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DESCRIPTION

FIELD OF INVENTION

[0001] The invention is in the field of transplantation, particularly, the invention allows to identify whether a kidney recipient subject is tolerant among the subjects treated with an immunosuppressive treatment.

BACKGROUND OF INVENTION

[0002] Solid-organ transplantation relies on the use of immunosuppressive treatment (IS) to prevent graft rejection. However, because of long-term IS side-effects, including cancers, cardiovascular diseases, infections and nephrotoxicity [1-3], physicians are encouraged to reduce IS exposition while still protecting the graft from immune aggression [4]. Ideally, achievement of allograft tolerance in solid-organ transplantation, *i.e.,* allograft acceptance in absence of immunosuppression (IS) treatment, would be a tremendous insight by avoiding IS side-effects. This would also decrease cost of transplantation maintenance [5], reduce cases of re-transplantation while improving recipients' quality of life. In this aim, several protocols of tolerance induction have been attempted with successes, mainly through transient chimera establishment via bone marrow or stem cells transfer [6-10]. However, so far, effectiveness is limited to living donors or zero-mismatch deceased-donor and only few cases of successful tolerance induction with HLA mismatches have been reported [6, 11]. Interestingly, tolerance has also been observed as a result of IS interruption for non-compliance or medical decision, especially post-transplant lymphoproliferative disorder (PTLD) [12, 13]. These patients display stable and good graft function for years, respond to immunological challenge [12] and do not harbour more opportunistic infections than healthy volunteers [12, 13]. From a clinical point of view, these patients, who are mainly discovered fortuitously [12, 14, 15], are comparable with renal recipients with stable graft function under standard IS (STA), with only few differences including increase proportion of graft from living donors and lower levels of HLA mismatch [12].

[0003] To date, no clinical parameter safely permitted to wean off IS, even in trials based on a drastic selection of non-sensitized recipients with highly stable graft function [16, 17] (Dugast E et al., Am J Transplant. 2016 Nov;16(11):3255-3261). Thus, it is clear that intentional replication of withdrawal of IS in renal transplantation in new trials requires clinical parameters but also new laboratory tests that recent reports have paved the way for [Sagoo et al., 2010. J Clin Invest. 120(6):1848-1861, WO2011138609, WO2011068829, EP1990425, WO2010136576, Sarwal et al., 2016 Clin Biochem. 49(4-5):404-410] Recently, an integrative meta-analysis of these different studies further highlighted 20 genes, mainly B-cell related, as the most significantly differentially expressed genes between TOL and STA [WO2016075232, [18]].

[0004] Interestingly, B-cells from these recipients harbour a specific phenotype with expression of inhibitory receptors [24], a unique differentiation profile [27] and display suppressive properties [28]. Collectively, these data suggest that B-cells may not only be potential biomarkers but may also actively regulate the immune response to the transplanted kidney, their induction and expansion being likely favoured by induction therapies [26]. Whereas the utility of such signatures is now clearly established and results from the effort of the scientific community in the last decades [29, 30], we now need to demonstrate their safety and reliability for minimization of IS and follow-up of patients in transplantation. First, we need a signature which is the most reliable while applicable easily to the higher number of patients. Second, we need a signature to be stable and not influenced by events occurring during transplanted patients' life, such as malignancy or immunosuppression. Third, while induction of tolerance is a promising therapeutic way, whether these protocols recapitulate what is observed in operationally tolerant patients or if there are different situations of so-called "tolerance" has not been solved yet, thus, a common signature for the two situations remains to be demonstrated.

SUMMARY

[0005] The present invention has been defined in the appended claims.

[0006] The present disclosure relates to a method for discriminating an operationally tolerant (TOL) kidney recipient subject from a non-operationally tolerant (STA) kidney recipient subject, comprising the following steps:

1. i) establishing a composite score of tolerance (cSoT) with the expression levels of six genes in a biological sample obtained from said subject and two clinical parameters; wherein said cSoT is established by the following formula:
$$cSoT = \sum_{i}^{n} = \beta_i \times Exprs + \beta_{test\ time} \times age_{test\ time} + \beta_{trans\ time} \times age_{trans\ time} + intercept - scaling\ coefficient$$
2. ii) comparing this cSoT with a predetermined reference value; and
3. iii) concluding that the subject is TOL when the cSoT is higher than the predetermined reference value or concluding that the subject is STA when the cSoT is lower than the predetermined reference value.

[0007] The six genes are *ID3, AKR1C3*, *CD40*, *CTLA4, TCL1A* and *MZB1.*

[0008] The two clinical parameters are the age of said subject at test time and the age of said subject at transplantation time.

[0009] In one embodiment, the predetermined reference value is the cSoT of a TOL subject. In another embodiment, the predetermined reference value is the cSoT of a STA subject. In one

embodiment, the subject is under immunosuppressive treatment.

[0010] In one embodiment, the subject is a human.

[0011] In one embodiment, said kidney recipient has further been grafted with the pancreas, and optionally with a piece of duodenum of the kidney donor.

[0012] The present disclosure further relates to a method of treating a kidney recipient subject with an immunosuppressive therapy comprising the steps of:

1. i) determining whether the subject is an operationally tolerant (TOL) subject or a non-operationally tolerant (STA) subject using the method for discriminating a TOL from a STA subject according to the present invention; and
2. ii) treating the subject with immunosuppressive therapy when the subject is STA.

[0013] The present disclosure also relates to a method for identifying a kidney recipient subject under immunosuppressive therapy as a candidate for immunosuppressive therapy weaning or minimization, comprising the steps of:

1. i) determining whether the subject is an operationally tolerant (TOL) subject or a non-operationally tolerant (STA) subject using the method for discriminating a TOL from a STA subject according to the present invention; and
2. ii) concluding that the subject is eligible to immunosuppressive therapy weaning or minimization when the subject is TOL.

DEFINITIONS

[0014] In the present invention, the following terms have the following meanings:
As used herein, the term "**AKR1C3**" refers to aldo-keto reductase family 1 member C3. The naturally occurring human *AKR1C3* gene has a nucleotide sequence as shown in Genbank Accession number NM_001253908.1 and the naturally occurring human AKR1C3 protein has an amino acid sequence as shown in Genbank Accession number NP_001240837.1.

[0015] As used herein, the term "**biological sample**" refers to any sample obtained from a subject, preferably from a transplanted subject, such as a serum sample, a plasma sample, a urine sample, a blood sample, a lymph sample, or a biopsy. In a particular embodiment, the biological samples for the determination of a gene expression level include samples such as a blood sample, a lymph sample, or a biopsy. In a particular embodiment, the biological sample is a blood sample, more particularly, peripheral blood mononuclear cells (PBMC). Typically, these cells can be extracted from whole blood using Ficoll, a hydrophilic polysaccharide that

separates layers of blood, with the PBMC forming a cell ring under a layer of plasma. Additionally, PBMC can be extracted from whole blood using a hypotonic lysis which will preferentially lyse red blood cells. Such procedures are known to the expert in the art.

[0016] As used herein, the term "**CD40**" refers to cluster of differentiation 40. CD40 is a costimulatory protein found on antigen presenting cells and is required for their activation. The naturally occurring human *CD40* gene has a nucleotide sequence as shown in Genbank Accession number NM_001250.5 and the naturally occurring human CD40 protein has an amino acid sequence as shown in Genbank Accession number NP_001241.1. The murine nucleotide and amino acid sequences have also been described (Genbank Accession numbers NM_011611.2 and NP_035741.2, respectively).

[0017] As used herein, the term "**CTLA4**" refers to cytotoxic T-lymphocyte-associated protein 4, also known as CD152 (cluster of differentiation 152). It is a protein receptor that, functioning as an immune checkpoint, downregulates immune responses. The naturally occurring human *CTLA4* gene has a nucleotide sequence as shown in Genbank Accession number NM_001037631.2 and the naturally occurring human CTLA4 protein has an amino acid sequence as shown in Genbank Accession number NP_001032720.1. The murine nucleotide and amino acid sequences have also been described (Genbank Accession numbers NM_001281976.1 and NP_001268905.1, respectively).

