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

[FR/FR]; 101, rue de Tolbiac, 75013 Paris (FR). UNIVERSITÉ PIERRE ET MARIE CURIE (PARIS 6) [FR/FR]; 4, place Jussieu, 75005 Paris (FR). ASSISTANCE PUBLIQUE-HÔPITAUX DE PARIS (APHP) [FR/FR]; 3, avenue Victoria, 75004 Paris (FR).

(72) Inventors: SAUCE-LARSEN, Delphine; INSERM

U1135 / Cimi-Paris, 91 Boulevard de l'hôpital, 75013 PARIS (FR). LARSEN, Martin; INSERM U1135 / Cimi-Paris 83 Boulevard de l'hôpital, 75013 PARIS (FR). BODDAERT, Jacques; Département de Gériatrie, UPOG CHU Pitié Salpêtrière, 47-83 Boulevard de l'Hôpital, 75013 PARIS (FR). APPAY, Victor; Inserm U1135 / CIMI-Paris, Hôpital Pitié-Salpêtrière, 91 Boulevard de l'Hôpital, 75013 Paris (FR).

(74) Agent: COLLIN, Matthieu; Inserm Transfert 7 rue Watt, 75013 PARIS (FR).

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(54) Title: METHODS FOR PREDICTING THE SURVIVAL TIME IN A SUBJECT

(57) Abstract: By analyzing the kinetics of the immunological parameters evocating the immune risk phenotype (IRP) in elderly subjects suffering from an acute event, the inventors have surprisingly identified that neopterin expressed highly in the subjects who has a short survival time after an acute event. Accordingly, the invention relates to a method for predicting the survival time in a subject suffering from an acute event comprising the following steps: i) determining neopterin level in a biological sample obtained from said subject; ii) comparing said level with its predetermined reference value and iii) concluding that the subject will have a short survival time when the level of neopterin is higher than its predetermined reference value or concluding that the subject will have a long survival time when the level of neopterin is lower than its predetermined reference value.



METHODS FOR PREDICTING THE SURVIVAL TIME IN A SUBJECT

FIELD OF THE INVENTION:

5 The invention is in the geriatric field, in particular the invention discloses a new biomarker, which predicts the survival time in the geriatric population.

BACKGROUND OF THE INVENTION:

 Aging is characterized by a progressive decline of physical and mental performances.
10 This geriatric syndrome, coined frailty, is increasing incrementally with advancing age, and more rapidly in older women and among those of lower socio-economic status. Frail older adults are at high risk of major adverse health outcomes, including disability, falls, institutionalization, hospitalization, and mortality. Autonomy and quality of life remains key considerations in geriatric population. One of the complications of frail elderly is hip fracture.
15 Hip fracture is a common condition associated with a poor outcome of 20-30% one-year mortality in the elderly. Factors causing the high morbidity burden in surgery for hip fractures commonly include older age and cardiorespiratory comorbidities as shown in the ESCORTE study analyzing the outcomes after hip fracture surgery in a large cohort of French patients [1]. However, inflammatory cytokines may play a major role in the patients' outcome. Indeed,
20 this trauma is known to be associated with major elevation of inflammatory markers and cytokines [2]. The high incidence of hip fracture in elderly (3%) raises an increased concern in a world with an aging population. In France, hip fracture in elderly corresponds to 80,000 hospitalizations/year (source: DREES, 2011), and has an estimated annual cost >€475 millions/year, for surgery only (DREES, 2011). This may reach €1.5 billion/year if including
25 the costs that can be incurred by rehabilitation/rehospitalization over a period of 1-2 years after HFS. Consequently, a major challenge is the management of the health and socioeconomic burden caused by this acute physical stress in the older population (+75 years). Thus, there is a need to establish which factors could influence and/or predict the outcome of an acute event in hip trauma in elderly patients.

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SUMMARY OF THE INVENTION:

 The present invention relates to a method for predicting the survival time in a subject suffering from an acute event comprising the following steps: i) determining neopterin level in a biological sample obtained from said subject; ii) comparing said level with its

predetermined reference value and iii) concluding that the subject will have a short survival time when the level of neopterin is higher than its predetermined reference value or concluding that the subject will have a long survival time when the level of neopterin is lower than its predetermined reference value. In particular, the present invention is defined by the
5 claims.

