevent late chronic active form of ABMR with transplant glomerulopathy.

In a particular embodiment, the invention relates to a method of treating early and to prevent late or active chronic form of ABMR with transplant glomerulopathy (TG) in a subject comprising following steps: i) identifying whether a subject is at risk of having or developing chronic active form of ABMR with transplant glomerulopathy (TG) as described

above and ii) administering to said subject as diagnosed a therapeutically effective amount of immunosuppressive drugs and an anti-ABMR therapy.

In another embodiment, the method according to the invention is suitable to treat the transplant recipients and prevent subsequently the progression of graft injury and fibrosis.

In a particular embodiment, the invention is suitable to treat an endothelial activation related disease.

In another embodiment, the method according to the invention is suitable to prevent the graft loss. Typically, the invention relates to a method of preventing the graft loss in subject, comprising: i) identifying whether a transplanted subject is at risk of graft loss according to the method as described above and ii) treating said subject with a therapeutically effective amount of immunosuppressive drugs. More particularly, such subject as identified can also be treated with an anti-ABMR therapy.

As used herein, the term "subject" refers to a grafted subject (also known as transplanted subject). Particularly, the subject who has received an organ transplantation. The method as described above are suitable to use in a subject no renal transplanted recipients and thrombotic microangiopathy or hepatitis C infection related renal pathologies can developed on their own kidneys.

As used herein, the terms "treating" or "treatment" refer to both prophylactic or preventive treatment as well as curative or disease modifying treatment, including treatment of subject at risk of contracting the disease or suspected to have contracted the disease as well as subject who are ill or have been diagnosed as suffering from a disease or medical condition, and includes suppression of clinical relapse. The treatment may be administered to a subject having a medical disorder or who ultimately may acquire the disorder, in order to prevent, cure, delay the onset of, reduce the severity of, or ameliorate one or more symptoms of a disorder or recurring disorder, or in order to prolong the survival of a subject beyond that expected in the absence of such treatment. By "therapeutic regimen" is meant the pattern of treatment of an illness, e.g., the pattern of dosing used during therapy. A therapeutic regimen may include an induction regimen and a maintenance regimen. The phrase "induction regimen" or "induction period" refers to a therapeutic regimen (or the portion of a therapeutic regimen) that is used for the initial treatment of a disease. The general goal of an induction regimen is to provide a high level of drug to a subject during the initial period of a treatment regimen. An induction regimen may employ (in part or in whole) a "loading regimen", which may include administering a greater dose of the drug than a physician would employ during a maintenance regimen, administering a drug more frequently than a physician would

administer the drug during a maintenance regimen, or both. The phrase "maintenance regimen" or "maintenance period" refers to a therapeutic regimen (or the portion of a therapeutic regimen) that is used for the maintenance of a subject during treatment of an illness, e.g., to keep the subject in remission for long periods of time (months or years). A maintenance regimen may employ continuous therapy (e.g., administering a drug at a regular intervals, e.g., weekly, monthly, yearly, etc.) or intermittent therapy (e.g., interrupted treatment, intermittent treatment, treatment at relapse, or treatment upon achievement of a particular predetermined criteria).

As used herein the terms "administering" or "administration" refer to the act of injecting or otherwise physically delivering a substance as it exists outside the body (e.g., immunosuppressive drug) into the subject, such as by mucosal, intradermal, intravenous, subcutaneous, intramuscular delivery and/or any other method of physical delivery described herein or known in the art. When a disease, or a symptom thereof, is being treated, administration of the substance typically occurs after the onset of the disease or symptoms thereof. When a disease or symptoms thereof, are being prevented, administration of the substance typically occurs before the onset of the disease or symptoms thereof.

A “therapeutically effective amount” is intended for a minimal amount of active agent which is necessary to impart therapeutic benefit to a subject. For example, a "therapeutically effective amount" to a subject is such an amount which induces, ameliorates or otherwise causes an improvement in the pathological symptoms, disease progression or physiological conditions associated with or resistance to succumbing to a disorder. It will be understood that the total daily usage of the compounds of the present invention will be decided by the attending physician within the scope of sound medical judgment. The specific therapeutically effective dose level for any particular subject will depend upon a variety of factors including the disorder being treated and the severity of the disorder; activity of the specific compound employed; the specific composition employed, the age, body weight, general health, sex and diet of the subject; the time of administration, route of administration, and rate of excretion of the specific compound employed; the duration of the treatment; drugs used in combination or coincidential with the specific compound employed; and like factors well known in the medical arts. For example, it is well within the skill of the art to start doses of the compound at levels lower than those required to achieve the desired therapeutic effect and to gradually increase the dosage until the desired effect is achieved. However, the daily dosage of the products may be varied over a wide range from 0.01 to 1,000 mg per adult per day. Typically, the compositions contain 0.01, 0.05, 0.1, 0.5, 1.0, 2.5, 5.0, 10.0, 15.0, 25.0, 50.0, 100, 250 and

500 mg of the active ingredient for the symptomatic adjustment of the dosage to the subject to be treated. A medicament typically contains from about 0.01 mg to about 500 mg of the active ingredient, preferably from 1 mg to about 100 mg of the active ingredient. An effective amount of the drug is ordinarily supplied at a dosage level from 0.0002 mg/kg to about 20 mg/kg of body weight per day, especially from about 0.001 mg/kg to 7 mg/kg of body weight per day.

As used herein, the term "immunosuppressive drugs" also known as immunosuppressive agents or antirejection medications are drugs that inhibit or prevent the activity of immune system. Typically, the subject is treated with immunosuppressive drugs or other drugs that are currently known in the art or that will be identified in the future. In a particular embodiment, the subject is under immunosuppressive treatment, which means that the subject is administered with one or more immunosuppressive drugs. Immunosuppressive drugs that may be employed in transplantation procedures include corticosteroids, calcineurin inhibitors (cyclosporin, tacrolimus), azathioprine, mycophenolate mofetil and tyrosin kinase inhibitors (everolimus, sirolimus). These drugs may be used in monotherapy or in combination therapies.