[0018] As used herein, the term **"discriminating"** refers to identify, observe a difference or distinguish two groups. Typically, the method according to the invention is suitable to identify or distinguish a subject who is tolerant among subjects treated with an immunosuppressive drug.

[0019] As used herein, the term "**ID3**" refers to inhibitor of DNA binding 3. The naturally occurring human *ID3* gene has a nucleotide sequence as shown in Genbank Accession number NM_002167.4 and the naturally occurring human ID3 protein has an amino acid sequence as shown in Genbank Accession number NP_002158.3. The murine nucleotide and amino acid sequences have also been described (Genbank Accession numbers NM_008321.2 and NP_032347.1, respectively).

[0020] As used herein, the terms **"immunosuppressive therapy"** or **"immunosuppressive treatment"** refer to the administration to a transplanted subject of one or more immunosuppressive drugs. Immunosuppressive drugs that may be employed in transplantation procedures include, but are not limited to, azathioprine, methotrexate, cyclophosphamide, FK-506 (tacrolimus), sirolimus, everolimus, rapamycin, corticosteroids, cyclosporins (such as, *e.g.*, cyclosporin A), mycophenolic acid, leflumacide, ascomycin and hydroxyurea. These drugs may be used in monotherapy or in combination therapies.

[0021] As used herein, the term **"immunosuppressive therapy weaning or minimization"** refers to the progressive reduction, and optionally eventually the suppression, of an immunosuppressive therapy in a transplanted subject being administered with immunosuppressive drugs.

[0022] As used herein, the term "**MZB1**" refers to marginal zone B and B1 cell-specific protein 1. The naturally occurring human *MZB1* gene has a nucleotide sequence as shown in Genbank Accession number NM_016459.3 and the naturally occurring human TCL1A protein has an amino acid sequence as shown in Genbank Accession number NP_057543.2. The murine nucleotide and amino acid sequences have also been described (Genbank Accession numbers NM_027222.3 and NP_081498.2, respectively).

[0023] As used herein, 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).

[0024] As used herein, the term **"predetermined reference value"** refers to a threshold value or a cut-off value. Typically, a **"threshold value"** or **"cut-off value"** can be determined experimentally, empirically, or theoretically. A threshold value can also be arbitrarily selected based upon the existing experimental and/or clinical conditions, as would be recognized by a person of ordinary skilled in the art. For example, retrospective measurement in properly banked historical subject samples may be used in establishing the predetermined reference value. The threshold value has to be determined in order to obtain the optimal sensitivity and specificity according to the function of the test and the benefit/risk balance (clinical consequences of false positive and false negative). Typically, the optimal sensitivity and specificity (and so the threshold value) can be determined using a Receiver Operating Characteristic (ROC) curve based on experimental data. For example, after determining the expression level of the selected peptide in a group of reference, one can use algorithmic analysis for the statistic treatment of the expression levels determined in samples to be tested, and thus obtain a classification standard having significance for sample classification. The full name of ROC curve is receiver operator characteristic curve, which is also known as receiver operation characteristic curve. It is mainly used for clinical biochemical diagnostic tests. ROC curve is a comprehensive indicator that reflects the continuous variables of true positive rate (sensitivity) and false positive rate (1-specificity). It reveals the relationship between sensitivity and specificity with the image composition method. A series of different cut-off values (thresholds or critical values, boundary values between normal and abnormal results of diagnostic test) are set as continuous variables to calculate a series of sensitivity and specificity values. Then sensitivity is used as the vertical coordinate and specificity is used as the horizontal coordinate to draw a curve. The higher the area under the curve (AUC), the higher the accuracy of diagnosis. On the ROC curve, the point closest to the far upper left of the coordinate diagram is a critical point having both high sensitivity and high specificity values. The AUC value of the ROC curve is between 1.0 and 0.5. When AUC > 0.5, the diagnostic result gets better and better as AUC approaches 1. When AUC is between 0.5 and 0.7, the accuracy is low. When AUC is between 0.7 and 0.9, the accuracy is moderate. When AUC is higher than 0.9, the accuracy is high. This algorithmic method is preferably done with a

computer. Existing software or systems in the art may be used for the drawing of the ROC curve, such as: MedCalc 9.2.0.1 medical statistical software, SPSS 9.0, ROCPOWER.SAS, DESIGNROC.FOR, MULTIREADER POWER.SAS, CREATE-ROC.SAS, GB STAT VI0.0 (Dynamic Microsystems, Inc. Silver Spring, Md., USA), etc.

[0025] As used herein, the term **"reagent for the determination of a gene expression level"** is meant a reagent which specifically allows for the determination of said gene expression level, *i.e.,* a reagent specifically intended for the specific determination of the expression level of a given gene, such as, *e.g.,* of the *ID3, AKR1C3*, *CD40*, *CTLA4, TCL1A* and/or *MZB1* genes. This definition therefore excludes generic reagents useful for the determination of the expression level of any gene, such as taq polymerase or an amplification buffer, although such generic reagents may be necessary, albeit not sufficient, to determine the expression level of a given gene, and may therefore also be included in a kit according to the invention.

[0026] As used herein, the term "**STA**" refers to a "**non-operationally tolerant subject**", *i.e.,* the subject is under immunosuppression with stable function, but would reject his/her graft if the immunosuppressive treatment was withdrawn. Such subject is considered as non-tolerant to the graft. In a particular embodiment, the subject "**STA**" is considered as having a high immunologic risk, *i.e.*, said subject has a higher risk of developing rejection (acute or chronic) and/or antibodies against the graft.

[0027] As used herein, the term "**subject**" refers to any mammals, such as a rodent, a feline, a canine, and a primate. Particularly, in the present invention, the subject is a human, also termed "**patient**". In a particular embodiment, the subject is a transplanted subject, also termed "**recipient**" or "**grafted subject**".

[0028] As used herein, the term "**TCL1A**" refers to T-cell leukaemia or lymphoma protein 1. The naturally occurring human *TCL1A* gene has a nucleotide sequence as shown in Genbank Accession number NM_001098725.1 and the naturally occurring human *TCL1A* protein has an amino acid sequence as shown in Genbank Accession number NP_001092195.1.

[0029] As used herein, the term "**TOL**" refers to an "**operationally tolerant subject**", *i.e.,* the subject is under immunosuppression with stable function for which immunosuppression regimen can be safely withdrawn. It means that the subject does not reject his/her graft in the absence of an immunosuppressive treatment with a well-functioning graft. Such subject is considered as tolerant to the graft. In a particular embodiment, the subject "**TOL**" is considered as having a low immunologic risk, *i.e.,* said subject has a lower risk of developing rejection (acute or chronic) and/or antibodies against the graft.

[0030] As used herein, the term "**transplanted subject**" (also called "**recipient**" or "**grafted subject**"), refers to a subject who has received an organ transplantation.

[0031] As used herein, the terms "**treating**" and "**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 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 [*e.g.*, pain, disease manifestation, etc.]).

[0032] As used herein, the term "**two clinical parameters"** refers to the age of the subject at the test time and the age of the subject at the transplantation time.

DETAILED DESCRIPTION

[0033] Based on the 20-gene signature from their published meta-analysis, Inventors used a sparse methodology to identify and validate the most informative genes that, in association with few demographic parameters, allow to construct a predictive score of tolerance applicable in clinical routine [31]. The Inventors thus identified and validated a score of 6 genes combined with 2 basic clinical parameters that allow identifying TOL from STA with excellent accuracy. They showed that this signature of tolerance is not compromised by the malignancy (PTLD) status of the patients, centre origin, neither by IS treatment. Operationally tolerant recipients and patients who benefit from a protocol of tolerance induction do not share a common "tolerance signature". Finally, they showed that this score was influenced by *de novo* anti-HLA antibody, including DSA and tolerance loss, which reinforced its potential to follow renal transplanted patients.

