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(54) Title: A NEW BIOMARKER OF ANTIBODY-MEDIATED REJECTION

(57) Abstract: The invention relates to a method for determining whether a transplanted patient has or is at risk of antibody-mediated rejection (ABMR), comprising a step of determining the expression level of the Notch3 gene in pericytes present on a transplant biopsy obtained from said patient. The invention also relates to a method for adjusting the immunosuppressive treatment administered to a transplanted recipient following its transplantation, comprising the following steps of: (i) performing the method for determining whether a transplanted patient is at risk of ABMR according to the invention, and (ii) adjusting the immunosuppressive treatment.



A NEW BIOMARKER OF ANTIBODY-MEDIATED REJECTION

FIELD OF THE INVENTION:

5 The invention relates to the field of medicine, and more particularly to the diagnosis or the prediction of transplant antibody-mediated rejection (ABMR).

BACKGROUND OF THE INVENTION:

10 In transplantation, the antibody-mediated rejection (ABMR) is currently a major thread for the long term graft survival. Although important advances have been made in the detection of preexistent donor-specific antibodies (DSA) and the use of desensitization protocols before transplantation avoids efficiently the hyperacute form of ABMR, the early/acute ABMR developed in the cross-match positive, but desensitized patients still occurs; and the late/chronic ABMR because of the presence of *de novo* DSA or the increased
15 activity of low preexisting title of DSA now emerges as a leading cause of late graft loss. This form of ABMR is usually at beginning indolent, but it destroys progressively the graft structure. When the graft function starts to be altered, the pharmaceutical intervention often has no efficiency because the chronic ABMR associated graft fibrosis is too extensive and is no longer reversible. However the diagnosis of the ABMR remains still difficult and mainly
20 based on a triad of criteria: 1) the presence in the recipient's plasma of donor specific antibodies (DSA) for HLA antigens; 2) inflammation in the micro-circulation of the allograft such as peri-tubular capillaritis (ptc) and glomerulitis; and 3) deposits of C4d (a fragment of complement) on the endothelial cells of peritubular capillaries.

25 The medical community stresses the necessity of developing new biomarkers in predicting and diagnosing ABMR because the current criteria are neither necessary nor sufficient: the presence of DSA in the plasma of patients is a pre-requisite for the diagnosis of ABMR, but it is not always associated with ABMR. In addition, some antibodies may be present in the context of accommodation that is without significant harmful effect even if C4d
30 is detected. In contrast, some anti-donor antibodies directed against MICA or MICB antigens or other anti-endothelial cell antibodies are not included in the panel of DSA detection. The presence of C4d on the peritubular capillaries is a good witness of complement activation thus strongly suggestive of ABMR. Yet, recent studies pointed out its poor sensitivity as a diagnostic tool, since it can detect only 50-60% of ABMR patients.

It results that it is likely that many patients with ABMR are underdiagnosed and not appropriately treated. Yet, early and accurate diagnosis of ABMR is thus important because a specific treatment, different from that of T cell-mediated rejection (TCMR), can be proposed for these patients including anti-CD20 antibodies (rituximab). Otherwise, in the absence of appropriate treatment, the patients can lose their graft rapidly. In addition, when the diagnostic can be made with certainty, undue over-immunosuppression will be prevented.

SUMMARY OF THE INVENTION:

In a first aspect, the invention relates to a method for determining whether a transplanted patient has or is at risk of antibody-mediated rejection (ABMR), comprising a step of determining the expression level of the Notch3 gene in pericytes present on a transplant biopsy obtained from said patient.

In a second aspect, the invention also relates to a method for adjusting the immunosuppressive treatment administered to a transplanted patient following its transplantation, comprising the following steps of: (i) performing the method for determining whether a transplanted patient has or is at risk of ABMR of the invention, and (ii) adjusting the immunosuppressive treatment.

In a third aspect, the invention also relates to a compound selected from the group consisting of monoclonal anti-CD20 antibodies, anti-thymocyte globulin (ATG), proteasome inhibitors, anti-C5 antibodies, for use in preventing ABMR or progression of ABMR in a transplanted patient, determined as having or being at risk of ABMR by an increase in the expression level of the Notch3 gene in pericytes present on a transplant biopsy obtained from said patient.

DETAILED DESCRIPTION OF THE INVENTION:

The invention addresses these needs, as it relates to methods and treatment approaches useful in the prediction, diagnosis and prevention of development and progression of antibody-mediated transplant rejection (e.g., in kidney transplantation).

The inventors have demonstrated that the expression level of Notch3 in pericytes present on a transplant biopsy is useful to specifically predict ABMR, until two years before said ABMR and even if said biopsy displays no classical sign of rejection. The inventors have thus shown that Notch3 expression is highly induced in the peritubular capillary pericytes in the renal transplanted patients with ABMR.

Prognostic methods of the invention:

In a first aspect, the invention relates to a method for determining whether a
5 transplanted patient has or is at risk of antibody-mediated rejection (ABMR), comprising a
step of determining the expression level of the Notch3 gene in pericytes present on a
transplant biopsy obtained from said patient.

As used herein, the term "risk" refers to the probability that an event will occur over a
10 specific time period, such as the onset of transplant rejection, and can mean a patient'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.
15 Relative risk refers to the ratio of absolute risks of a patient 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.

As used herein, the term "risk determination" encompasses making a prediction of the
probability, odds, or likelihood that an event may occur. Risk determination can also comprise
prediction of future clinical parameters, traditional laboratory risk factor values, such age, sex
mismatch, HLA-testing, etc ...; either in absolute or relative terms in reference to a previously
25 measured population. The methods of the invention may be used to make categorical
measurements of the risk of transplant rejection, thus defining the risk spectrum of a category
of transplanted patient defined as being at risk of transplant rejection.