As used herein the term "anti-ABMR therapy" includes plasmapheresis in association with high dose of intravenous immunoglobulins and rituximab (antibody against B lymphocyte surface antigen CD20), Bortezomib, (a proteasome inhibitor for depleting DSA producing cells (plasma cells) to reduce DSA level; eculizumab for complement C5 inhibition to reduce DSA associated graft injury as well as splenectomy.

Kit

In a sixth aspect, the present invention relates to a kit for predicting of chronic active form of ABMR with transplant glomerulopathy TG in a subject comprising at least one reagent for the determination of an expression level of fibronectin and/or collagen IV.

In particular embodiment, the invention relates to a kit for use in predicting of chronic active form of ABMR with transplant glomerulopathy TG comprising antibodies specific for fibronectin and/or collagen IV. Said kit is optimized for immunohistochemistry. The kit according to the invention further comprises a component for antigen retrieval, and immunohistochemistry visualization.

In another aspect, the present invention relates to a kit for early diagnosing of chronic active form of ABMR with transplant glomerulopathy TG in a subject comprising at least one reagent for the determination of an expression level of fibronectin and/or collagen IV.

In particular embodiment, the invention relates to a kit for use in diagnosing of chronic active form of ABMR with transplant glomerulopathy TG comprising antibodies specific for fibronectin and/or collagen IV.

As used herein, the term "a reagent for the determination of an expression level" is meant a reagent which specifically allows for the determination of said expression level, i.e. a reagent specifically intended for the specific determination of the expression level of fibronectin and/or collagen IV comprised in the expression profile. This definition excludes generic reagents useful for the determination of the expression level of any gene, such as taq polymerase or an amplification buffer, although such reagents may also be included in a kit according to the invention.

In some embodiments, the kit according to the invention may comprise instructions for determining whether a subject is at risk of having or developing chronic active ABMR, more particularly a caABMR with TG. The instructions for determining whether a subject has ABMR may include at least one reference expression profile.

In a particular embodiment, at least one reference expression profile is a stable expression profile. Alternatively, at least one reference expression profile may be a graft non-tolerant expression profile (e.g. expression profile obtained from a healthy subject).

The kit as described above is also used to predict the risk of having graft loss.

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: The level of expression of fibronectin and coll4 in GBM was significantly higher in the graft with ABMR ($p<0.0001$), and the highest in the graft with chronic ABMR ($p<0.0001$).

Figure 2: The expression of fibronectin (figure 2A) or coll4 (figure 2B) was determinant for the late graft dysfunction in the groups of patients with or without ABMR.

Figure 3: A predictive value of fibronectin detected in the GBM in the grafts with or without ABMR for graft loss.

Figure 4: Expression of pattern of fibronectin and collagen IV in a subject having healthy graft and a subject having an active ABMR.

EXAMPLES:

Example 1: Assessment of the expression of fibronectin and collagen type IV for the early diagnosis of chronic antibody mediated rejection in human renal engrafted patients and their predictive value for long term graft loss

In the present example, 203 biopsies patients containing 59 with ABMR and 144 without ABMR from 153 patients in a renal transplant center (Tenon hospital in Paris, France) were included in a study.

By immunohistochemistry, the fibronectin and collagen type IV (coll4) as well as fascin expression pattern in the glomeruli was analyzed and the importance of expression in the glomerular basement membrane (GBM) was semi quantified in all biopsies. And then, the expression was compared with that from control groups including 3 normal kidney samples obtained from the healthy part of kidneys removed because of a renal cancer, and 66 protocol biopsies for the graft surveillance without morphological lesions included in this studied cohort.

The results showed that in the normal kidneys or in the protocol renal graft biopsies without morphological lesions, the expression of fibronectin and coll4 in the glomeruli was limited in the mesangial area (Figure 4). No evident expression was detected in the GBM by immunohistochemistry with the technical sensibility of the method we used. However, the pattern of expression in the glomeruli was changed with an up-regulated and linear expression level in the GBM in the renal graft with antibody mediated rejection (ABMR)(Figure 4), especially in those with a chronic active form of ABMR (cABMR) with transplant glomerulopathy (TG)(Figure 4). The expression of fascin was limited in the peri-nuclear area of glomerular endothelial cells in normal kidney (Figure 4) or in the protocol renal graft biopsies without morphological lesions. But in the grafts with ABMR, we can see a strong and diffused cytoplasmic expression of fascin in the glomerular endothelial cells in the glomeruli and in the peri-tubular capillaries (Figure 4). The level of expression of FN and coll4 in the GBM was significantly correlated by a spearman's correlation test with the level of glomerular endothelial fascin expression (with rho=0.52 $p<0.0001$, and rho=0.42, $p<0.0001$ for FN and coll4 respectively). The level of expression of these early glomerular endothelial activation markers was also significantly correlated with the ABMR related Banff scores, such as the micro vascular inflammation in the glomeruli (glomerulitis: g), in the peri-tubular capillaries (peri-tubular capillaritis: ptc) and the glomerular morphological changes with double contour: cg, a typical morphological lesion for the TG diagnosis (with rho=0.44, $p<0.0001$; rho=0.41, $p<0.0001$; rho=0.61, $p<0.0001$ for fibronectin; rho=0.34, $p<0.0001$; rho=0.3, $p<0.0001$; rho=0.43, $p<0.0001$ for coll4; and rho=0.43, $p<0.0001$; rho=0.29,