[0034] The disclosure relates to methods for discriminating an operationally tolerant (TOL)

kidney recipient subject from a non-operationally tolerant (STA) kidney recipient subject, comprising the following steps:

1. i) establishing a composite score of tolerance (cSoT) with the expression level of at least two, at least three, at least four, at least five, at least six or more genes in a biological sample obtained from said subject and at least one, at least two or more clinical parameters; wherein, said score is established by the following formula:
$$cSOT = \sum_{i}^{n} = \beta_i \times Exprs + \beta_{test\ time} \times age_{test\ time} + \beta_{trans\ time} \times age_{trans\ time} + intercept - scaling\ coefficient$$
2. ii) comparing said cSoT with a predetermined reference value; and
3. iii) concluding that the subject is TOL when said cSoT is higher than the predetermined reference value or concluding that the subject is STA when said cSoT is lower than the predetermined reference value.

[0035] In one embodiment, the genes are selected from the group consisting of *ID3, AKR1C3*, *CD40*, *CTLA4, TCL1A, MZB1, CD22, BLK, MS4A1, CD79B, BLNK, FCRL2, IRF4, HINT1, RFC4, ANXA2R, FCER2, AKIRIN2, EPS15* and *PLBD1.* Preferably, the genes are selected from the group consisting of *ID3, AKR1C3*, *CD40*, *CTLA4, TCL1A* and *MZB1.*

[0036] In one embodiment, the clinical parameters are selected from the group consisting of the age of said subject at test time, the age of the subject at transplantation time, the donor age, the recipient gender, the donor gender, the donor type, the graft order, the number of HLA mismatches, the induction treatment, the recipient's creatinemia, the recipient's proteinuria, the presence in the recipient of anti-HLA Ab at test time and the presence in the recipient of DSA at test time. Preferably, the clinical parameters are selected from the group consisting of the age of said subject at test time and the age of the subject at transplantation time.

[0037] In one embodiment, the method for discriminating a kidney recipient subject TOL from STA comprises the following steps:

1. i) establishing a composite score of tolerance (cSoT) with the expression level of at least two, at least three, at least four, at least five, at least six or more genes selected from the group consisting of *ID3, AKR1C3*, *CD40*, *CTLA4, TCL1A* and *MZB1* in a biological sample obtained from said subject and at least one, at least two or more clinical parameters selected from the group consisting of the age of said subject at test time and the age of the subject at transplantation time; wherein, said score is established by the following formula:
$$cSOT = \sum_{i}^{n} = \beta_i \times Exprs + \beta_{test\ time} \times age_{test\ time} + \beta_{trans\ time} \times age_{trans\ time} + intercept - scaling\ coefficient$$

2. ii) comparing said cSoT with a predetermined reference value; and
3. iii) concluding that the subject is TOL when said cSoT is higher than the predetermined reference value or concluding that the subject is STA when said cSoT is lower than the predetermined reference value.

[0038] The method for discriminating a kidney recipient subject TOL from STA comprises the following steps:

1. i) establishing a composite score of tolerance (cSoT) with the expression level of six genes *ID3*, *AKR1C3*, *CD40*, *CTLA4*, *TCL1A, MZB1* in a biological sample obtained from said subject and two clinical parameters selected from the age of said subject at test time and the age of the subject at transplantation time; wherein, said score is established by the following formula:

$$cSOT = \sum_{i}^{n} = \beta_i \times Exprs + \beta_{test\ time} \times age_{test\ time} + \beta_{trans\ time} \times age_{trans\ time} + intercept - scaling\ coefficient$$

2. ii) comparing said cSoT with a predetermined reference value; and
3. iii) concluding that the subject is TOL when said cSoT is higher than the predetermined reference value or concluding that the subject is STA when said cSoT is lower than the predetermined reference value.

[0039] In one embodiment, a TOL subject maintains stable graft function off immunosuppressive drugs. In one embodiment, a STA subject does not maintain stable graft function off immunosuppressive drugs.

[0040] In one embodiment, the subject is a mammal. In a particular embodiment, the subject is a human.

[0041] In one embodiment, the subject is a transplanted subject. 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.

[0042] In one embodiment, 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, *i.e.,* the subject is administered with one or more immunosuppressive drugs.

[0043] Immunosuppressive drugs that may be employed in transplantation procedures include, but are not limited to, azathioprine, methotrexate, cyclophosphamide, FK-506 (tacrolimus),

sirolimus, everolimus, rapamycin, corticosteroids, cyclosporins (such as, *e.g.*, cyclosporin A), mycophenolic acid, leflumacide, ascomycin and hydroxyurea.

[0044] In one embodiment, immunosuppressive drugs are used in monotherapy. In another embodiment, immunosuppressive drugs are used in combination therapies.

[0045] In the case of renal transplantation, the following immunosuppressive protocols are usually used. Subjects with primary renal transplantation generally receive an induction treatment consisting of 2 injections of basiliximab (Simulect®, a chimeric murine/human monoclonal anti-IL2-Rα antibody commercialized by Novartis), in association with tacrolimus (Prograf™, Fujisawa Pharmaceutical, 0.1 mg/kg/day), mycophenolate mofetil (Cellcept™, Syntex Laboratories, Inc., 2 g/day) and corticoids (1 mg/kg/day), the corticoid treatment being progressively decreased by 10 mg every 5 days until end of treatment, 3 months post-transplantation. Subjects with secondary or tertiary renal transplantation, or subjects considered at immunological risk (percentage of anti-T panel reactive antibodies (PRA) previously peaking above 25% or cold ischemia for more than 36 hours), generally receive a short course of anti-thymocyte globulin (ATG) (for, *e.g.,* 7 days), in addition from day 0 with mycophenolate mofetil (Cellcept™, Syntex Laboratories, Inc, 2 g/day), and corticosteroids (1 mg/kg/day), then the steroids are progressively tapered by 10 mg every 5 days until end of treatment and finally stopped around 3 months post-transplantation. Tacrolimus (Prograf™, Fujisawa Pharmaceutical) is introduced in a delayed manner (at day 6) at a dose of 0.1 mg/kg/day.

[0046] As used herein, the term "**composite score of tolerance (cSoT)**", also referred to as "**score**", refers to a value obtained from the following formula:

$$cSOT = \sum_{i}^{n} = \beta_i \times Exprs + \beta_{test\ time} \times age_{test\ time} + \beta_{trans\ time} \times age_{trans\ time} + intercept - scaling\ coefficient$$

[0047] This formula was obtained by using the Bolasso algorithm, a lasso (Least Absolute Selection and Shrinkage Operator) analysis performed by bootstrap resampling (10,000 times) followed by multiple testing (false discovery rate < 0.05), which allows to identify the eight parameters (six genes and two clinical parameters) of the present invention.

[0048] Within the meaning of the invention, the term "**β**" refers to a coefficient for each gene according to the invention.

[0049] "**β_i**" represent the regression β coefficient for each gene. Typically, the regression β coefficients are determined by the skilled man in the art for each gene using the Bolasso method as described in Erickson, K.F., *et al.*, 2016. Typically, a blood sample can be obtained from a subject transplanted and treated under IS; and the expression of 6 genes can be determined by conventional methods.

[0050] Two clinical parameters (the subject's age at the test time [$age_{test\ time}$] and the subject's age at the transplantation time [$age_{trans\ time}$]) are taken into consideration. The score is then compared with a cut-off value that distinguishes TOL or STA. The cut-off value can be determined by analysing the expression of the same genes via the same analysis method in non-transplanted subjects and in transplanted subjects. For example, it can be the middle point between the score of non-transplanted subjects and that of transplanted subj ects.

[0051] As used herein, the term "$\boldsymbol{\beta_{test\ time}}$" refers to the β coefficient of the age of the subject at the test time.

[0052] As used herein, the term "$\boldsymbol{\beta_{test\ time}}$" refers to the β coefficient of the age of the subject at the transplantation time.