As used herein, the term "transplant rejection" or "graft rejection" or encompasses
30 both acute and chronic transplant rejection. "Acute rejection" is the rejection by the immune
system of a tissue transplant recipient when the transplanted tissue is immunologically
foreign. Acute rejection is characterized by infiltration of the transplant tissue by immune
cells of the recipient, which carry out their effector function and destroy the transplant tissue.
The onset of acute rejection is rapid and generally occurs in humans within a few weeks after

transplant surgery. Generally, acute rejection can be inhibited or suppressed with immunosuppressive drugs such as rapamycin, cyclosporin, anti-CD40L monoclonal antibody and the like. "Chronic transplant rejection" generally occurs in humans within several months to years after engraftment, even in the presence of successful immunosuppression of acute rejection. Fibrosis is a common factor in chronic rejection of all types of organ transplants.

The term "transplantation" and variations thereof refers to the insertion of a transplant (also called graft) into a recipient, whether the transplantation is syngeneic (where the donor and recipient are genetically identical), allogeneic (where the donor and recipient are of different genetic origins but of the same species), or xenogeneic (where the donor and recipient are from different species). Thus, in a typical scenario, the host is human and the graft is an isograft, derived from a human of the same or different genetic origins. In another scenario, the graft is derived from a species different from that into which it is transplanted, including animals from phylogenically widely separated species.

As used herein, the term "Notch3 gene" belongs to the family of highly conserved Notch/LIN-12 receptors, which includes 4 members in vertebrates. It encodes a protein of 2321 amino acids that includes all canonical Notch motifs, i.e., an extracellular domain containing 34 tandem EGF-like repeats, 3 cysteine-rich Notch/LIN-12 repeats, a single transmembrane domain, and an intracellular domain containing 6 tandem ankyrin repeats. Mutations in the NOTCH3 gene have been found to cause cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy, commonly known as CADASIL. The naturally occurring human Notch3 receptor protein has an amino acid sequence of 2321 amino acids provided in the UniProt database under accession number Q9UM47 and is shown as follows (SEQ ID NO: 1):

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MGPGARGRRRRRRPMSPPPPPPVRLPLLLLLAGPGAAAPPCLDGSPCANGGRCTQ
LPSREAACLCPPGWVGERCQLEDPCHSGPCAGRGVCQSSVVAGTARFSCRCPRGFRG
PDCSLPDPCLSSPCA HGARCSVGP DGRFLCSCPPGYQGRSCRSDVDECRVGEPCRHG
GTCLNTPGSFRCQCPAGYTGPLCENPAVPCAPSPCRNGGTCRQSGDLTYDCACLPGF
EGQNCEVNVD DCPGHRCLNNGGTCVDGVNTYNCQCPPEWTGQFCTEDVDECQLQPN
ACHNGGTCFNTLGGHSCV CVNGWTGESCSQNIDDCATAVCFHGATCHDRVASFYCA
CPMGKTGLLCHLDDACVSNPCHEDAICDTNPVNGRAICTCPPGFTGGACDQDVDECS
IGANPCEHLGRCVNTQGSFLCQCGRGYTGPRCETDVNECLSGPCR NQATCLDRIGQF
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TCICMAGFTGTYCEVDIDECQSSPCVNGGVCKDRVNGFSCTCPSGFSGSTCQLDVDE
CASTPCRNGAKCVDQPDGYECRAEGFEGTLCDRNVDDCSPDPCHHGRCVDGIASF
CACAPGYTGTRCESQVDECRSQPCRHHGGKCLDLVDKYLRCRPSGTTGVNCEVNIDD
CASNPCTFGVCRDGINRYDCVCQPGFTGPLCNVEINECASSPCGEGGSCVDGENGFRC
5 LCPPGSLPPLCLPPSHPCAHEPCSHGICYDAPGGFRCVCEPGWSGPRCSQSLARDACES
QPCRAGGTCSSDGMGFHCTCPPGVQGRQCELLSPCTPNPCEHGGRCESAPGQLPVCS
CPQGWQGPRCQQDVDECAGPAPCGPHGICTNLAGSFSTCHGGYTGPSCDQDINDC
DPNPCLNGGSCQDGVGSFSCSCLPGFAGPRCARDVDECLSNPCGPGTCTDHVASFTC
TCPPGYGGFHCEQDLPCSPSSCFNGGTCVDGVNSFSCLCRPGYTGAHCQHEADPCL
10 SRPCLHGGVCSAAHPGFRCTCLESFTGPQCQTLVDWCSRQPCQNGGRCVQTGAYCL
CPPGWSGRLCDIRSLPCREAAAQIGVRLEQLCQAGGQCVDEDSSHVCVCEGRTGSH
CEQEVDPCLAQPCQHGGTCRGYMGGYMCECLPGYNGDNCEDDVDECASQPCQHGG
SCIDLVARLYLCSCPPGTLGLVLCIINEDDCGPGPLDSGPRCLHNGTCVDLVGGFRCTC
PPGYTGLRCEADINECRSGACHAAHTRDCLQDPGGGFRCLCHAGFSGPRCQTVLSPC
15 ESQPCQHGGQCRPSPGPGGGLTFTCHCAQPFWGPRCERVARSRELQCPVGVPCQQT
PRGPRCACPPGLSGPSCRSFPGSPPGASNASCAAAPCLHGGSCRPAPLAPFFRCACAQ
GWTGPRCEAPAAPEVSEEPRCPRAACQAKRGDQRCDRECNSPGCGWDGGDCSLSV
GDPWRQCEALQCWRLFNNSRCDPACSSPACLYDNFDCHAGGRERTCNPVYEKYCA
DHFADGRCDQGCNTEECGWDGLDCASEVPALLARGVLVLTVLLPPEELLRSSADFLQ
20 RLSAILRTSLRFRDLAHGQAMVFPYHRPSPGSEPRARRELAPEVIGSVVMLEIDNRLCL
QSPENDHCFPDAQSAADYLGALSAVERLDFPYPLRDVRGEPEPEPSVPLLPLLVAG
AVLLLVLVLGVMVARRKREHSTLWFPEGFSLHKDVASGHKGRREPVGQDALGMK
NMAKGESLMGEVATDWMDETECEAKRLKVEEPMGAEEAVDCRQWTQHHLVAA
DIRVAPAMALTTPQGDADADGMDVNVRGPDGFTPLMLASFCCGALEPMPTEEDEAD
25 DTSASIISDLICQGAQLGARTDRTGETALHLAARYARADAAKRLLDAGADTNAQDHS
GRTPLHTAVTADAQGVFQILIRNRSTDLDARMADGSTALILAAARLAVEGMVEELIAS
HADVNAVDELGKSALHWAAAVNNVEATLALLKNGANKDMQDSKEETPLFLAARE
GSYEAALKLLLDHFANREITDHLDRLPDVAQERLHQDIVRLLDQPSGPRSPPGPHGLG
PLLCPPGAFLPGLKAAQSGSKKSRRPPGKAGLGPQGPRGRGKKLTLACPGPLADSSVT
30 LSPVDSLDSRPFGGPPASPGGFLEGPYAAATATAVSLAQLGGPGRAGLGRQPPGGC
VLSLGLLNPAVPLDWARLPAPPGPSFLLPLAPGPQLLNPGTPVSPQERPPPYLAVP
GHGEEYPAAGAHSSPPKARFLRVPSEHPYLTPSPESPEHWASPPPSLSDWSESTPSA
TATGAMATTTGALPAQPLPLSVPSLAQAQTQLGPQPEVTPKRQVLA