p<0.0001; rho=0.37, p<0.0001 for fascin in glomeruli respectively). Moreover, the expression level of fibronectin, coll4 and fascin was significantly correlated with the level of donor specific antibodies (DSA) detected in the recipients at time of biopsy with rho=0.32, p<0.0001, and rho=0.22, p=0.0014 for the correlation of fibronectin expression with the level of DSA against class II and class I of HLA antigens respectively; and rho=0.22, p=0.003, and rho=0.3, p<0.0001 for the correlation of coll4 with the level of DSA against class II and class I of HLA antigens and rho=0.31, p<0.0001, and rho=0.25, p<0.0001 for the correlation of fascin expression in the glomeruli with the level of DSA against class II and class I of HLA antigens respectively. In addition, the level of expression of fibronectin was also significantly correlated with the level of c4d, a member of complement, detected at the peri tubular capillaries, another important diagnostic criteria of ABMR, with rho=0.244, p=0.0004. The level of expression of fibronectin and coll4 in GBM was significantly high in the graft with ABMR, and particularly higher in the graft with chronic ABMR (figure 1).

Moreover, we analyzed the relationship of the expression of fibronectin and coll4 in the GBM in the renal grafts with graft dysfunction at time of biopsy, and in the long term. We found a significant and negative correlation of expression in the GBM of these two extra cellular matrix with the graft function presented here with estimated glomerulo filtration rate (eGFR) at different time points (with rho=-0.35, p<0.0001; rho=-0.4, p<0.0001; rho=-0.43, p<0.0001; rho=-0.46, p<0.0001; rho=-0.49, p<0.0001; rho=-0.57, p<0.0001 for the correlation of fibronectin in the GBM with eGFR at time of biopsy or one, two, three, four and five years after biopsy, and with rho=-0.29, p<0.0001; rho=-0.24, p=0.0017; rho=-0.28, p=0.0008; rho=-0.33, p=0.0007; rho=-0.35, p=0.0037; rho=-0.4, p=0.0032 for the correlation of coll4 in the GBM with eGFR at time of biopsy, or one, two, three, four and five years after biopsy respectively). More interestingly, as we know that the persistent ABMR is the most important risk for the development of TG and for the long term renal graft loss, we studied the value of fibronectin and coll4 detection in the GBM for renal graft function loss prediction (which could be due to the late development of TG) in the groups of patients with ABMR or without in their renal grafts. The expression of fibronectin (figure 2a) or coll4 (figure 2b) was determinant for the late graft function loss in the groups of patients with or without ABMR. We found that the patients who had finbronectin and/or coll4 in the GBM, especially in the group of patients with ABMR lost very quickly their graft function during the transplant course. However the patients without fibronection and/or expression in the GBM kept very well their graft function up to 5 years post biopsy. More impressionably, in the group of patients with ABMR, a disease well known for its association with bad graft outcome, no

detectable fibronectin and/or coll4 in the GBM predicted a very good renal graft outcome in the long term. Logistic regression model showed fibronectin expression in the GBM to be a strong and independent predictor for late graft loss before death with an odds ratio (OR) of 4.17 (95% CI:1.5-11.6, p=0.006) after adjustment for almost all known risk factor for graft loss such as: capillaritis or glomeruloritis, c4d deposit in peri-tubular capillaries, presence of DSA against HLA II at time of biopsy, expanded criteria donor, donor's age, transplant duration and graft fibrosis score ci (table1). These results are in agreement with our knowledge that the development of TG is a major risk for late graft loss. This suggests also a predictive value of fibronectin and/or coll4 expression in the GBM for late TG development (studied in next paragraph) and points out the importance of using these interesting markers to detect the early occurrence of TG and to guide an accurate treatment in the early course of disease for the patients at risk, in order to avoid their graft loss.

Example 2: A predictive value of fibronectin and/or coll4 in the GBM for late occurrence of TG

To study the value of detection of fibronectin and/or coll4 expression in GBM for late caABMT prediction, we followed the patients who had not TG at time of biopsy examined by light microscope, and who had the subsequent biopsies to assess the late TG development during their transplant course, according to the fibronectin and/or coll4 GBM expression in their first biopsies. We found that amount 18 patients who had fibronectin and/or coll4 expression in their first biopsies, 9 out of 18 patients developed TG late; but TG development in late biopsies was observed only in 3 out of 51 patients who had no fibronectin and/or coll4 expression in their first studied biopsies, $p<0.0001$ by Fisher's exact test. Analysis by COX regression model for the patients without TG detected at time of biopsy showed that the expression of FN or coll4 in the GBM was the most important independent determinant for the late occurrence of TG and/or the graft loss with a Haz. ratio (HR) 2.96, (95% CI:1.28-6.8, p=0.011) after adjustment for the known risk factors for TG development or graft loss such as: capillaritis or glomeruloritis, presence of DSA against HLA I or II at time of biopsy, expanded criteria donor, transplant duration and graft fibrosis score ci (table2). These results demonstrate that fibronectin and/or coll4 expression in the GBM can predict a late occurrence of TG and could be an interesting biomarker for renal graft surveillance.

risk factors	HR	p	95% IC
fibronectin/coll4	4.14	0.002	1.65-10.4
Microvasculature inflammation g+ptc score	2.96	0.038	1.06-8.27
DSA for HLA class I or II	0.95	0.78	0.66-1.36
c4d in ptc	0.82	0.74	0.25-2.69
expanded criteria donor	2.66	0.095	0.84-8.38
donor's age	0.99	0.5	0.96-1.02
duration of transplant	1.002	0.39	0.9998-1.0006
graft fibrosis	1.7	0.022	1.08-2.68

Table 1: Independent risk factors for renal graft loss by Logistic regression model analysis. Fibronectin and/or coll4 expression in the GBM, microvasculature inflammation measured by g+ptc scores and pre-existent graft interstitial fibrosis ci score at time of biopsy are 3 independent risk factors associated with renal graft loss.

risk factors	HR	p	95% IC
fibronectin/coll4 c2	2.96	0.011	1.28-6.8
Microvasculature inflammation g+ptc score	2.64	0.04	1.05-6.6
DSA for HLA class I or II	0.99	0.92	0.75-1.3
expanded criteria donor	2.5	0.008	1.27-4.9
duration of transplant	1.0003	0.07	0.99998-1.0006
graft fibrosis	1.47	0.035	1.03-2.1

5

Table 2: Independent risk factors for late TG occurrence and/or renal graft loss in the group of patients without Tg at time of biopsy by COX regression model analysis. Fibronectin and/or coll4 expression in the GBM, microvasculature inflammation measured by g+ptc scores, expanded criteria donor and pre-existent graft interstitial fibrosis ci score at time of biopsy are 4 independent determinants for late occurrence of TG or graft loss in the group of patients without TG examined by light microscope at time of biopsy.