DETAILED DESCRIPTION OF THE INVENTION:

Elderly subjects are susceptible to have accidents, burn or traumas. The impact of the accidents or traumas influences the life style of elderly subjects and particularly, the economic
10 system (e.g.hospitalization). Thus, there is a need to identify new biomarkers and methods to determine the survival time and monitor the elderly subjects after an acute event. Neopterin is synthesised by human macrophages upon stimulation with the cytokine interferon-gamma. Neopterin is considered as a non-specific marker of activated cell mediated immunity (C.Murr et al 2002; Curr Drug Metab. 2002 Apr;3(2):175-87.). By analyzing the kinetics of
15 the immunological parameters evocating the immune risk phenotype (IRP) [3] in elderly subjects suffering from an acute event, the inventors have surprisingly identified that neopterin is expressed highly in the subjects who has a short survival time after an acute event.

Methods for predicting the survival time of a subject suffering from an acute event

Accordingly, the invention relates to a method for predicting the survival time in a
20 subject suffering from an acute event comprising the following steps: i) determining neopterin level in a biological sample obtained from said subject; ii) comparing said level with its predetermined reference value and iii) concluding that the subject will have a short survival time when the level of neopterin is higher than its predetermined reference value or
25 concluding that the subject will have a long survival time when the level of neopterin is lower than its predetermined reference value

As used herein, the term “predicting the survival time” refers to the duration of the disease or disease-free. In particular, the term “predicting the survival time” refers to whether the subject will have or not any complications after a post-acute-event. In the context of the
30 invention, the method is suitable for determining whether the subject suffering from an acute event will have any complications after treatment or a surgical operation. Typically, complications can be: invalidity, loss of independence, serious injury or death. In the context of the invention, the subject suffers from a hip fracture. The skilled person in the art can determine whether the subject suffering from hip fracture will have any complications after a

surgical operation. In a particular embodiment, the complication is loss of independence. When the skilled in the art determined that the level of neopterin is higher than its predetermined reference value, it means that the subject will have one of the complications after a surgical operation and thus will have a short survival time.

5 As used herein, the expression “short survival time” indicates that the subject will have a survival time that will be lower than the median (or mean) observed in the general population of subjects suffering from said disease. When the subject will have a short survival time, it is meant that the subject will have a “poor prognosis”. Inversely, the expression “long survival time” indicates that the subject will have a survival time that will be higher than the
10 median (or mean) observed in the general population of subjects suffering from said disease. When the subject will have a long survival time, it is meant that the subject will have a “good prognosis”.

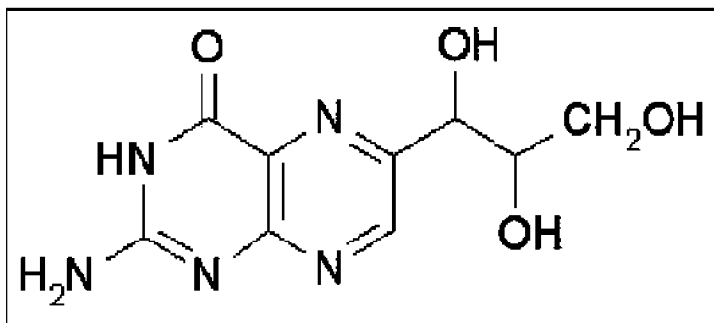
As used herein, the term “subject” refers to any mammals, such as a rodent, a feline, a canine, and a primate. Particularly, in the present invention, the subject is a human. In a
15 particular embodiment, the subject is an elderly. As used herein, the term “elderly” refers to a subject more than 70 years-old. In particular embodiment, the subject suffers or is susceptible to suffer from a hip fracture.