As used herein, the term "gene" refers to a DNA sequence that codes for or corresponds to a particular sequence of amino acids which comprise all or part of one or more proteins or enzymes, and may or may not include regulatory DNA sequences, such as promoter sequences, which determine for example the conditions under which the gene is expressed. Some genes, which are not structural genes, may be transcribed from DNA to RNA, but are not translated into an amino acid sequence. Other genes may function as regulators of structural genes or as regulators of DNA transcription. In particular, the term gene may be intended for the genomic sequence encoding a protein, i.e. a sequence comprising regulator, promoter, intron and exon sequences.

A "coding sequence" or a sequence "encoding" an expression product, such as a RNA, polypeptide, protein, or enzyme, is a nucleotide sequence that, when expressed, results in the production of that RNA, polypeptide, protein, or enzyme, i.e., the nucleotide sequence encodes an amino acid sequence for that polypeptide, protein or enzyme. A coding sequence for a protein may include a start codon (usually ATG) and a stop codon.

As used herein, the term "determining" includes qualitative and/or quantitative determination (i.e. detecting and/or measuring the expression level) with or without reference to a control or a predetermined value. As used herein, "detecting" means determining if Notch3 is present or not in a biological sample and "measuring" means determining the amount of Notch3 in a biological sample. Typically the expression level may be determined for example by immunohistochemistry performed on a transplant biopsy obtained from the recipient (e.g., a transplant renal biopsy).

As used herein, the term "transplant biopsy" has its general meaning in the art and refers to any biopsy sample which may be obtained from a transplanted patient. A preferred biological sample is a renal biopsy that can be used for immunohistochemistry (IHC).

As used herein, the terms "pericyte" or "microvascular perivascular cell" are well known in the art and refer to small ovoid shaped cell with many finger-like projections that parallel the capillary axis and partially surround an endothelial cell in a microvessel. Pericytes are associated with capillaries and post-capillary vessels. In fact, there are pericytes in all tissues containing capillaries. Pericytes are elongated cells with the power of contraction that have been observed to have a variety of functional characteristics.

In one embodiment, the pericytes are pericytes of peritubular capillaries.

In the setting of transplantation, it is envisioned that that ABMR of non-renal
5 transplants would as well be diagnosed using the invention because the microvascular damage induced by anti-donor HLA antibodies is broad and not organ-specific. Accordingly, the invention also encompasses various types of transplants including, but not limited to, a renal allograft a heart transplant, a lung allograft, a liver allograft or a pancreas allograft.

10 In one embodiment, the transplanted patient is a transplanted renal patient.

In one embodiment, said method comprises a step of (i) determining the expression level of the Notch3 gene in a transplant biopsy obtained from said patient, and (ii) comparing said expression level with a predetermined reference value, wherein an increase in the
15 expression level of the Notch3 gene is indicative of having or being at risk of ABMR.

As used herein, the term "predetermined reference value" refers to the amount of Notch3 in biological samples obtained from the general population or from a selected population of subjects. The predetermined reference value can be a threshold value or a range.
20 For example, the selected population may be comprised of apparently healthy transplanted patient, such as individuals who have not previously had any sign or symptoms indicating the outcome of a graft rejection, more particularly ABMR.

Methods for determining the expression level of the biomarker of the invention:

25 Determination of the expression level of *Notch3* gene may be performed by a variety of techniques. Generally, the expression level as determined is a relative expression level. For example, the determination comprises contacting the biological sample with selective reagents such as probes or ligands, and thereby detecting the presence, or measuring the amount, of nucleic acids or polypeptides of interest originally in said biological sample.
30 Contacting may be performed in any suitable device, such as a plate, microtiter dish, test tube, well, glass, column, and so forth. In specific embodiments, the contacting is performed on a substrate coated with the reagent, such as a nucleic acid array or a specific ligand array. The substrate may be a solid or semi-solid substrate such as any suitable support comprising glass, plastic, nylon, paper, metal, polymers and the like. The substrate may be of various forms and

sizes, such as a slide, a membrane, a bead, a column, a gel, etc. The contacting may be made under any condition suitable for a detectable complex, such as a nucleic acid hybrid or an antibody-antigen complex, to be formed between the reagent and the nucleic acids or polypeptides of the biological sample.