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. Kamal L, Broin PO, Bao Y, Ajaimy M, Lubetzky M, Gupta A, de Boccardo G, Pullman J, Golden A, Akalin E: Clinical, Histological, and Molecular Markers Associated With Allograft Loss in Transplant Glomerulopathy Patients. Transplantation 2015;99:1912-1918.
2. Cosio FG, Gloor JM, Sethi S, Stegall MD: Transplant glomerulopathy. Am J Transplant 2008;8:492-496.
3. Haas M, Sis B, Racusen LC, Solez K, Glotz D, Colvin RB, Castro MC, David DS, David-Neto E, Bagnasco SM, Cendales LC, Cornell LD, Demetris AJ, Drachenberg CB, Farver CF, Farris AB, 3rd, Gibson IW, Kraus E, Liapis H, Loupy A, Nickeleit V, Randhawa P, Rodriguez ER, Rush D, Smith RN, Tan CD, Wallace WD, Mengel M: Banff 2013 meeting report: inclusion of c4d-negative antibody-mediated rejection and antibody-associated arterial lesions. Am J Transplant 2014;14:272-283.
4. Loupy A, Haas M, Solez K, Racusen L, Glotz D, Seron D, Nankivell BJ, Colvin RB, Afrouzian M, Akalin E, Alachkar N, Bagnasco S, Becker JU, Cornell L, Drachenberg C, Dragun D, de Kort H, Gibson IW, Kraus ES, Lefaucheur C, Legendre C, Liapis H, Muthukumar T, Nickeleit V, Orandi B, Park W, Rabant M, Randhawa P, Reed EF, Roufosse C, Seshan SV, Sis B, Singh HK, Schinstock C, Tambur A, Zeevi A, Mengel M: The Banff 2015 Kidney Meeting Report: Current Challenges in Rejection Classification and Prospects for Adopting Molecular Pathology. Am J Transplant 2017;17:28-41.
5. Haas M, Loupy A, Lefaucheur C, Roufosse C, Glotz D, Seron D, Nankivell BJ, Halloran PF, Colvin RB, Akalin E, Alachkar N, Bagnasco S, Bouatou Y, Becker JU, Cornell LD, van Huyen JPD, Gibson IW, Kraus ES, Mannon RB, Naesens M, Nickeleit V, Nickerson P, Segev DL, Singh HK, Stegall M, Randhawa P, Racusen L, Solez K, Mengel M: The Banff 2017 Kidney Meeting Report: Revised diagnostic criteria for chronic active T cell-mediated rejection, antibody-mediated rejection, and prospects for integrative endpoints for next-generation clinical trials. Am J Transplant 2018;18:293-307.
6. Haas M, Mirocha J: Early ultrastructural changes in renal allografts: correlation with antibody-mediated rejection and transplant glomerulopathy. Am J Transplant 2011;11:2123-2131.
7. Xu-Dubois YC, Peltier J, Brocheriou I, Suberbielle-Boissel C, Djamali A, Reese S, Mooney N, Keuylian Z, Lion J, Ouali N, Levy PP, Jouanneau C, Rondeau E, Hertig A: Markers of Endothelial-to-Mesenchymal Transition: Evidence for Antibody-

Endothelium Interaction during Antibody-Mediated Rejection in Kidney Recipients. J Am Soc Nephrol 2015;27:324-332.

8. Discher DE, Mooney DJ, Zandstra PW: Growth factors, matrices, and forces combine and control stem cells. Science 2009;324:1673-1677.

9. Geiger B, Spatz JP, Bershadsky AD: Environmental sensing through focal adhesions. Nat Rev Mol Cell Biol 2009;10:21-33.

CLAIMS:

1. A method for predicting whether a transplant subject having the anti-donor specific antibodies (DSA) and/or suffering from an acute form of antibody-mediated rejection (ABMR), is at risk of having or developing a chronic form of ABMR with transplant glomerulopathy (TG), comprising the steps of: i) measuring the expression of fibronectin and/or type IV collagen in a biological sample obtained from said subject; ii) comparing the expression pattern and level measured at step i) with its predetermined reference value, and iii) concluding that the subject is at risk of having or developing chronic form of ABMR with transplant glomerulopathy (TG) when a linear expression of fibronectin and/or type IV collagen is observed along the GBM and the level of expression is higher than its predetermined reference value or concluding that the subject is not at risk of developing a chronic form of ABMR with transplant glomerulopathy (TG) when the expression pattern and level of fibronectin and/or type IV collagen is the same as its predetermined reference value.

2. A method for early diagnosing a chronic active form of antibody mediated rejection (caABMR) with transplant glomerulopathy (TG) for the renal transplanted patients having the anti-donor specific antibodies (DSA) and/or suffering from an acute form of ABMR, comprising the steps of: i) measuring the expression of fibronectin and/or type IV collagen in a biological sample obtained from said subject; ii); comparing the expression pattern and level measured at step i) with its predetermined reference value, and iii) concluding that the subject is having chronic active form of ABMR with transplant glomerulopathy (TG) when a linear expression of fibronectin and/or type IV collagen is observed along the GBM and the level of expression is higher than its predetermined reference value or concluding that the subject is not at risk of having chronic active form of ABMR with transplant glomerulopathy (TG) when the expression pattern and level of fibronectin and/or type IV collagen is the same as its predetermined reference value.