As used herein, the term “acute event” refers to any events from which a subject, typically an elderly suffers. Typically, the acute event refers to any accidents, burn or
20 traumas. The term “accident” includes any and unforeseen and unplanned event or circumstance resulting in damage inflicted on the body by an external force including resulting from fall or physical violence. The term “trauma” as used herein encompasses a damage which interrupts the integrity of the body skin or bone like a wound, particularly a wound that may be associated with tearing, cutting, piercing, or breaking of the tissue. In a
25 particular embodiment, the acute event is selected from the group consisting of: hip fracture, infectious diseases (e.g viral infections, bacterial infections, parasitic infections), autoimmune diseases or transplantation. As used herein, the term “hip fracture” refers to fractures of the proximal portion of the femur generally include femoral neck fractures and intertrochanteric fractures. As used herein, the term “infectious diseases” refers to diseases caused by
30 pathogenic microorganisms, such as bacteria, viruses, parasites or fungi; the diseases can be spread, directly or indirectly, from one person to another. Infectious diseases including, but not limited to, those caused by hepatitis viruses (e.g. hepatitis B virus, hepatitis C virus), human immunodeficiency virus (HIV), papillomaviruses, herpesviruses, respiratory viruses (e.g. influenza viruses, respiratory syncytial virus, rhinovirus, metapneumovirus,

parainfluenzavirus, SARS), West Nile virus, tuberculosis, bacterial pneumonia, aspergillosis, histoplasmosis, candidosis, pneumocystosis, leprosy, chlamydia, cryptococcal disease, cryptosporidiosis, toxoplasmosis, leishmania, malaria, and trypanosomiasis. As used herein, the term “autoimmune diseases” refers to a pathological state arising from an abnormal immune response of the body to substances and tissues that are normally present in the body. Autoimmune diseases including, but not limited to rheumatoid arthritis, psoriatic arthritis, systemic lupus erythematosus, Sjogrens disease, ankylosing spondylitis, scleroderma, dermatomyositis, diabetes, graft rejection, including graft-versus-host disease, inflammatory bowel diseases including, but not limited to, Crohn's disease and ulcerative colitis.

As used herein, the term “transplantation” refers to the procedure of replacing diseased organs, parts of organs, or tissues by healthy organs or tissues. The transplanted organ or tissue can be obtained either from the subject himself (= autograft), from another human donor (= allograft) or from an animal (= xenograft). Transplanted organs may be artificial or natural, whole (such as kidney, heart and liver) or partial (such as heart valves, skin and bone). In particular, said renal transplanted subject may further have been grafted with the pancreas, and optionally a piece of duodenum, of the kidney donor. Said subject is treated with immunosuppressive drugs or another drugs that are currently known in the art or that will be identified in the future. In a particular embodiment, the subject is under immunosuppressive treatment, which means that the subject is administered with one or more immunosuppressive drugs. Immunosuppressive drugs that may be employed in transplantation procedures include azathioprine, methotrexate, cyclophosphamide, FK-506 (tacrolimus), rapamycin, corticosteroids, and cyclosporins. These drugs may be used in monotherapy or in combination therapies.

As used herein, the term “neopterin” also known as 2-amino-4-hydroxy-6-(D-erythro-1', 2', 3'-trihydroxypropyl) has the following formula I and belongs to the group of pteridines. Neopterin represents a precursor molecule of biopterin that is an essential cofactor in neurotransmitter synthesis, and it is also involved in a variety of oxydation/reduction reactions in the body. Neopterin is derived in vivo from guanosine triphosphate (GTP). The enzyme GTP-cyclohydrolase-I (GTPCH I) catalyses this reaction in activated monocytes, macrophages, dendritic cells, and endothelial cells and to a lesser extent in renal epithelial cells, fibroblasts, and vascular smooth muscle cells upon stimulation mainly by interferon gamma and to a lesser extent by interferon alpha and beta with its release being enhanced by tumor necrosis factor [4, 5].