5

In a particular embodiment, the expression level of the *Notch3* gene may be determined by determining the quantity of mRNA.

Methods for determining the quantity of mRNA are well known in the art. For
10 example the nucleic acid contained in the biological samples (e.g., biopsy prepared from the patient) is first extracted according to standard methods, for example using lytic enzymes or chemical solutions or extracted by nucleic-acid-binding resins following the manufacturer's instructions. The extracted mRNA is then detected by hybridization (e.g., Northern blot analysis) and/or amplification (e.g., RT-PCR). Quantitative or semi-quantitative RT-PCR is
15 preferred. Real-time quantitative or semi-quantitative RT-PCR is particularly advantageous.

In a particular embodiment, the methods of the invention comprise a step of carrying out an *in situ* hybridization (ISH).

20 In the context of the invention, "hybridization" relates to the fact of obtaining a close interaction of the nucleotide probe and the target region that is expected to be revealed by the detection of the nucleotide probe. Such an interaction can be achieved by the formation of hydrogen bonds between the nucleotide probe and the target sequence, which is typical of the interactions between complementary nucleotide molecules capable of base pairing. Hydrogen
25 bonds can be found, for example, in the annealing of two complementary strands of DNA.

"*In situ* hybridization" (ISH) thus refers to a type of hybridization that uses a labelled complementary DNA or RNA strand (i.e., probe) to localize a specific DNA or RNA sequence in a portion or section of tissue (in situ). RNA ISH (RNA in situ hybridization) is
30 used to measure and localize RNAs (e.g., mRNAs) within tissue sections. Preferably, the integrity of the structure and/or content of the biological material is maintained. Therefore, in order to achieve the invention, the biological material is preferably fixed.

Nucleic acids having at least 10 nucleotides and exhibiting sequence complementarity or homology to the mRNA of interest herein find utility as hybridization probes. It is understood that such nucleic acids need not be identical, but are typically at least about 80% identical to the homologous region of comparable size, more preferably 85% identical and even more preferably 90-95% identical. Probes typically comprise single-stranded nucleic acids of between 10 to 1000 nucleotides in length, for instance of between 10 and 800, more preferably of between 15 and 700, typically of between 20 and 500. The probes and primers are "**specific**" to the nucleic acids they hybridize to, i.e. they preferably hybridize under high stringency hybridization conditions (corresponding to the highest melting temperature T_m , e.g., 50 % formamide, 5x or 6x SCC. SCC is a 0.15 M NaCl, 0.015 M Na-citrate).

In a particular embodiment, the expression level of the *Notch3* gene may be determined by determining of the quantity of protein encoded by the *Notch3* gene.

Such methods comprise contacting the biological sample with a binding partner capable of selectively interacting with the protein present in said sample. The binding partner is generally an antibody that may be polyclonal or monoclonal, preferably monoclonal. Antibodies directed against Notch3 are well known from the skilled man in the art such as the antibodies commercialized by Abcam (ab23426, rabbit polyclonal). Antibodies that bind specifically to human Notch3 and which are useful in the detection and/or diagnosis of Notch3 related diseases are disclosed in the US patent application N° US2012142899

As used herein, the term "monoclonal antibody" refers to a population of antibody molecules that contains only one species of antibody combining site capable of immunoreacting with a particular epitope. A monoclonal antibody thus typically displays a single binding affinity for any epitope with which it immunoreacts. A monoclonal antibody may therefore contain an antibody molecule having a plurality of antibody combining sites, each immunospecific for a different epitope, e.g. a bispecific monoclonal antibody. Although historically a monoclonal antibody was produced by immortalization of a clonally pure immunoglobulin secreting cell line, a monoclonally pure population of antibody molecules can also be prepared by the methods of the invention.

Laboratory methods for preparing monoclonal antibodies are well known in the art (see, for example, Harlow et al., 1988). Monoclonal antibodies (mAbs) may be prepared by

immunizing purified Notch3 into a mammal, e.g. a mouse, rat, human and the like mammals. The antibody-producing cells in the immunized mammal are isolated and fused with myeloma or heteromyeloma cells to produce hybrid cells (hybridoma). The hybridoma cells producing the monoclonal antibodies are utilized as a source of the desired monoclonal antibody. This
5 standard method of hybridoma culture is described in Kohler and Milstein (1975).

While mAbs can be produced by hybridoma culture the invention is not to be so limited. Also contemplated is the use of mAbs produced by an expressing nucleic acid cloned from a hybridoma of this invention. That is, the nucleic acid expressing the molecules
10 secreted by a hybridoma of this invention can be transferred into another cell line to produce a transformant. The transformant is genotypically distinct from the original hybridoma but is also capable of producing antibody molecules of this invention, including immunologically active fragments of whole antibody molecules, corresponding to those secreted by the hybridoma. See, for example, U.S. Pat. No. 4,642,334 to Reading; European Patent
15 Publications No. 0239400 to Winter et al. and No. 0125023 to Cabilly et al.

Antibody generation techniques not involving immunisation are also contemplated such as for example using phage display technology to examine naive libraries (from non-immunised animals); see Barbas et al. (1992), and Waterhouse et al. (1993).