3. A method for predicting whether a subject suffering from thrombotic microangiopathy has the glomerular involvement and/or is at risk of having or developing a glomerular sclerosis and/or glomerular obsolescence, comprising the steps of: i) measuring the expression of fibronectin and/or type IV collagen in a biological sample obtained from

said subject; ii) comparing the expression pattern and level measured at step i) with its predetermined reference value, and iii) concluding that the subject is at risk of having or developing glomerular sclerosis and/or glomerular obsolescence when a linear expression of fibronectin and/or type IV collagen is observed along the GBM and level of expression is higher than its predetermined reference value or concluding that the subject is not at risk of developing glomerular sclerosis and/or glomerular obsolescence when the expression pattern and level of fibronectin and/or type IV collagen is the same as its predetermined reference value.

4. A method for predicting whether a subject suffering from hepatitis C infection has the glomerular involvement and/or is at risk of having or developing a glomerular sclerosis and/or glomerular obsolescence, comprising the steps of: i) measuring the expression of fibronectin and/or type IV collagen in a biological sample obtained from said subject; ii) comparing the expression pattern and level measured at step i) with its predetermined reference value, and iii) concluding that the subject is at risk of having or developing glomerular sclerosis and/or glomerular obsolescence when a linear expression of fibronectin and/or type IV collagen is observed along the GBM and level of expression is higher than its predetermined reference value or concluding that the subject is not at risk of developing a glomerular sclerosis and/or glomerular obsolescence when the expression pattern and level of fibronectin and/or type IV collagen is the same as its predetermined reference value.

5. A method for predicting whether a transplanted subject is at risk of graft loss comprising the steps of: i) measuring the expression pattern and level of fibronectin and/or collagen IV in a biological sample obtained from said subject; ii) comparing the expression pattern and level measured at step i) with its predetermined reference value, and iii) concluding that the subject is at risk of graft loss when a linear expression of fibronectin and/or type IV collagen is observed along the GBM and level of expression is higher than its predetermined reference value or concluding that the subject is not at risk of graft loss when the expression pattern and level of fibronectin and/or collagen IV is not different from its predetermined reference value.

6. The method according to claims 1 and 5, wherein, the subject is renal transplanted subject.

7. The method according to claims 1 to 5, wherein, the biological sample is a biopsy.

8. The method according to claims 1 to 5, wherein, the expression pattern and level of fibronectin and/or collagen IV are measured in the glomeruli.

9. A method of treating chronic active form of ABMR with transplant glomerulopathy (TG) in a subject comprising a step of administering to said subject a therapeutically effective amount of immunosuppressive drugs and anti-ABMR therapy.

10. The method according to claim 8 wherein, the method comprising following steps: i) identifying whether a subject having anti-donor specific antibodies (DSA) and/or suffering from ABMR is at risk of having or developing chronic active form of ABMR with transplant glomerulopathy (TG) according to claims 1 to 2 and ii) administering to said subject a therapeutically effective amount of immunosuppressive drugs and anti-ABMR therapy.

11. A kit for use in vitro diagnosing of chronic active form of ABMR with transplant glomerulopathy (TG) comprising antibodies specific for fibronectin and/or collagen IV.

12. A kit for early diagnosing of chronic active form of ABMR with transplant glomerulopathy TG in a subject comprising at least one reagent for the determination of an expression level of fibronectin and/or collagen IV.

13. The kit according to claims 11 to 12 comprises a component for antigen retrieval, and immunohistochemistry visualization.

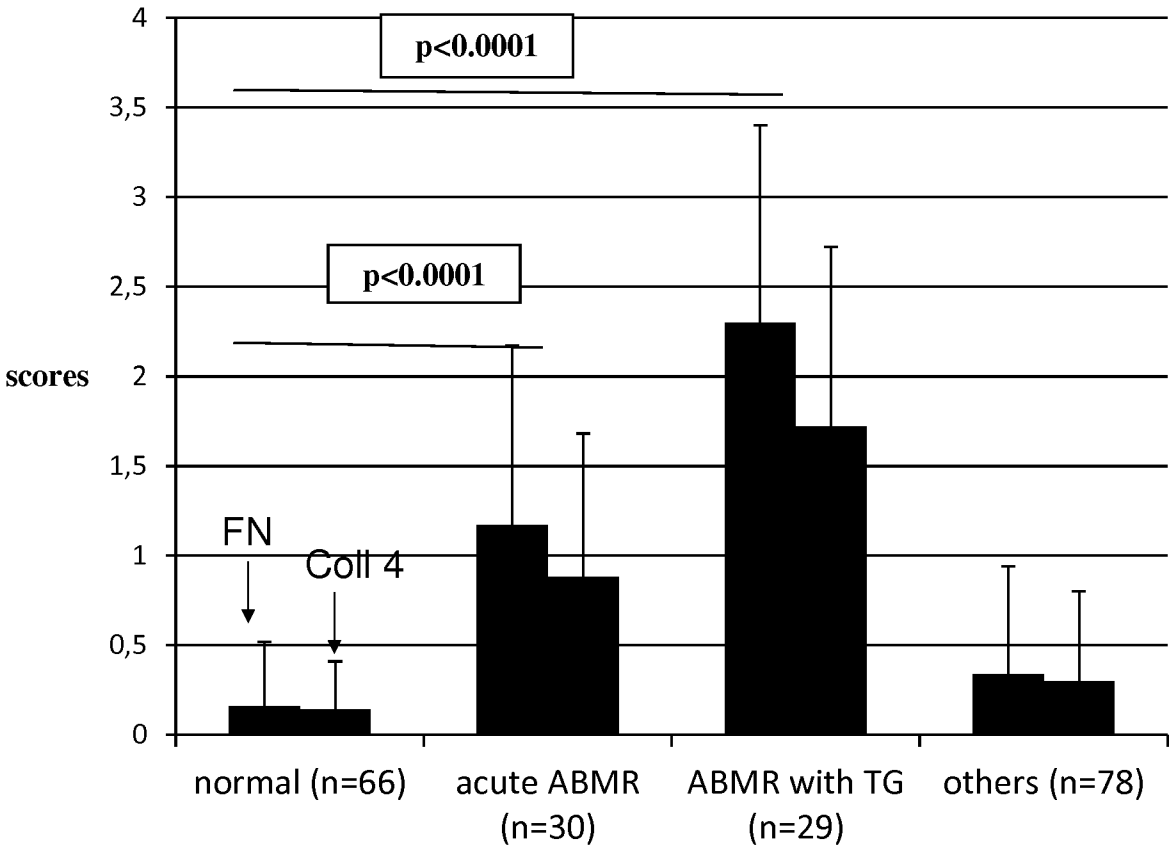
4
3,5
3
2,5
2
1,5
1
0,5
0
scores
p<0.0001
p<0.0001
FN
Coll 4
normal (n=66)
acute ABMR (n=30)
ABMR with TG (n=29)
others (n=78)