[0053] As used herein, the term **"intercept"** refers to a fixed value used to correct the equation (refers to the interception of the regression curve to the Y axis).

[0054] As used herein, the term **"scaling coefficient"** refers to a value used to centre the score in order to associate positive and negative scores with TOL and STA diagnosis, respectively.

[0055] As used herein, the term "**Exprs**" refers to the expression level of each gene. The "**gene expression profile"** corresponds to a group of at least 2, 3, 4, 5, 6 or more values corresponding to the gene expression level of each of at least 2, 3, 4, 5, 6 or more genes selected from the group consisting of *ID3, AKR1C3*, *CD40*, *CTLA4, TCL1A, MZB1, CD22, BLK, MS4A1, CD79B, BLNK, FCRL2, IRF4, HINT1*, *RFC4, ANXA2R, FCER2, AKIRIN2, EPS15* and *PLBD1*, optionally with further other values corresponding to the clinical parameters. Preferably, the gene expression profile corresponds to a group of 6 values corresponding to the gene expression level of each of the *ID3, AKR1C3*, *CD40*, *CTLA4, TCL1A* and *MZB1* gene, optionally with further other values corresponding to the clinical parameters. Typically, the expression level of the genes, preferably of the 6 genes, may be determined by any technology known by a person skilled in the art. In particular, each gene expression level may be measured at the genomic and/or nucleic and/or protein level. In a particular embodiment, the expression level of gene is determined by measuring the amount of nucleic acid transcripts of each gene. In another embodiment, the gene expression level is determined by measuring the amount of each gene corresponding protein. The amount of nucleic acid transcripts can be measured by any technology known by a man skilled in the art. In particular, the measure may be carried out directly on an extracted messenger RNA (mRNA) sample, or on retrotranscribed complementary DNA (cDNA) prepared from extracted mRNA by technologies well-known in the art. From the mRNA or cDNA sample, the amount of nucleic acid transcripts may be measured using any technology known by a man skilled in the art, including nucleic microarrays, quantitative PCR, microfluidic cards, and hybridization with a labelled probe. In a particular embodiment, the gene expression level is determined using quantitative PCR. Quantitative, or real-time, PCR is a well-known and easily available technology for those skilled in the art and

does not need a precise description. Methods for determining the quantity of mRNA are well known in the art. For example, the nucleic acid contained in the biological sample 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). Preferably quantitative or semi-quantitative RT-PCR is preferred. Real-time quantitative or semi-quantitative RT-PCR is particularly advantageous.

[0056] Other methods of amplification include ligase chain reaction (LCR), transcription-mediated amplification (TMA), strand displacement amplification (SDA) and nucleic acid sequence based amplification (NASBA).

[0057] Nucleic acids having at least 10 nucleotides and exhibiting sequence complementarity or homology to the mRNA of interest herein find utility as hybridization probes or amplification primers. 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, even more preferably 90% identical, even more preferably 95% or more identical to the homologous region of comparable size. In certain embodiments, it will be advantageous to use nucleic acids in combination with appropriate means, such as a detectable label, for detecting hybridization. A wide variety of appropriate indicators are known in the art including, fluorescent, radioactive, enzymatic or other ligands (*e.g.*, avidin/biotin).

[0058] 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. Primers typically are shorter single-stranded nucleic acids, of between 10 to 25 nucleotides in length, designed to perfectly or almost perfectly match a nucleic acid of interest, to be amplified. 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 Tm, *e.g.,* 50% formamide, 5x or 6x SCC buffer. 1x SCC buffer comprises 0.15 M NaCl and 0.015 M Na-citrate, adjusted to pH 7.0 with HCl).

[0059] The nucleic acid primers or probes used in the above amplification and detection method may be assembled as a kit. Such a kit includes consensus primers and molecular probes. A kit also includes the components necessary to determine if amplification has occurred. The kit may also include, for example, PCR buffers and enzymes; positive control sequences, reaction control primers; and instructions for amplifying and detecting the specific sequences.

[0060] In a particular embodiment, the method of the invention comprises the steps of providing total RNAs extracted from a biological sample and subjecting the RNAs to amplification and hybridization to specific probes, more particularly by means of a quantitative or semi-quantitative RT-PCR.

[0061] In another embodiment, the gene expression level is determined by DNA chip analysis. Such DNA chip or nucleic acid microarray consists of different nucleic acid probes that are chemically attached to a substrate, which can be a microchip, a glass slide or a microsphere-sized bead. A microchip may be constituted of polymers, plastics, resins, polysaccharides, silica or silica-based materials, carbon, metals, inorganic glasses, or nitrocellulose. Probes comprise nucleic acids such as cDNAs or oligonucleotides that may be about 10 to about 60 base pairs. To determine the gene expression level, a biological sample from a test subject, optionally first subjected to a reverse transcription, is labelled and contacted with the microarray in hybridization conditions, leading to the formation of complexes between target nucleic acids that are complementary to probe sequences attached to the microarray surface. The labelled hybridized complexes are then detected and can be quantified or semi-quantified. Labelling may be achieved by various methods, *e.g.,* by using radioactive or fluorescent labelling. Many variants of the microarray hybridization technology are available to the man skilled in the art (see *e.g.* the review by Hoheisel, Nature Reviews, Genetics, 2006, 7:200-210).

[0062] The present disclosure also relates to a method of treating a kidney recipient subject with an immunosuppressive therapy comprising the steps of:

1. i) determining whether the subject is an operationally tolerant (TOL) subject or a non-operationally tolerant (STA) subject according to the invention; and
2. ii) treating the subject with one or more immunosuppressive drugs when the subject is STA.

[0063] The present disclosure also relates to a method for identifying a kidney recipient subject under immunosuppressive therapy as a candidate for immunosuppressive therapy weaning or minimization, comprising the steps of:

1. i) determining whether the subject is an operationally tolerant (TOL) subject or a non-operationally tolerant (STA) subject according to the invention; and
2. ii) concluding that the subject is eligible to immunosuppressive therapy weaning or minimization when the subject is TOL.

[0064] In another aspect, the present disclosure also relates to a kit for discriminating an operationally tolerant (TOL) kidney recipient subject from a non-operationally tolerant (STA) kidney recipient subject, comprising at least one reagent for the determination of a gene expression profile corresponding to a group of at least 2, 3, 4, 5, 6 or more values corresponding to the gene expression level of each of at least 2, 3, 4, 5, 6 or more genes selected from the group consisting of *ID3, AKR1C3*, *CD40*, *CTLA4, TCL1A, MZB1, CD22, BLK, MS4A1, CD79B, BLNK, FCRL2, IRF4, HINT1*, *RFC4, ANXA2R, FCER2, AKIRIN2, EPS15* and *PLBD1*, optionally with further other values corresponding to the clinical parameters. Preferably, the kit according to the present invention comprises at least one reagent for the

determination of a gene expression profile corresponding to a group of 6 values corresponding to the expression level of each of the 6 following genes: *ID3, AKR1C3*, *CD40*, *CTLA4, TCL1A* and *MZB1.*

[0065] In one embodiment, the kit may further comprise at least one reagent for the determination of a gene expression level of at least one reference gene. Examples of reference genes include, but are not limited to, *ACTB, B2M, GAPDH* and *HPRT1.* In one embodiment, determination of a gene expression level of at least one reference gene is used to normalize and/or calculate the relative expression of each genes according to the present invention.

[0066] In some embodiments, the kit may further comprise instructions for discriminating a kidney recipient subject TOL from STA. The instructions for the discrimination of a subject TOL from STA may include at least one reference gene expression profile. In a particular embodiment, at least one reference gene expression profile is a graft-tolerant expression profile, *i.e.*, a group of 6 values corresponding to the expression level of each of the 6 following genes: *ID3, AKR1C3*, *CD40*, *CTLA4, TCL1A* and *MZB1,* in an operationally tolerant (TOL) subject. Alternatively, at least one reference gene expression profile may be a graft-non-tolerant expression profile, *i.e.*, a group of 6 values corresponding to the expression level of each of the 6 following genes: *ID3, AKR1C3*, *CD40*, *CTLA4, TCL1A* and *MZB1,* in a non-operationally tolerant (STA) subj ect.