Alternatively, binding agents other than antibodies may be used for the purpose of the invention. These may be for instance aptamers, which are a class of molecule that represents an alternative to antibodies in term of molecular recognition. Aptamers are oligonucleotide or oligopeptide sequences with the capacity to recognize virtually any class of target molecules
25 with high affinity and specificity. Such ligands may be isolated through Systematic Evolution of Ligands by EXponential enrichment (SELEX) of a random sequence library, as described in Tuerk C. and Gold L., 1990. The random sequence library is obtainable by combinatorial chemical synthesis of DNA. In this library, each member is a linear oligomer, eventually chemically modified, of a unique sequence. Possible modifications, uses and advantages of
30 this class of molecules have been reviewed in Jayasena S.D., 1999. Peptide aptamers consists of a conformationally constrained antibody variable region displayed by a platform protein, such as E. coli Thioredoxin A that are selected from combinatorial libraries by two hybrid methods (Colas et al., 1996).

As used herein, the term "labelled" with regard to the antibody or aptamer, is intended to encompass direct labelling of the antibody or aptamer by coupling (i.e., physically linking) a detectable substance, such as a radioactive agent or a fluorophore (e.g. fluorescein isothiocyanate (FITC) or phycoerythrin (PE) or indocyanine (Cy5), to the antibody or aptamer, as well as indirect labelling of the probe or antibody (e.g., horseradish peroxidase, HRP) by reactivity with a detectable substance. An antibody or aptamer may be also labelled with a radioactive molecule by any method known in the art. For example, radioactive molecules include but are not limited radioactive atom for scintigraphic studies such as I^{123} , I^{124} , In^{111} , Re^{186} and Re^{188} .

More particularly, an immunohistochemistry (IHC) method may be used.

IHC specifically provides a method of detecting a target protein in a biological sample or tissue specimen *in situ*. The overall cellular integrity of the sample is maintained in IHC, thus allowing detection of both the presence and location of the protein of interest. Typically a biological sample is fixed with formalin, embedded in paraffin and cut into sections for staining and subsequent inspection by light microscopy. Current methods of IHC use either direct labelling or secondary antibody-based or hapten-based labelling. Examples of known IHC systems include, for example, EnVision™ (DakoCytomation), Powervision® (Immunovision, Springdale, AZ), the NBA™ kit (Zymed Laboratories Inc., South San Francisco, CA), HistoFine® (Nichirei Corp, Tokyo, Japan).

Accordingly, the invention also relates to a kit for performing a method above-mentioned, wherein said kit comprises means for determining the expression level of the *Notch3* gene in a biopsy sample obtained from said transplanted patient.

In another aspect, the invention relates to the use of Notch3 as a biomarker of ABMR.

The term "biomarker", as used herein, refers generally to a molecule, i.e., a gene (or nucleic acid encoding said gene), protein, the expression of which in a biological sample from a patient can be detected by standard methods in the art (as well as those disclosed herein), and is predictive or denotes a condition of the patient from which it was obtained.

In one embodiment, the invention relates to the use of Notch3 as a biomarker of ABMR in a transplanted renal patient.

Methods for adjusting an immunosuppressive treatment:

The invention further provides methods for developing personalized treatment plans.

5 Information gained by way of the methods described above can be used to develop a personalized treatment plan for a transplant recipient.

10 Accordingly, in a further aspect, the invention relates to a method for adjusting the immunosuppressive treatment administered to a transplanted patient following its transplantation, comprising the following steps of: (i) performing the method for determining whether a transplanted patient has or is at risk of ABMR of the invention, and (ii) adjusting the immunosuppressive treatment.

15 The methods can be carried out by, for example, using any of the methods for determining risk described above and, in consideration of the results obtained, designing a treatment plan for the transplant recipient. If Notch3 is present in pericytes on a kidney biopsy obtained from a patient of interest, this indicates that said patient is at risk for an undesirable clinical outcome (e.g., ABMR). Therefore, said patient is a candidate for treatment with an effective amount of an immunosuppressive treatment (e.g. by an anti-rejection agent). On the
20 contrary, the absence of Nocth3 is indicative of a reduced risk of transplant rejection. Moreover, depending on the expression level Notch3 (i.e. low level or high level of Nocth3 in the analyzed biological sample), the patient may require a treatment regime that is more or less aggressive than a standard regimen, or it may be determined that the patient is best suited for a standard regimen. For instance, a patient without Notch3 expressed in pericytes may
25 avoid an immunosuppressive treatment (or require a less aggressive regimen) and their associated side effects.

Reducing the level and the production of the DSA and/or protecting the allograft may be achieved using any suitable medical means known to those skilled in the art.

30

In one embodiment, such reduction and protection comprise a therapeutic intervention with the patient such as administration of anti-thymocyte globulin (ATG), monoclonal anti-CD20 antibodies (rituximab), proteasome inhibitor (bortezomib), anti-C5 antibodies (eculizumab), intravenous administration of immunoglobulins and plasmapheresis.

In one embodiment, the transplanted patient is a transplanted renal patient.

Therapeutic methods and uses:

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In a further aspect, the invention relates to a method for preventing ABMR or progression of ABMR in a transplanted patient, comprising the following steps of: (i) performing the method for determining whether a renal transplanted patient has or is at risk of ABMR of the invention, and (ii) administering to said patient a therapeutically effective amount of a compound selected from the group consisting of anti-thymocyte globulin (ATG),
10 monoclonal anti-CD20 antibodies (rituximab), proteasome inhibitor (bortezomib), anti-C5 antibodies (eculizumab).