Formula I

As used herein, the term “biological sample” refers to sample obtained from a subject, for example blood, saliva, breast milk, urine, semen, blood plasma, synovial fluid or serum. In a particular embodiment, the biological sample is blood sample. The term “blood sample” means any blood sample derived from the subject. Typically, the level of neopterin is measured in a blood sample obtained when the subject arrived at hospital. In another embodiment, the blood sample is cryo-preserved at -80°C and the level of neopterin may be measured in the 18 months from the cryo-preservation. Typically, the serum has been obtained after collecting blood by venipuncture, allowing clotting. The clot is removed by centrifugation at room temperature and the resulting supernatant, designated serum, is carefully removed using Pasteur pipette. Plasma is produced when whole blood is collected into tubes that are treated with anticoagulant (heparine). . In a particular embodiment, the biological sample is a urine sample. In a particular embodiment the neopterin level is measured by competitive enzyme-linked immunosorbent assay (ELISA) by following commercial kits (e.g. DRG Diagnostics, Biomnis) using horseradish peroxidase (HRPO)-labeled neopterin. Particularly, ELISA is suitable for measuring neopterin level in saliva, urine and blood sample. In another embodiment the neopterin level is assayed by high pressure liquid chromatography (HPLC; see, e.g., Huber et al., J. Chromatography B: Biomed. Sci. App. 666(2): 223-232 (April 1995) regarding HPLC of neopterin in serum) with fluorescence detection after appropriate sample clean-up. Particularly, HPLC is performed to measure neopterin level in urine or plasma samples.

In a particular embodiment, the neopterin level is determined in dark conditions. As used herein, the term “dark conditions” refers to the conditions where all experiments and analysis related to neopterin measurement are performed with dark opaque material (i.e. material can be covered by aluminium foil or black plate) and in the absence of the light (<1 lux).

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 skill 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. 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: R, MedCalc 9.2.0.1 medical statistical software, SPSS 9.0, ROCPOWER.SAS, DESIGNROC.FOR, MULTIREADER POWER.SAS, CREATE-ROC.SAS, GB STAT VI0.0 (Dynamic Microsystems, Inc. Silver Spring, Md., USA), etc. In the context of the invention, the predetermined reference value is 20 nmol/L. Accordingly, when the level of neopterin is higher than its predetermined reference value obtained (e.g. higher than 20 nmol/L), concluding that the subject will have a

short survival time or when the level of neopterin is lower than its predetermined reference value (e.g lower than 20nmol/L), concluding that the subject will have a long survival time.

In a particular embodiment, the invention relates to a method for predicting the risk of having the mortality of the subject as identified having a short time survival according the invention comprising the following steps: i) determining neopterin level in a biological sample obtained from said subject; ii) comparing said level with its reference standard curve and iii) concluding that the subject is at risk of having a mortality when the level of neopterin is higher than its reference standard curve or concluding that the subject is not at risk of having a mortality when the level of neopterin is lower than its reference standard curve.

As used herein, the term “predicting” means that the subject to be analyzed by the method of the invention is allocated either into the group of subjects who will have risk of mortality, or into a group of subjects who will not have high risk of mortality. Typically, said risk is elevated as compared to the average risk in a cohort of subjects suffering from an acute event. In the context of the invention, the risk of having high mortality in a subject suffering from an acute event shall be predicted. The term “predicting the risk”, as used herein, refers to assessing the probability according to which the subject as referred to herein will die within the first year following the acute event.

In a particular embodiment, the duration of survival is predicted according to the level of neopterin measured in a said subject. Typically, higher the level of neopterin, shorter the said duration of survival. In the context of the invention, the duration of survival in a subject suffering from an acute event shall be predicted. The term “predicting the duration” as used herein refers to assessing the time to death of a subject.

As used herein, the term “reference standard curve” refers to a regression between neopterin levels and survival time. The reference standard curve can be established using retrospective measurements in properly banked historical subject samples. In one embodiment such standard curve describes a negative linear correlation between survival time and neopterin levels. More precisely, for each unit change of neopterin an individual will change survival time with approximately 4 days.

The inventors have shown that the predictive capacity may be improved by including another marker, the percentage of CD56+NKG2C+ NK cells. Indeed, they have shown that the sum of the neopterin level and the percentage of CD16+CD56dimNKG2C+ NK cells allows to predict the survival time of the subjects suffering from an acute event.