[0067] 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.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1: Composite score of tolerance (cSoT)

[0068] (**A**) cSoT model: left axis displays coefficients of selected genes and clinical parameters (False Discovery Rate [fdr] < 0.05) (black bars) and right axis represents the number of times, *i.e.,* the occurrence, of selected genes and clinical parameters among the 100 times 10-fold cross-validations (grey bars). (**B**) Receiver operating characteristic (ROC) curves of cSoT [cSoT], combination of the 6 genes (*ID3, AKR1C3*, *CD40*, *CTLA4, TCL1A* and *MZB1*) [6 genes], age of the patient at test time [Age at collection], age of the patient at transplantation time [Age at Tx] and creatinemia [creatinemia]. (**C**) cSoT values for 231 patients (42 TOL [grey] and 189 STA [black]). The dashed line represents the centred best threshold of the ROC curve (Youden index). The grey zone (on both side of the dashed line) represents the inconclusive zone defined by values with specificity and specificity below 90%.

Figure 2: cSoT validated using qPCR

[0069] (A) Using qPCR, cSoT was still differential between TOL and STA (n= 9 and 12; mean= 2.89 ± 5.41 and -2.58 ± 2.37, respectively) and (B) displayed an AUC of 0.86 $IC_{95\%}$ [0.66-1]).

EXAMPLES

Material & Methods

Meta-analysis dataset

[0070] Gene expression dataset was obtained from Gene Expression Ominbus (GEO) database (accession number GSE28456) previously described [18]. This dataset was the result of a meta-analysis from 5 independent studies gathering 596 samples [14, 15, 21, 50, 51].

[0071] Briefly, datasets were renormalized using a Lowess procedure, log-transformed, and median-centred according to the STA group as previously described and composed of 1,846 merged genes [18]. In addition to Nantes' collection and thanks to the European IoT and the American ITN networks, we were able to identify 344 unique non-redundant patients among the different studies: 46 individual operationally tolerant patients (TOL) out of 96 TOL samples and 266 patients with stable graft function (STA) out of 311 STA samples. Demographic description of available clinical parameters from TOL and STA patients are given in **Table 1** and **Table 2**. Mean expression for each of the 20 genes was calculated in case of technical replicates (identical blood sample time) and earliest time point was selected in case of time replicates.

Table 1: demographic parameters of TOL (n=46) and STA (n=199)

	TOL			STA			T.test p.value
	Mean	sd	n	Mean	sd	n	
Age of the patient at test time	49.89	13.00	45	52.94	13.64	190	0.175
Time post-transplantation	18.30	9.302	42	9.32	3.948	198	< 0.0001
Time post-treatment	7.914	9.018	45	NA	NA	NA	NA
Age of the patient at transplantation time	31.66	13.25	42	43.71	14.00	190	< 0.0001
Creatinemia	106	30.62	44	118.8	31.06	190	0.0144
Proteinuria	0.213	0.247	12	0.207	0.207	178	0.915

	TOL			STA			T.test p.value
	Mean	sd	n	Mean	sd	n	
Donor age	36.84	19.19	25	35.54	14.36	167	0.684

Table 2: demographic parameters of TOL (n=46) and STA (n=199).

	TOL		STA		Fisher p.value
		n		n	
Recipient gender (M/F)	29/16	45	116/75	191	0.734
Donor gender (M/F)	20/12	32	125/45	167	0.192
Donor type (NLD/LD)	15/7	22	191/0	191	< 0.0001
Graft order (1/2)	16/6	32	191/0	191	< 0.0001
HLA MM (0,1, 2, 3, 4, 5, 6)	14, 3, 5, 7, 5, 1, 1	36	5, 7, 31, 41, 45, 30, 8	167	< 0.0001
Induction treatment (Y/N)	10/6	16	125/42	167	0.371
Presence of anti-HLA Ab at test time (Y/N)	7/8	15	24/ 137	161	0.0013
Presence of DSA at test time (Y/N)	9/4	13	141 / 14	168	0.036
M/F: male/female					
NLD/LD: non-living donor/living donor					
1/2: primary/secondary					
HLA MM: human leukocyte antigen mismatch (0 to 6 mismatches)					
Y/N: Yes/No					
DSA: donor-specific antibodies					

Additional microarray datasets

[0072] Three publicly available microarray datasets were collected from GEO: GSE14630 [34], GSE22224 [35] and GSE45593[8] and normalized with the robust multi-array average method (RMA) using the *affy* package [52] in the R software. Normalized collected expression values from dataset GSE45218 [33] were used for *in silico* cross-validation. For all datasets, gene expressions of the 6 genes of interest were centred/scaled before applying coefficients of the cSoT.

Validation cohort

[0073] Additional 21 kidney recipients from Nantes' Hospital were enrolled to perform qPCR

validation, including 9 TOL and 12 STA. Local Ethic Committee approved all aspects of this study and all patients gave their written informed consent.

qPCR validation

[0074] Venous blood samples were collected in EDTA vacutainers and processed for analysis within 4 hours. Peripheral Blood Mononuclear Cells (PBMC) were separated on a Ficoll layer (Eurobio, Les Ulis, France) and frozen in TRIzol® reagent (Thermo Fisher Scientific, Waltham, MA USA) at -80°C. RNA was extracted from peripheral blood using the TRIzol method (Thermo Fisher Scientific). RNA quality and quantity were determined using an Agilent 2100 BioAnalyzer (Palo Alto, CA, USA) and a Nanodrop (Labtech, Palaiseau, France), respectively. RNA was reverse-transcribed using poly-dT oligonucleotides and *Maloney leukaemia virus* reverse transcription (Thermo Fisher Scientific). Real-time quantitative PCR was performed on a StepOnePlus instrument (Thermo Fisher Scientific) using commercially available primer and probe sets (Taqman) for the 6 tested genes and 4 reference genes: *AKR1C3*: Hs00366267_m1, *CD40*: Hs00374176_m1, *CTLA4*: Hs00175480_m1, *ID3*: Hs00171409_m1, *MZB1* (or MGC29506): Hs00414907_m1, *TCLIA*: Hs00951350_m1; and 4 reference genes: *ACTB*: Hs99999903_m1, *B2M*: Hs00984230_m1, *GAPDH*: Hs99999905_m1, *HPRT1*: Hs99999909_m1 (Thermo Fisher Scientific). The geometric mean of the 4 reference genes was used to normalize for RNA amounts and to calculate the relative expression of each genes according to the $2^{-\Delta\Delta}$Cq method [53].

cSoT construction

[0075] Parameters associated with TOL compared to STA in logistic univariate analysis (using *glm* package in R) were used for cSoT construction. To identify the most discriminative combination between the 24 parameters associated with tolerance in univariate analysis (20 genes and 4 demographic parameters), we used the Bolasso method [5] which performs bootstrap resampling (10,000 fold) combined with a lasso (least absolute shrinkage and selection operator) regression analysis followed by multiple testing to select the significant variables associated with the model (False Discovery Rate (FDR) <0.05) using the *mht* package (version 3.2.2) in R [2]. Coefficients obtained from *mht* were used to calculate the score from other datasets after scaling, as performed by the *mht* package. For qPCR data, scaled-dCq were used.

Statistical analysis

[0076] Statistical analyses were performed using R software version 3.2.2 or GraphPrism v.4 software. Parametric student T test, ANOVA test or Khi^2 test were used for group comparisons. Differences were defined as statistically significant when $p < 0.05$.