By "therapeutically effective amount" is meant an amount sufficient to achieve a
15 concentration of compound which is capable of preventing or slowing down the disease to be treated. Such concentrations can be routinely determined by those of skilled in the art. The amount of the polypeptide actually administered will typically be determined by a physician or a veterinarian, in the light of the relevant circumstances, including the condition to be treated, the chosen route of administration, the actual compound administered, the age,
20 weight, and response of the patient, the severity of the subject's symptoms, and the like. It will also be appreciated by those of skilled in the art that the dosage may be dependent on the stability of the administered compound.

In one embodiment, said compound is selected from the group consisting of anti-
25 thymocyte globulin (ATG), monoclonal anti-CD20 antibodies (rituximab), proteasome inhibitor (bortezomib), anti-C5 antibodies (eculizumab).

In one embodiment, the transplanted patient is a transplanted renal patient.

30 The compounds of the invention may be administered by any means that achieve the intended purpose. For example, administration may be achieved by a number of different routes including, but not limited to subcutaneous, intravenous, intradermal, intramuscular, intraperitoneal, intracerebral, intrathecal, intranasal, oral, rectal, transdermal, buccal, topical, local, inhalant or subcutaneous use. Parenteral and topical routes are particularly preferred.

Dosages to be administered depend on individual needs, on the desired effect and the chosen route of administration. It is understood that the dosage administered will be dependent upon the age, sex, health, and weight of the recipient, concurrent treatment, if any, frequency of treatment, and the nature of the effect desired. The total dose required for each treatment may be administered by multiple doses or in a single dose.

The doses used for the administration can be adapted as a function of various parameters, and in particular as a function of the mode of administration used, of the relevant pathology, or alternatively of the desired duration of treatment. For example, it is well within the skill of the art to start doses of the compounds 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 polypeptides may be varied over a wide range from 0.01 to 1,000 mg per adult per day. Preferably, 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 10 mg/kg of body weight per day.

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: Notch3 expression in peritubular capillary pericytes is strongly increased in the renal transplanted patients with ABMR compared to other types of graft rejection.

Figure 2: Notch3 expression in peritubular capillary pericytes in the different groups of transplanted patients. The level of Notch3 expression was significantly higher in the patients with ABMR than that in other groups

EXAMPLE:**Material & Methods****PATIENTS AND SAMPLES COLLECTION:**

We analyzed retrospectively the expression of Notch3 in 55 biopsies taken from 50 renal transplanted patients during 2010 to 2012 in Tenon hospital. As negative and positive controls, Notch3 expression was also studied in 2 “healthy” kidneys in the vicinity of renal carcinomas and in 3 renal grafts de-transplanted due to repeated antibody-mediated rejection ABMR and graft function loss.

The expression of Notch3 was evaluated in the pericytes of peritubular capillaries in 14 biopsies diagnosed with ABMR, and compared with those in 41 non-ABMR biopsies including 6 biopsies with T cell mediated rejection (TCMR) or borderline lesions, 19 with diagnoses other than rejection (6 biopsies with acute tubular necrosis, 9 with graft fibrosis, 4 for no classified graft diseases such as minimum graft inflammation) and 16 biopsies with no lesions. ABMR diagnosed by medical staff was based on the biopsies showing peribubular capillaritis with or without c4d staining and anti class I or II antibodies determined by Luminex. All patients had given their oral informed consent to participate in the study. Forty of these patients had received a graft from a deceased donor and the other 10 from living relatives. 60% of biopsies were taken for clinical evaluation due to delayed or declining graft function, suspicion of rejection, occurring 6 days to 10 years after transplantation. 40% were taken for graft surveillance. The mean recipient age was 50 years at the time of biopsy, and 57.41 % of them were male. The mean donor age was 52.4 years. The mean cold ischemia time was 15.5 hours. 28% of patients had delayed graft function. 63% of patients had received basiliximab as induction therapy. All patients received triple-therapy including a calcineurin inhibitor (CNI). Renal histopathological analysis was scored according to the recently (2011) updated Banff working classification for renal graft pathology [11-13]. Mean i score was 0.32, t score was 0.23, g score was 0.39 and ptc score was 0.53. Seven biopsies displayed acute tubular necrosis lesions. Chronic Banff score for ci was 0.79; and ct was 0.66; cv was 0.7; ah was 0.72.

IMMUNOHISTOCHEMISTRY FOR NOTCH3 DETECTION:

Notch3 expression was detected in paraffin tissue by immunohistochemistry. Target retrieval was carried out by heating the tissue in citrate buffer. The sections were incubated overnight at 4°C with phosphate-buffered saline containing anti-Notch3 antibodies (gift kindly offered by Dr. Anne Joutel as described in Joutel et al, JCI 2000). The immunoreactive Notch3 expression was visualized using Envision + HRP system (AEC). For the negative controls, the primary antibodies were replaced by an equal concentration of mouse IgG. Notch3 expression in each biopsy was semi-quantified 3 times in a blind fashion in the pericytes of peritubular capillaries according to the following scale score: 0: no staining; 1: <10%; 2: 10-24%; 3: 25-50%; 4: >50%.

STATISTICAL ANALYSIS:

The score of Notch3 staining in the pericytes in the patients with ABMR was compared with that in different control groups by t test. Statistical significance was confirmed and p was presented by Mann-Whitney non parametric test by two sides. The association of Notch3 expression in pericytes with Banff histological scores, graft function and proteinuria was evaluated by linear correlation analysis. The level of significance was set up $p < 0.05$. Data analysis was performed using the Stata 8 software.

Results

Notch3 expression in normal kidneys is mainly observed in vascular smooth cells and in glomerular mesangial cells, but not in the peritubular capillary pericytes.

Notch3 expression is highly induced in the peritubular capillary pericytes in the renal transplanted patients with ABMR.