Accordingly, the method of the invention, comprises a further step of quantifying the percentage of CD16+CD56dimNKG2C+ NK cells in a biological sample obtained from said subject and concluding that the subject will have a short survival time when the sum of the level of neopterin and the percentage of CD16+CD56dimNKG2C+ NK cells is higher than their predetermined reference value or concluding that the subject will have a long survival time when the sum of the level of neopterin and the percentage of CD16+CD56dimNKG2C+ NK cells is lower than their predetermined reference value.

As used herein, the term “CD16+CD56dimNKG2C+ NK cells” refers to natural killer cells which express NKG2C receptor. As used herein, the term “NKG2C” also known as killer cell lectin-like receptor, refers to subfamily C, member 2, is a type II transmembrane protein with extracellular C-type lectin domain.

In some embodiments, the percentage of CD16+CD56dimNKG2C+ NK cells is quantified by flow cytometry. Typically, cells are incubated with a fluorescently labelled antibody which recognizes a polypeptide present on the surface on the target cells population. The cells are then forced into a small nozzle of a flow cytometer one at a time. They are then scanned by a fluorescence laser, separated according to their fluorescence and the target population can be collected. For example, the quantification of these cells is performed by contacting the blood sample with a binding partner (e.g antibody) for a cell marker of said cells. Typically, the quantification of these cells is performed by contacting the blood sample with several binding partners (e.g antibody) specific for following cell surface markers CD45, CD16, CD3, CD56, NKG2A and NKG2C. These binding partners are coupled with fluorescent agents known in the art, such as fluorescein, isothiocyanate, phycoerythrin etc by flow cytometry.

Methods for determining whether a subject will achieve a response to a treatment

Neopterin is considered as a non-specific marker of activated cell mediated immunity involving release of interferon gamma (C.Murr et al 2002; Curr Drug Metab. 2002 Apr;3(2):175-87.). Indeed, increased neopterin concentrations in body-fluids (such as serum or urine) has been described to be connected with diseases linked with cellular immune reaction, e.g. viral infections, including HIV infection and infections by intracellularly living bacteria or parasites, autoimmune diseases, inflammatory diseases, rejection episodes following organ transplantation and certain malignant diseases.

The method according to the invention is suitable for determining whether a subject will achieve a response to a treatment respected by change in neopterin level.

Accordingly, in a second aspect, the invention relates to a method for determining whether a subject will achieve a response to a treatment comprising: i) determining neopterin level in a biological sample obtained from said subject; ii) comparing said level with its predetermined reference value and iii) concluding that the subject will achieve a response to the treatment when the level of neopterin is higher than its predetermined reference value or concluding that the subject will not achieve a response to the treatment when the level of neopterin is lower than its predetermined reference value.

As used herein, the term “respond” refers to the response to a treatment of the subject suffering from a disorder. Typically such treatment induces, ameliorates or otherwise causes an improvement in the pathological symptoms, disease progression or physiological conditions associated with or resistance to succumbing to a disorder. Accordingly, the survival time of the subject is increased with said treatment. In particular, in the context of the invention, the term “respond” refers to the ability of the treatment in the improvement of the pathological symptoms, thus, the subject presents a clinical improvement compared to the subject who does not receive the treatment. The said subject is considered as a “responder” to the treatment. The term “not respond” refers to a subject who does not present any clinical improvement to the treatment. This subject is considered as a “non-responder” to the treatment. Accordingly, the subject as considered “non-responder” has a particular monitoring in the therapeutic regimen.

As used herein, the term “subject” refers to a human. Particularly, the subject suffers from one of the acute event as described above.