Figure 1

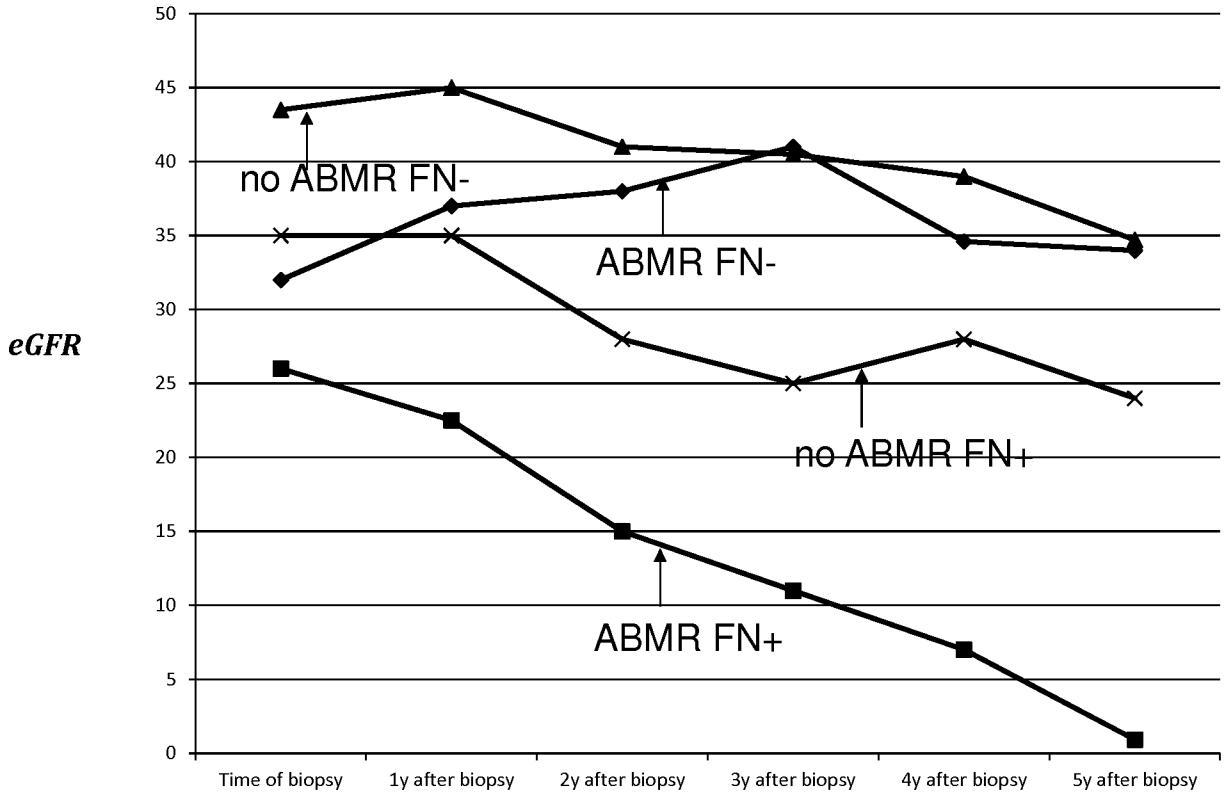

Figure 2A

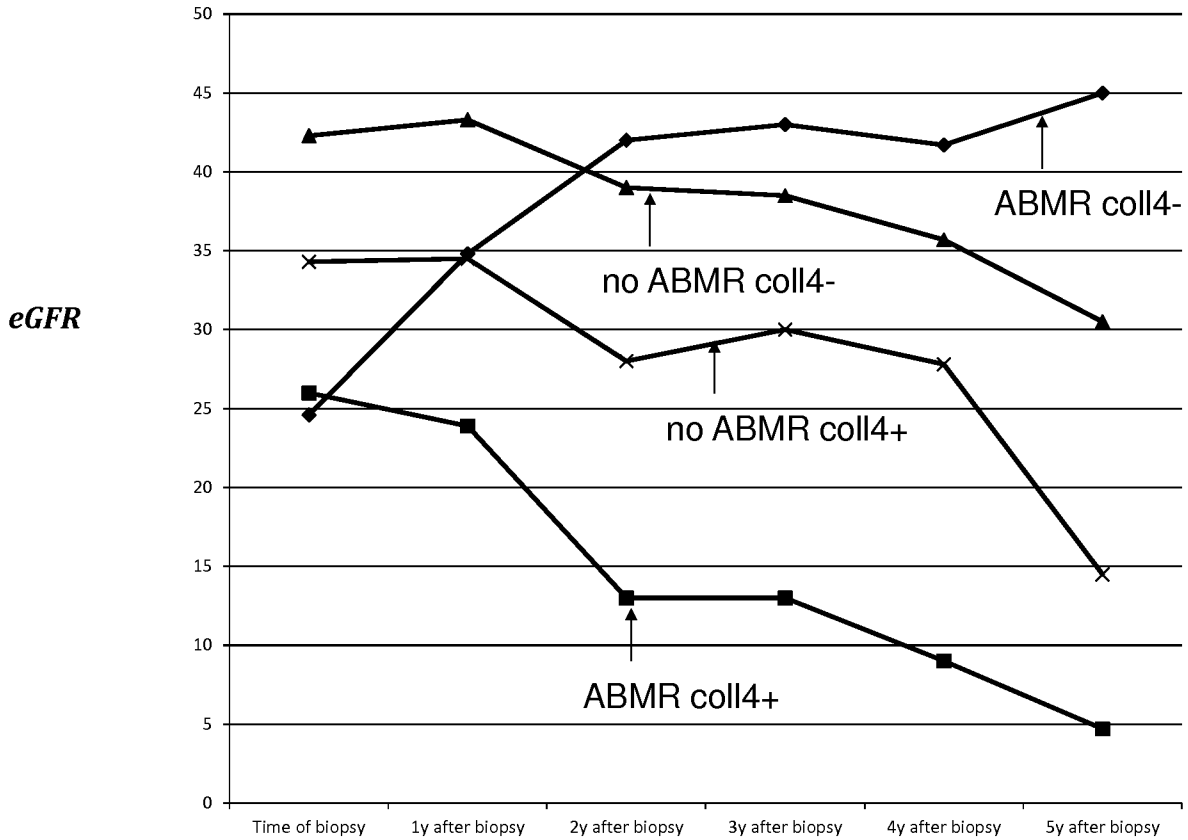

Figure 2B

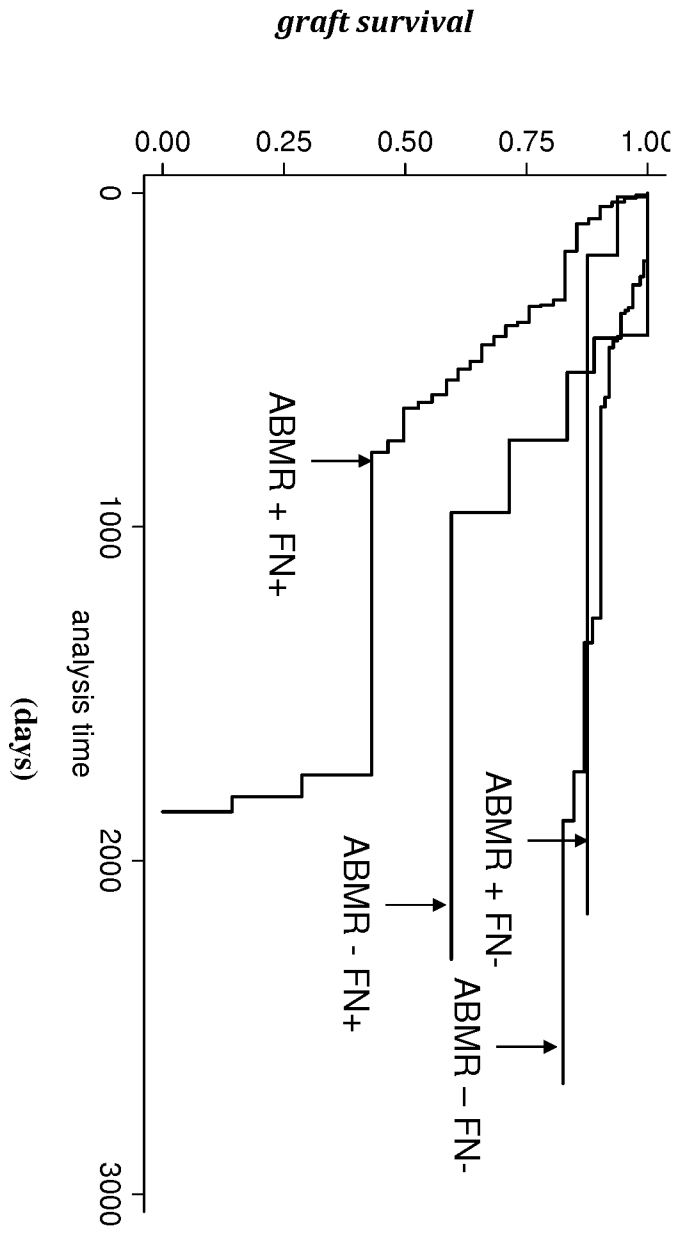
graft survival
0.00
0.25
0.50
0.75
1.00
0
1000
2000
3000
analysis time
(days)
ABMR + FN+
ABMR - FN+
ABMR + FN-
ABMR – FN-