Table 1: Correlation of Notch3 expression in peritubular capillary pericytes with Banff scores. The Notch3 expression level in the pericytes of peritubular capillaries was significantly correlated with microcirculation infiltration scores g, ptc and v (found in ABMR), but not with interstitial or tubular inflammation score (for TCMR). Notch3 expression level was significantly correlated with graft function (up to 2 years post biopsy) and proteinuria (two important clinical manifestations during ABMR).

correlation of Notch3 with:	R	p
g	0.56	<0.0001
ptc	0.56	<0.0001
i	0.18	0.18
t	-0.022	0.9
v	0.53	<0.0001
cg	0.24	0.072
ci	0.27	0.046
ct	0.2	0.15
cv	0.2	0.16
ah	-0.04	0.76
eGFR at time of biopsy	-0.5	0.0001
eGFR 1y after biopsy	-0.42	0.0016
eGFR 2y after biopsy	-0.513	0.0003
proteinuria at time of biopsy	0.34	0.012
proteinuria 1y after biopsy	0.36	0.0136
proteinuria 2y after biopsy	0.22	0.22

REFERENCES:

5 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.

10 Joutel A, Andreux F, Gaulis S, Domenga V, Cecillon M, Battail N, Piga N, Chapon F, Godfrain C, Tournier-Lasserre E; The ectodomain of the Notch3 receptor accumulates within the cerebrovasculature of CADASIL patients; J Clin Invest. 2000 Mar;105(5):597-605.

CLAIMS:

- 5 1. A method for determining whether a transplanted patient has or is at risk of antibody-mediated rejection (ABMR), comprising a step of determining the expression level of the Notch3 gene in pericytes present on a transplant biopsy obtained from said patient.
2. The method according to claim 1, further comprising a step of comparing said expression level with a predetermined reference value, wherein an increase in the expression level of the Notch3 gene is indicative of having or being at risk of ABMR.
- 10 3. The method according to claim 1 or 2, wherein the transplanted patient is a renal transplanted patient.
4. The method according to any one claims 1 to 3, wherein the expression level of the Notch3 gene is determined by measuring the amount of Notch3 by immunohistochemistry (IHC).
- 15 5. The method according to any one claims 1 to 4, wherein the pericytes are pericytes of peritubular capillaries.
6. The method according to any one claims 1 to 5, wherein the ABMR is acute ABMR.
7. A method for adjusting the immunosuppressive treatment administered to a transplanted recipient following its transplantation, comprising the following steps of :
 - 20 (i) performing the method for determining whether a transplanted patient has or is at risk of ABMR according to any one claims 1 to 6, and
 - (ii) adjusting the immunosuppressive treatment.
- 25 8. A compound selected from the group consisting of monoclonal anti-CD20 antibodies, anti-thymocyte globulin (ATG), anti-C5 antibodies, for use in preventing ABMR or progression of ABMR in a transplanted patient, determined as having or being at risk of ABMR by an increase in the expression level of the Notch3 gene in pericytes present on a transplant biopsy obtained from said patient.

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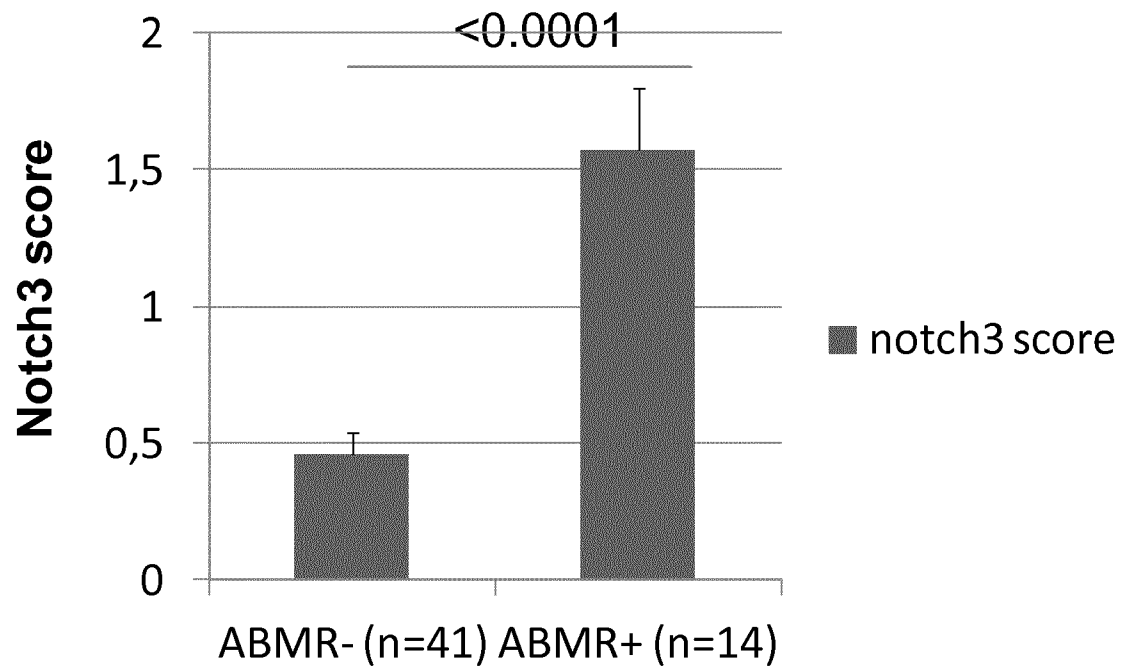