Results

Selection of clinical parameters associated with operational tolerance status

[0077] From the meta-dataset we previously described [18], in addition to Nantes' collection and thanks to the European Indice of Tolerance (IoT) and the American Immune Tolerance Networks (ITN), we were able to identify 312 non-redundant patients among the different studies: 46 individual operationally tolerant patients (TOL) out of 96 TOL samples and 266 patients with stable graft function (STA) out of 311 STA samples. Demographic description of available clinical parameters from TOL and STA patients are given in **Table 1** and **Table 2**. In order to construct a predictor score, we selected among demographic clinical parameters of the patients only intrinsic and non-variant patient-related ones and known for at least half of the TOL (**Table 1** and **Table 2**). 4 parameters were associated with tolerance status using univariate logistic regression ($p < 0.20$) and were selected for the composite score: age of the patient at transplantation time ($p < 0.0001$), age of the patient at test time ($p = 0.176$) (**Table 1**), number of HLA mismatches ($p < 0.0001$) and donor gender ($p = 0.154$) (**Table 2**).

Composite Score of Operational Tolerance (cSoT) combining genes and clinical parameters

[0078] Mean expression for each of the 20 genes was calculated in case of technical replicates (identical blood sample times) and earliest time point was selected in case of time replicates. Expressions of the 20 genes that were previously reported as differential between TOL and STA [1] were confirmed in univariate analysis in this large cohort of 312 patients (46 TOL and 266 STA; $p < 0.0001$).

[0079] To identify the most discriminative combination between the 20 original genes and the 4 clinical parameters selected above to include in the cSoT, we used the Bolasso method [5] which perform bootstrap resampling (10,000 fold) combined with a lasso (least absolute shrinkage and selection operator) regression analysis followed by multiple testing to select the significant variables associated with the model (False Discovery Rate [FDR] < 0.05) [2].

[0080] We identified a combination of 6 genes and 2 clinical parameters - *AKR1C3*, *CD40*, *CTLA4, ID3, MZB1, TCL1A,* age of the patient at test time and age of the patient at transplantation time **(Figures 1A and 1B)** - that enabled to establish a cSoT discriminating TOL and STA (mean cSoT = 6.43 ± 4.73 (SD) for 42 TOL and -4.04 ± 2.81 for 189 STA; $p < 0.0001$) with an AUC of 0.973 ($IC_{95\%}$ [0.939-1.00]), with negative and positive predictive values of 0.989 and 0.800 respectively **(Figures 1B and 1C)**.

[0081] The computed cSoT score has been centred using the best threshold of the ROC curve (Youden index) to associate positive and negative scores with TOL and STA diagnosis respectively, and an inconclusive zone (also called "grey zone") has been defined by values with specificity and specificity below 90% (predictive tolerance of 10%) for ease of interpretation **(Figure 1)** [32]. The consistency of selection of these 8 parameters was validated through a 10-fold cross-validation (randomly one tenth of TOL and STA) repeated 100 times. We found that the 8 selected parameters were present in at least 80% out of the 1,000 models **(Figure 1A).**

[0082] Finally, the robustness of the cSoT score was further validated through 100 times 10-fold cross-validation with a mean AUC for test sets of 0.967 $IC_{95\%}$ [0.966-0.968]. As attempted, the cSoT score discriminated TOL from STA better than the 2 demographic parameters separately **(Figure 1C**): age at transplantation time (AUC = 0.737 $IC_{95\%}$ [0.655-0.819]) and age at test time (AUC = 0.564, $IC_{95\%}$ [0.474-0.655]; $p < 0.0001$ for both comparisons), better than the combination of the 6 genes only (AUC = 0.947 $IC_{95\%}$ [0.902-0.992]; p = 0.38) and better then the function of the patients (creatinemia alone (AUC = 0.615, $IC_{95\%}$ [0.519-0.711]; $p < 0.0001$).

[0083] Finally, for cross-validation, we took advantage of a recent microarray dataset performed on 16 TOL and 9 patients with chronic allograft nephropathy (CAN) [33]. Since individual clinical information were not provided, we could calculate only the combination of the expression 6 genes. Despites our gene combination was not designed to discriminate TOL from CAN but TOL from STA, we could observe significantly different score values (p = 0.0061) and a good discrimination between the 2 populations (AUC = 0.825 $IC_{95\%}$ [0.636- 0.1.014]).

Center origin, immunosuppressive regimen, PTLD, does not influenced the cSoT

[0084] Despite TOL samples were coming from 3 different origin (Nantes, IoT and ITN), cSoT was not associated with patient origin (p = 0.13). One of the main reasons for IS cessation in patients with stable graft function is the appearance of severe side effects such as PTLD. However, cSoT was not influenced by PTLD experience (PTLD, n = 4, p = 0.19).

[0085] Since the two groups of patients who were used to create the cSoT differed by their immunosuppressive regimen status - STA are under IS whereas TOL received no more IS - we assessed whether IS could impact cSoT values. Regarding the TOL patients, we observed that previous IS regimen before IS withdrawal, including cyclosporine A (CsA), azathioprine, mycophenolic acid (MPA) and use of an induction therapy was not influencing cSoT values (p = 0.74, p = 0.61, p = 0.81 and p = 0.51, respectively; 29 TOL). We then analyzed the effect of current IS regimen on the cSoT in the STA population (n = 189). Similarly, cSoT was not influenced neither by induction therapy (p = 0.97), nor by CsA or Tacrolimus (p = 0.64), corticosteroids (p = 0.42) and antimetabolite agents (p = 0.92). We then further tested the effect of IS regimen on the 6 genes from the cSoT separately or in combination in two

independent cohorts of renal transplant recipients with stable graft function with available microarray datasets [34, 35]:

1. 1) a first cohort of patients under CsA (n = 14) or rapamycin (Rapa, n = 23) monotherapy [7]; and
2. 2) a second cohort of patients after conversion from azathioprine to MPA (n = 5 paired before and 3 months after MPA conversion) [6].

[0086] In both cohorts, neither the combination of the 6 genes nor the 6 genes independently (data not shown) were modified according to IS regimen (p = 0.99 and 0.77, respectively).

[0087] Altogether, these data showed that neither the cSoT, nor the genes independently are influenced by previous or maintenance IS treatment.

cSoT is predictive of graft dysfunction and de novo antibody appearance

[0088] One of the main question is the stability and the outcome of the cSoT in time. We previously reported that some cases of loss of graft function may be observed in this cohort of TOL [36], with increase of creatinemia (> 150 µmol/L) or proteinuria (> 1 g/24h). Among the 15 TOL form Nantes' cohort, for which most clinical information were available, 9 decline their function in time (17.09 ± 3.46 years post-transplantation). In addition, among these 15 TOL, 8 develop *de novo* anti-HLA antibodies (14.67 ± 1.13 years post-transplantation) and among them, 4 patients develop donor-specific antibodies (DSA; 13.41 ± 0.21 years post-transplantation). Moreover, the presence of *de novo* anti-HLA antibodies was associated with tolerance loss (p = 0.034).

[0089] We found that, at the test time, when patients exhibited a good graft function (creatinemia < 150 µmol/L and proteinuria < 1 g/24h), cSoT was significantly lower in patients who decline their function (test time 2.29 ± 2.7 years before function decrease) (p = 0.047, cSoT = 3.79 ± 3.66 and 8.52 ± 4.67) and who develop anti-HLA antibodies and DSA (p = 0.016 and p = 0.013, respectively) (test time 1.13 ± 1.78 and 0.21 ± 1.10 years before antibody detection).

cSoT is specific of operational tolerance state

[0090] To assess the specificity of the cSoT according to operational or protocol-induced tolerance profiles, we tested the cSoT in a trial of tolerance induction, in which renal recipients of HLA-identical from living donors siblings were followed up for 4 years after transplantation [9][8]. The protocol consists in a lymphodepletive alemtuzumab treatment with tacrolimus and MPA with early sirolimus conversion and infusions of donor hematopoietic $CD34^+$ stem cells.

Immunosuppression was withdrawn 2 years after transplantation. These patients had a normal biopsy (*i.e.*, no sign of subclinical rejection) and a good renal function at least 1 year after complete IS cessation.