Figure 3

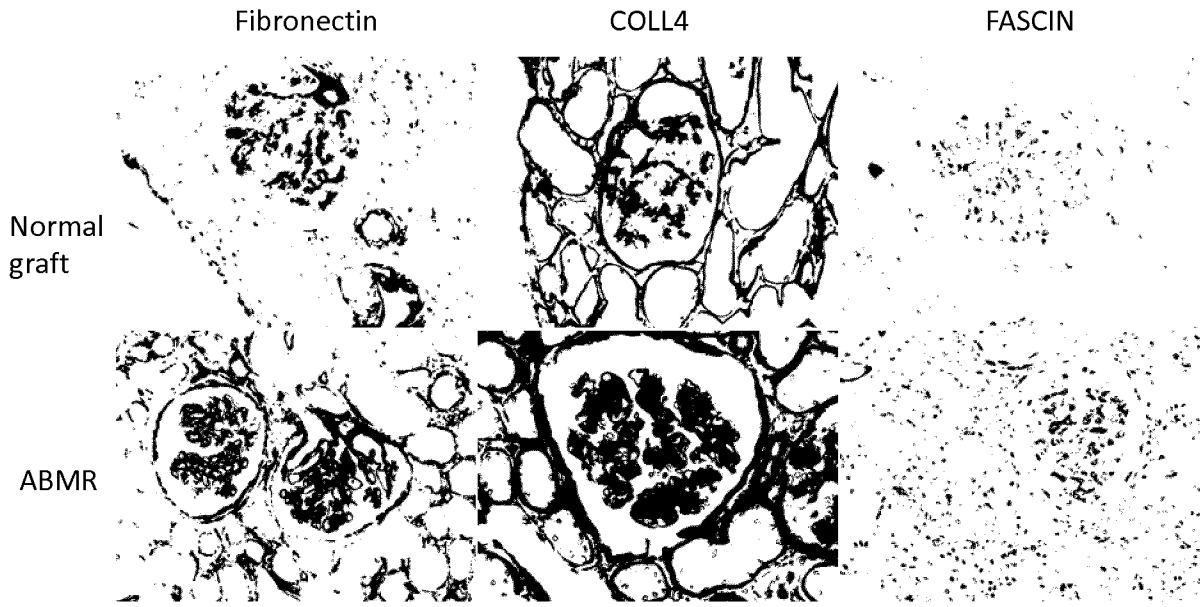

Figure 4

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2018/078795

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	D Koya ET AL: "Amelioration of accelerated diabetic mesangial expansion by treatment with a PKC beta inhibitor in diabetic db/db mice, a rodent model for type 2 diabetes", FASEB journal : official publication of the Federation of American Societies for Experimental Biology, 1 March 2000 (2000-03-01), pages 439-447, XP055429697, UNITED STATES Retrieved from the Internet: URL:http://www.fasebj.org/content/14/3/439.full.pdf [retrieved on 2017-12-13] p 441, col 1, para 1 ----- -/--	12,13

[X] 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	Date of mailing of the international search report
8 November 2018	16/01/2019

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Bigot-Maucher, Cora

1

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ANGASWAMY N ET AL: "Immune responses to collagen-IV and fibronectin in renal transplant recipients with transplant glomerulopathy", AMERICAN JOURNAL OF TRANSPLANTATION, WILEY INTERSCIENCE, vol. 14, no. 3, 28 February 2014 (2014-02-28), pages 685-693, XP009502016, ISSN: 1600-6143, DOI: 10.1111/AJT.12592 [retrieved on 2014-01-10] (p 685, col 2, para 1)(p 685, col 2, para 2); abstract	1,6-8
X	JEONG H J ET AL: "Alterations in extracellular matrix components in transplant glomerulopathy", VIRCHOWS ARCHIV, SPRINGER INTERNATIONAL, BERLIN, DE, vol. 437, no. 1, 1 July 2000 (2000-07-01), pages 69-73, XP009501989, ISSN: 0945-6317, DOI: 10.1007/S004280000193 abstract	1,6-8, 12,13
X	JEFFREY H MINER ED - RUAS JORGE ET AL: "The glomerular basement membrane", EXPERIMENTAL CELL RESEARCH, ELSEVIER, AMSTERDAM, NL, vol. 318, no. 9, 24 February 2012 (2012-02-24), pages 973-978, XP028413003, ISSN: 0014-4827, DOI: 10.1016/J.YEXCR.2012.02.031 [retrieved on 2012-03-05] abstract	1,6-8
X	CHIHARA RAY K ET AL: "Fibronectin from alpha 1,3-galactosyltransferase knockout pigs is a xenoantigen", JOURNAL OF SURGICAL RESEARCH, ACADEMIC PRESS INC., SAN DIEGO, CA, US, vol. 184, no. 2, 30 September 2013 (2013-09-30), pages 1123-1133, XP009502018, ISSN: 0022-4804, DOI: 10.1016/J.JSS.2013.04.012 abstract	1,6-8

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2018/078795

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1(completely); 6-8, 12, 13(partially)

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1(completely); 6-8, 12, 13(partially)

   method for predicting whether a transplant subject having the anti/donor specific antibodies and/or suffering from an acute form of antibody mediated rejection is at risk of having or developing a chronic form of antibody mediated rejection with transplant glomerulopathy and kit comprising antibodies specific for fibronectin and type IV collagen.

   ---

2. claims: 2, 9-11(completely); 6-8, 12, 13(partially)

   method for early diagnosis of a chronic active form of antibody mediated rejection (caABMR) with transplant glomerulopathy (TG) for the renal transplanted patients having anti-donor specific antibodies; treating the active chronic form of antibody mediated rejection with transplant glomerulopathy and kit for use in in vitro diagnosing of chronic active form of ABMR with transplant glomerulopathy

   ---

3. claims: 3(completely); 6-8, 12, 13(partially)

   method for predicting whether a subject suffering from thrombotic microangiopathy is at risk of having or developing a glomerular sclerosis and/or obsolescence and kit comprising antibodies specific for fibronectin and type IV collagen.

   ---

4. claims: 4(completely); 6-8, 12, 13(partially)

   method for predicting whether a subject suffering from hepatitis C infection is at risk of having or developing a glomerular sclerosis and/or glomerular obsolescence and kit comprising antibodies specific for fibronectin and type IV collagen.

   ---

5. claims: 5(completely); 6-8, 12, 13(partially)

   method for predicting whether a transplanted subject is at risk of graft loss and kit comprising antibodies specific for fibronectin and type IV collagen.