Figure 1

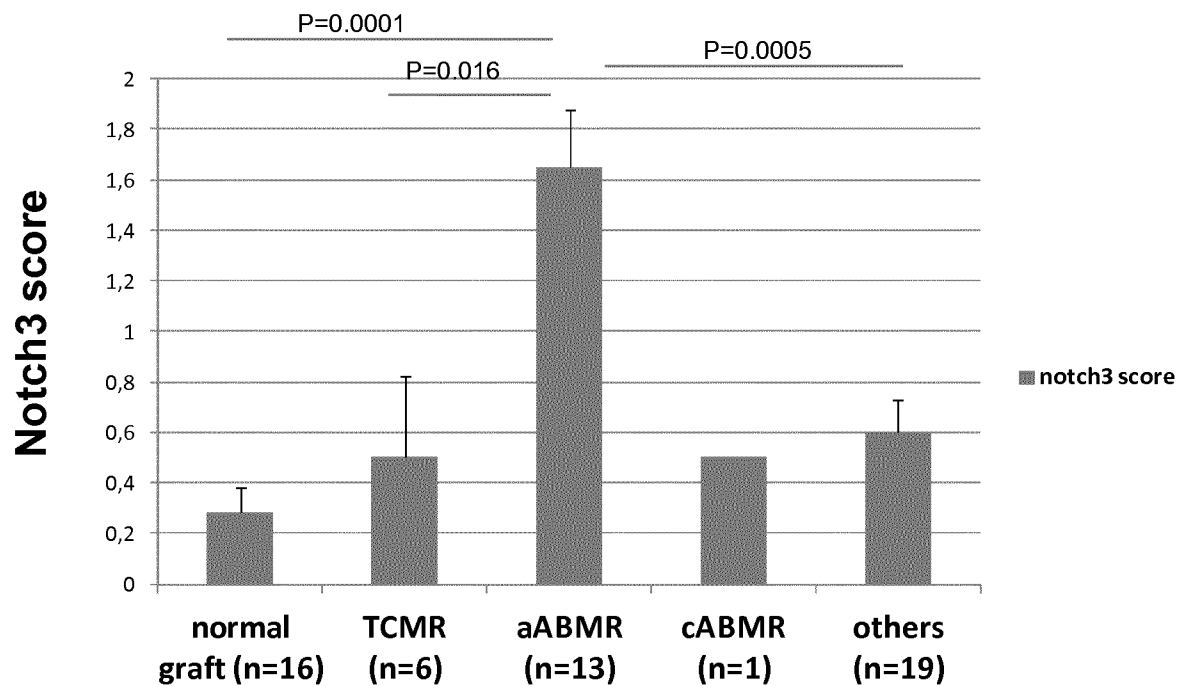


Figure 2

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2015/057551

A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N33/68 A61K38/00 A61K39/00 G01N33/50
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 C12Q A61K

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, BIOSIS, EMBASE, 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	JOUDEH AMANI ET AL: "Pathologic basis of antibody-mediated organ transplant rejection: from pathogenesis to diagnosis", CURRENT OPINION IN ORGAN TRANSPLANTATION,, vol. 18, no. 4, 1 August 2013 (2013-08-01) , pages 478-485, XP009179732, ISSN: 1531-7013 the whole document ----- -/--	1-7



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

10 June 2015

Date of mailing of the international search report

22/06/2015

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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2015/057551

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	ROSANA ROSA MIRANDA CORRÊA ET AL: "The Importance of C4d in Biopsies of Kidney Transplant Recipients", CLINICAL AND DEVELOPMENTAL IMMUNOLOGY, vol. 12, no. 3, 1 January 2013 (2013-01-01), pages 574-8, XP055135935, ISSN: 1740-2522, DOI: 10.1111/j.1600-6143.2011.03757.x the whole document -----	1-7
A	CORNELL LYNN D: "Renal allograft pathology in the sensitized patient", CURRENT OPINION IN ORGAN TRANSPLANTATION,, vol. 18, no. 3, 1 June 2013 (2013-06-01), pages 327-336, XP009179733, ISSN: 1531-7013 the whole document -----	1-7
A	SONJA DJUDJAJ ET AL: "Notch-3 receptor activation drives inflammation and fibrosis following tubulointerstitial kidney injury", THE JOURNAL OF PATHOLOGY, vol. 228, no. 3, 7 November 2012 (2012-11-07), pages 286-299, XP055135932, ISSN: 0022-3417, DOI: 10.1002/path.4076 the whole document -----	1-7
A	T. QUILLARD ET AL: "Impaired Notch4 Activity Elicits Endothelial Cell Activation and Apoptosis: Implication for Transplant Arteriosclerosis", ARTERIOSCLEROSIS, THROMBOSIS, AND VASCULAR BIOLOGY, vol. 28, no. 12, 1 December 2008 (2008-12-01), pages 2258-2265, XP055135895, ISSN: 1079-5642, DOI: 10.1161/ATVBAHA.108.174995 the whole document -----	1-7
A	Béatrice Charreau ET AL: "Crosstalk between inflammation, ADAM10 proteolytic activity and Notch signaling in antibody-mediated rejection and endothelial dysfunction", 7 November 2013 (2013-11-07), XP055135906, Retrieved from the Internet: URL:https://tech4pco.com/bsm2013/user_agendas/open_view/143 [retrieved on 2014-08-21] abstract -----	1-7

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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2015/057551

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SADAKA BASMA ET AL: "Management of antibody-mediated rejection in transplantation", THE SURGICAL CLINICS OF NORTH AMERICA,, vol. 93, no. 6, 1 December 2013 (2013-12-01), pages 1451-1466, XP009179730, the whole document -----	8
X	A. NICHOLAS R. BARNETT ET AL: "Rituximab in renal transplantation", TRANSPLANT INTERNATIONAL, vol. 26, no. 6, 18 June 2013 (2013-06-18), pages 563-575, XP055135916, ISSN: 0934-0874, DOI: 10.1111/tri.12072 abstract -----	8