[0091] Among the 15 patients, blood transcriptome was followed up to 4 years for 9 of them, 5 who became tolerant and 4 who did not. Either cSoT or the 6 genes only failed to classify the 5 tolerant patients as TOL, before and after arrest of IS, whatever the time post-transplantation. This result thus supports the fact that tolerance from this induction protocol did not share the operational and spontaneous tolerance-related signature, probably because of different mechanisms involved in the two situations, and that this signature is specific of operational tolerance state only.

<u>A qPCR cSoT applicable in clinic</u>

[0092] Since the cSoT score is based on gene microarray measures, we validated it and confirmed its usefulness using quantitative PCR in order to endorse its possible use in routine. The qPCR was performed into 5 independent TOL samples, not included in the meta-dataset, and 4 TOL from the meta-dataset at different times.

[0093] We showed that the cSoT score was discriminating TOL and STA ($p = 0.0054$, $n = 9$ and 12, mean = 2.89 ± 5.41 and -2.58 ± 2.37, respectively) with an AUC of 0.861 $IC_{95\%}$ [0.66-1]) (**Figure 2**). Furthermore, bootstrap procedure (1,000 times) confirmed the robustness of this validation with a mean AUC of 0.852 ($IC_{95\%}$ [0.845-0.859]).

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[0094] Throughout this application, various references describe the state of the art to which this invention pertains.

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Patentkrav

1. Fremgangsmåde til skelnen mellem en operationstolerant (TOL) nyrerecipient og en ikke-operationstolerant (STA) nyrerecipient, hvilken fremgangsmåde omfatter følgende trin:

5 i) etablering af en komposit tolerancescore (cSoT) med ekspressionsniveauer for seks gener i en biologisk prøve, der er opnået fra nyrerecipienten, og to kliniske parametre;

hvor de seks gener er *ID3, AKR1C3*, *CD40*, *CTLA4, TCL1A* og *MZB1*; og

hvor cSoT fastsættes ved hjælp af følgende formel:

$$cSoT = \sum_{i}^{n} = \beta_i \times Ekspr + \beta_{testtidspunkt} \times alder_{testtidspunkt} + \beta_{trans.tidspunkt} \times alder_{trans.tidspunk} + skæringspunkt - skaleringskoefficient$$

10 ii) sammenligning af denne cSoT med en forudbestemt referenceværdi; og

iii) konklusion om, at nyrerecipienten er TOL, når cSoT er højere end den forudbestemte referenceværdi, eller konklusion om, at nyrerecipienten er STA, når cSoT er lavere end den forudbestemte referenceværdi.

15 **2.** Fremgangsmåde ifølge krav **1**, hvor de to kliniske parametre er nyrerecipientens alder på testtidspunktet og nyrerecipientens på transplantationstidspunkter.

3. Fremgangsmåde ifølge krav **1** eller **2**, hvor den forudbestemte referenceværdi er en TOL-nyrerecipients cSoT.

20

4. Fremgangsmåde ifølge krav **1** eller **2**, hvor den forudbestemte referenceværdi er en STA-nyrerecipients cSoT.

5. Fremgangsmåde ifølge et hvilket som helst af kravene 1 til **4**, hvor nyrerecipienten er i

25 immunsuppressiv behandling.

6. Fremgangsmåde ifølge et hvilket som helst af kravene 1 til **5**, hvor nyrerecipienten er et menneske.

30 **7.** Fremgangsmåde ifølge et hvilket som helst af kravene 1 til **6**, hvor nyrerecipienten endvidere har fået transplanteret pancreas, og eventuelt et stykke af duodenum, fra nyredonoren.

8. Immunsuppressiv terapi til anvendelse ved behandling af en nyrerecipient, hvor nyrerecipienten er blevet bestemt som værende en ikke-operationstolerant (STA) nyrerecipient ved fremgangsmåden ifølge et hvilket som helst af kravene 1 til **7**.

5

9. Fremgangsmåde til identifikation af en nyrerecipient i immunsuppressiv terapi som kandidat til immunsuppressiv terapifravænning eller -minimering, hvilken fremgangsmåde omfatter følgende trin:

i) bestemmelse af, om personen er en operationstolerant (TOL) nyrerecipient eller en

10 ikke-operationstolerant (STA) nyrerecipient ved hjælp af fremgangsmåden ifølge et hvilket som helst af kravene 1 til **7**; og

ii) konklusion om, at nyrerecipienten er kvalificeret til immunsuppressiv terapifravænning eller -minimering, når personen er TOL.