As used herein, the term “treatment” refers to the treatment which could be used to treat the patient suffering from one of the acute event. Typically, the treatment is selected from the group consisting of: antiviral, antibacterial, antiparasite, anti-inflammatory, immunomodulatory or adoptive immunotherapy treatment. As used herein, the term “antiviral treatment” refers to drugs used for treating viral infections such as infections from adenoviridae, herpesviridae, papillomaviridae, polymoviridae, poxviridae, poxviridae etc. As used herein, the term “antibacterial treatment” refers to drugs used for treating bacterial infections such as infections from escherichia, enterococcus, haemophilus influenza etc. As used herein, the term “antiparasite treatment” also called as parasitic treatment, refers to drugs used for treating the parasitic infections such as infections from leishmaniasis, malaria, Isosporiasis etc. As used herein, the term “anti-inflammatory treatment” refers to the treatment with drugs having the property of to reduce inflammation or swelling. Anti-inflammatory drugs make up about half of analgesics, remedying pain by reducing

inflammation as opposed to opioids, which affect the central nervous system. As used herein, the term “adoptive immunotherapy treatment” refers to the transfer of cells into a patient, typically, T cells are extracted from the patient, potentially genetically modified and cultured in vitro and returned to the same patient.

5 In a particular embodiment, the method of the invention is suitable for determining the immune system state before a vaccination. For example, the physician could measure the level of neopterin before the vaccination to avoid the vaccination. As used herein, the term “vaccination” refers to the administration of an antigen in a subject to stimulate its immune system.

10 In a particular embodiment, the method of the invention is suitable for determining whether a subject will achieve a response to a vaccination. Typically, the invention relates to a method for determining whether a subject will achieve a response to a vaccination comprising: i) determining neopterin level in a biological sample obtained from said subject; ii) comparing said level with its predetermined reference value and iii) concluding that the
15 subject will achieve a response to the to a vaccination when the level of neopterin is higher than its predetermined reference value or concluding that the subject will not achieve a response to the to a vaccination when the level of neopterin is lower than its predetermined reference value.

20 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:

25 **Figure 1. (A)** Variables capable of predictive death were identified using a regularized logistic regression model with lasso penalty. To render the analysis more robust we performed cross-validation by repetitive (n=500 bootstraps) selection (training, 2/3 of cohort) and validation (test, 1/3 of cohort) of predictive variables. Bar diagram depicts the frequency of bootstrapped models identifying a given variable. Only variables being identified in 2/3 of the
30 bootstrapped models were selected for further analysis (dashed line). **(B)** Scatter plot of the first two components of a Partial Least Square Discriminant Analysis (PLS-DA) identifying the degree of variance of the predicted variable (death) the two predictive variables identified by regularized logistic regression (neopterin concentration in nmol/l and % CD16+CD56dimNKG2C+ NK cells) are able to model. The explained variance is 41.2% and

0.2% for component 1 and 2 (PLS-C1 & PLS-C2), respectively. Symbols discriminate between alive (white symbols) and dead (black symbols) hip fracture patients one-year post-surgery. (C) Correlation plot between the 2 predictive variables, % NK cells CD16+CD56dimNKG2C+ and neopterin concentration. The measures are conducted at D0 (pre-surgery). (D) Scatter plot depicting a mortality score mathematically derived from the PLS component 1 stratified according to survival status. The mortality score is calculated as the sum of neopterin concentration (nmol/l) and the % CD16+CD56dimNKG2C+ NK cells. A proposed cut-off value (24.7) is indicated with a dotted line ; this gives a test with 100% sensitivity and 70% specificity. Statistical significance is calculated with Mann-Whitney t-test. (E) Receiver operating characteristic (ROC) curves of logistic regression models of death occurrence predicted by the two predictive variables in combination or individually (dashed line for % CD16+CD56dimNKG2C+ NK cells at D0; dotted line for plasmatic concentration of neopterin (nmol/l) at D0 and black line for the 2 variables in combination). Area under curve (AUC) for each curve is indicated.

Figure 2: Neopterin levels in elderly (A) The concentration of neopterin at arrival to hospital (in nmol/l) was measured for the control group of healthy elderly > 75 years-old (black circles, n=42) and was analyzed for the hip fracture patients according to 2 clinical outcomes at one-year post fracture: survivors (black squares, n=50) or deceased (DCD; black triangles, n=10). p values are indicated as significant after performing a non-parametric Mann-Whitney test. Dashed line shows the threshold value of neopterin concentration defined according to the upper 95% confidence interval of the mean of the neopterin concentration obtained from the elderly control cohort included in our study (n=42). (B) Distribution of the number of patients within each clinical subgroup (control healthy elderly/ survivors post hip-fracture/ deceased post hip-fracture), according to the upper 95% confidence interval of the mean of the neopterin concentration obtained from the elderly control cohort included in our study (n=42). This value of 20 nmol/l separates individuals in two groups according to their neopterin level; low (white bar) vs high (black bar).