DRAWINGS

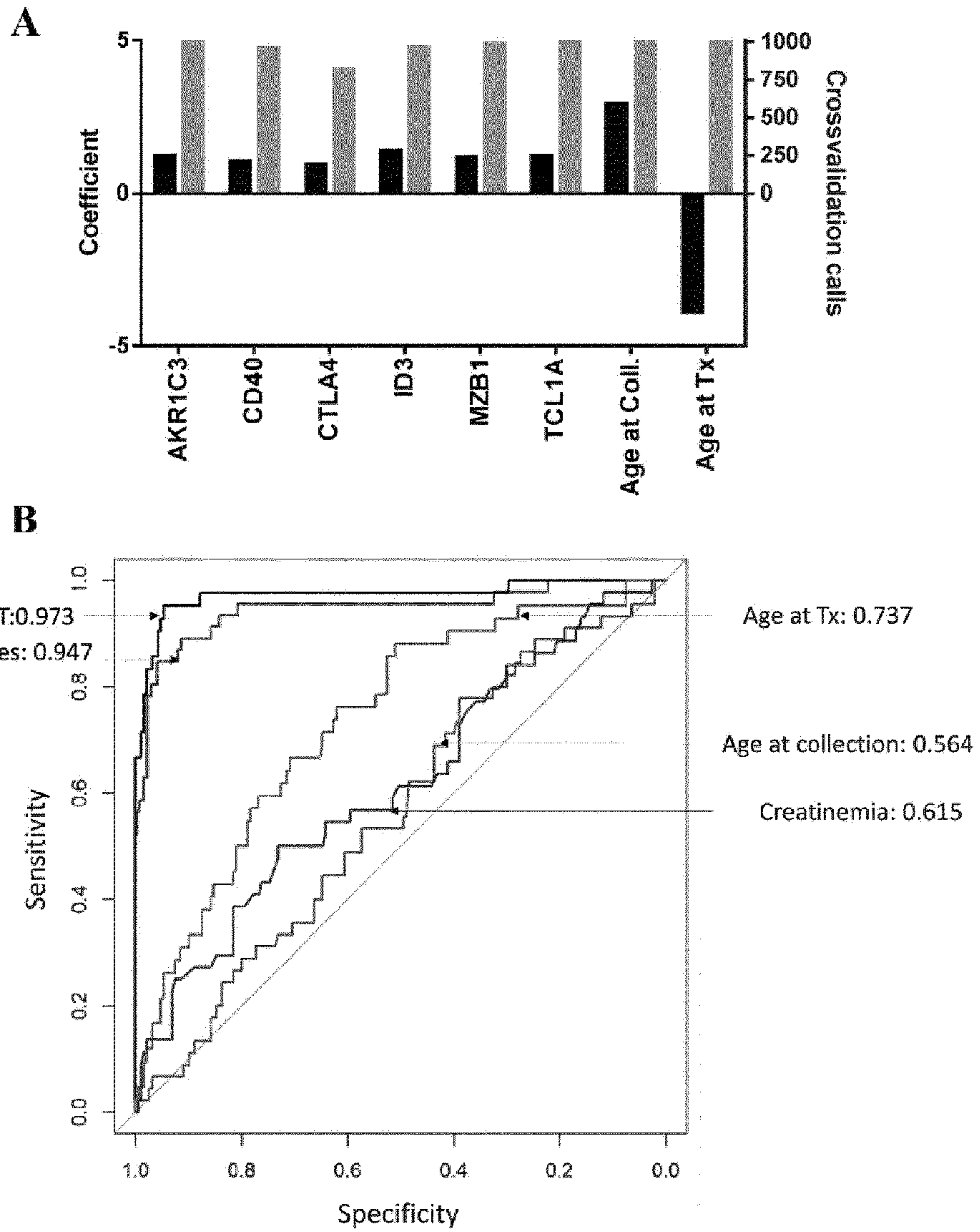

FIG. 1 A-B

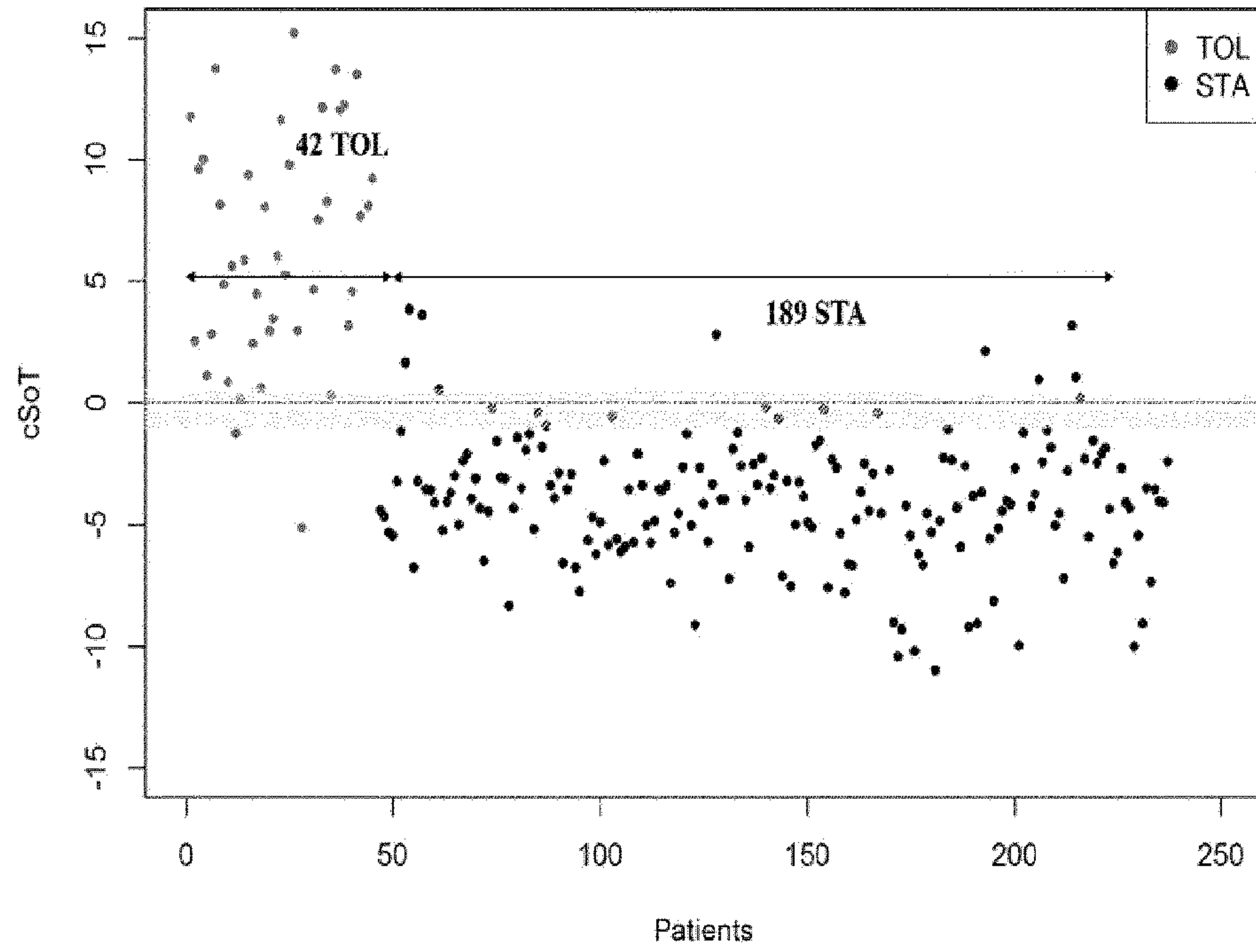

FIG. 1 C

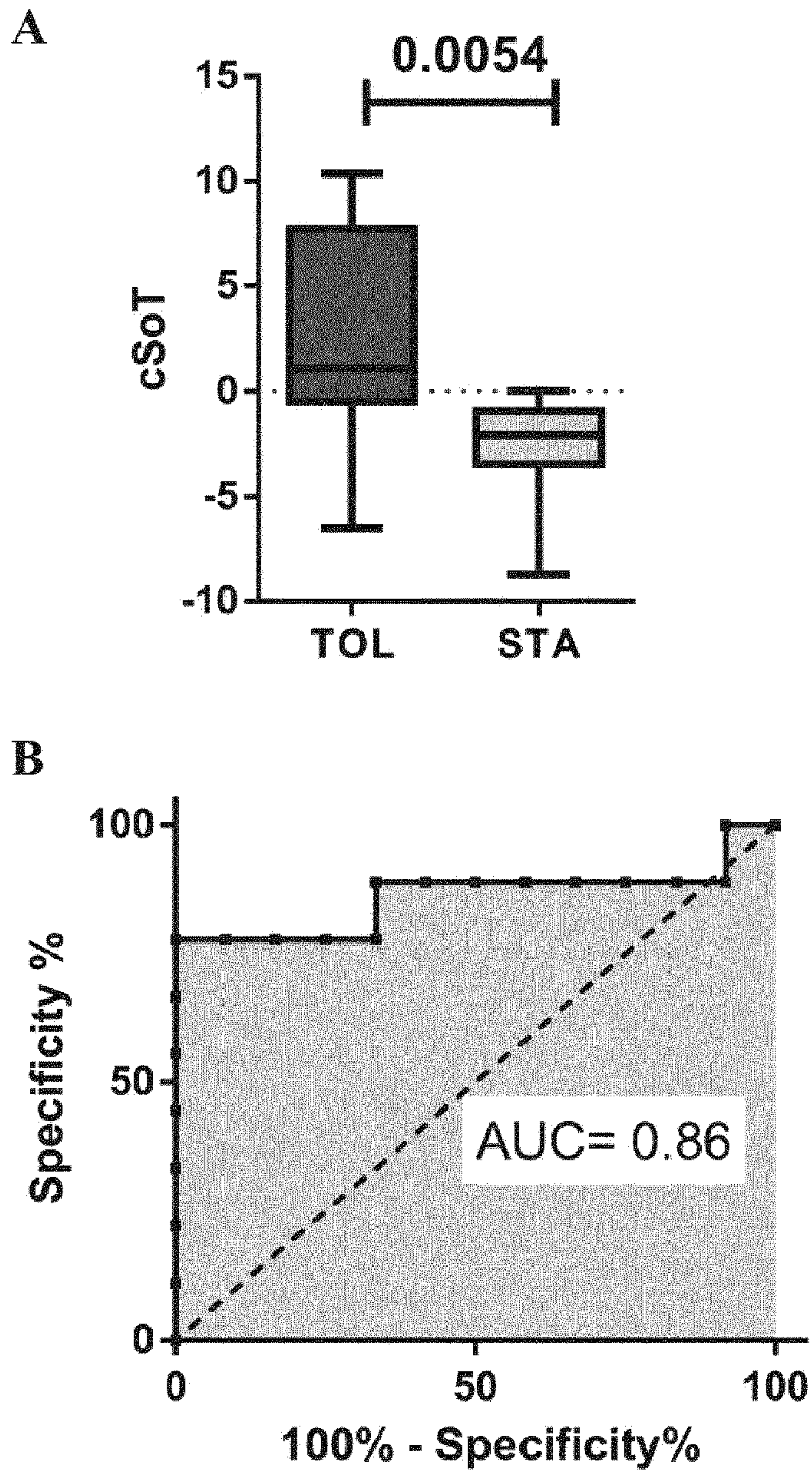

FIG. 2