Figure 3: Probability of one-year post fracture survival. (A) Kaplan Maier curves showing the percent of survival one-year post fracture according to the level of plasmatic neopterin measured at D0, which corresponds to the time of arrival to the hospital. Statistical differences are assessed with Mantel-Cox test. p value is indicated on the graph. (B) Correlation between the level of neopterin measured at D0 (nmol/l) and the time of survival

(days). Correlation statistics was assessed with a Spearman test. The coefficient of correlation and p value are indicated on the graph.

Figure 4: human NK-cell phenotype. Gating strategy to determine the differentiation profile of NK-cell compartment, looking at the expression of CD16, CD56, NKG2A and NKG2C. CD45+CD3-CD16+CD56+NKG2C+NKG2A- cells are referred as CD16+CD56dim NKG2C+ NK cells.

EXAMPLE:**Material & Methods**

Experiments to measure neopterin level were performed using a commercially available kit from alpha diagnostic (3410/150722A) by following manufacturer's instructions
5 (described below)

PRINCIPLE OF THE TEST

Neopterin ELISA kit is based on competitive binding of human Neopterin from serum samples and enzyme-labeled Neopterin to Neopterin specific antibodies immobilized on
10 microtiter plates. After a washing step, chromogenic substrate is added and color developed. The enzymatic reaction (blue color) is inversely proportional to the amount of Neopterin present in the sample. The reaction is terminated by adding stopping solution (converts blue to yellow). Absorbance is then measured on an ELISA reader at 450 nm and the concentration of Neopterin in samples and control is read off the standard curve.

SENSITIVITY

The lower detection limit is calculated from the standard curve by determining the resulting concentration of the mean OD of Calibrator A (based on 10 replicate analyses) minus 2 SD. The sensitivity of the Neopterin ELISA kit is 0.7 nmol/L.

SPECIFICITY (CROSS REACTIVITY)

The following compounds were tested for cross-reactivity with the Direct Neopterin
20 ELISA kit. No significant interference was detected at the following concentration. Hemoglobin 35 mg/dL, Bilirubin 2.25 mg/dL, Triglyceride 125 mg/dL.

SPECIMEN COLLECTION AND HANDLING

Collect blood by venipuncture, allow to clot, and separate serum by centrifugation at
25 room temperature. Do not heat inactivate the serum. If sera cannot be immediately assayed, these could be stored at -20°C for up to six months. Avoid repeated zing and thawing of samples. Do not use specimens containing NaN₃. Samples appearing turbid should be centrifuged before testing to remove any particulate material. Plasma, urine or other biological fluids are compatible with the kit upon manual optimization. Our experiments were
30 performed on frozen human plasma. The plasma samples have been aliquoted and cryopreserved at -80°C for up to eighteen months before usage. Thawing has been done by putting samples in a fridge allowing slow thawing. Thereafter, thawed plasma have been centrifuged at 1000 rpm during 10 min in the dark.

REAGENTS PREPARATION

Dilute stock Wash buffer 1:20 with water. Store diluted buffer at 4°C for 1 month. Dilution of samples: If necessary, diluted sample with assay buffer 1:101. All reagents must be at room temperature prior to their use. In our hands, human plasma aliquots were used undiluted and manipulated in the dark (covered by aluminium foil or stored in black plastic tubes from Heathrow Scientific or Sarstedt).

STORAGE AND STABILITY

The microtiter well plate and all other reagents are stable at 2-8°C until the expiration date printed on the label. The whole kit stability is usually 6 months from the date of shipping under appropriate storage conditions. The unused portions of the standards should be stored at 2-8°C or stored frozen in small aliquots.

TEST PROCEDURE

Remove required number of coated strips and arrange them on the plate. Store unused strips in the bag. 1). Label or mark the microtiter well strips to be used on the plate. Dilute the wash buffer with water (1:20). 2). Pipet 20 µl of standards, controls, and samples into appropriate wells in duplicate. 3). Add 100 µl of ready-to-use enzyme conjugate into each well. Pipet 50 µl of ready-to-use Neopterin antiserum into each well. Mix gently for 5-10 seconds. Cover the plate and incubate for 90 minutes at 18-25 °C temp on orbital shaker (500 rpm) in the dark. 4). Aspirate the well contents and blot the plate on absorbent paper. Immediately, wash the wells 4 times with 300 µl of 1X wash buffer. We recommend using an automated ELISA plate Washer for better consistency. Failure to wash the wells properly will lead to high blank or zero values. If washing manually, plate must be tapped over paper towel between washings to ensure proper washing. 5). Add 150 µl TMB substrate solution. Mix gently for 5-10 seconds. Cover the plate and incubate for 10 mins in the dark at 18-25 °C temp. Note: incubation time may be varied + 3 mins to achieve maximum ($A_{450} \approx 2.00$). 6). Stop the reaction by adding 150 µl of stop solution to all wells at the same timed intervals as in step 5. Mix gently for 5-10 seconds to have uniform color distribution (blue color turns yellow). 7). Measure the absorbance at 450 nm using an ELISA reader within 15 min.

CALCULATION OF RESULTS

A. Average the absorbance of all duplicates. Subtract the averaged non-specific binding (NSB) absorbance from the average obtained above. This yields the net absorbance. Divide the net absorbance by the net zero standard absorbance (B_0) to obtain the percent bound ($\%B/B_0$).

B. FORMULA:

Abs. (sample) – Abs. (NSB)

% B/Bo = x 100

Abs. (zero standard) – Abs. (NSB)

Abs. = average absorbance of duplicate wells

5 NSB = non-specific binding (also known as the blank)

Sample = particular serum or standard being calculated

Zero Standard = 0 nmol/L standard or 100% binding wells

10 C. Construct a plot of the percent bound (Y-axis) versus the concentration of the neopterin standards ((X-axis) starting with the 0.5 nmol/L point. Either logit-log or semi-log graph paper may be used. This yields the standard curve.

D. Using the standard curve, determines the neopterin concentration of each sample.

NOTE: Values that bind either higher or lower than the standard curve should not be determined by extrapolation.

15 Flow cytometry experiments to measure the frequency of CD16+CD56dim NKG2C+ NK cells were performed using directly conjugated antibodies: BD Biosciences (San Jose, CA): CD16 (APC-H7), CD56 (PE-Cy7); Beckman Coulter (Pasadena, CA): CD45 (KO), NKG2A (APC); BioLegend (San Diego, CA): CD57 (PB), CD3 (BV650); R&D systems (Abingdon, UK): NKG2C (PE). Staining for cell surface markers was performed with standard method:

20 PBMCs were first exposed at 4°C to the combination of human antibodies (described above) for 20 minutes, then washed with PBS and then fixed with 2% paraformaldehyde. Cells were analysed on a Fortessa flow cytometer (Becton Dickinson). Data were analyzed using FlowJo v8.2 (Tree Star, Inc) and DIVA softwares (BD Biosciences). Exhaustive phenotypic analysis of NK cells was conducted with a beta version of the “FunkyCells ToolBox” software

25 (www.FunkyCells.com) developed by Dr Martin Larsen (INSERM U1135, Paris, France).

Results

30 More than one hundred elderly individuals > 75 years old were screened to participate in our study. Half of the cohort was constituted of healthy individuals (n=42) whereas the other half was admitted in emergency department for hip fracture. Of note, patients suffering from osteoporosis were not included; therefore only accidental hip fracture patients were included in our cohort (n=60). Longitudinal follow-up post fracture was designed with blood samples taken at their arrival to hospital (D0; PRE), after surgery (POST), at their discharge from the hospital (D7-D10; EXIT) and at long- term post fracture (M12; FOLLOW UP).

Plasma as well as PBMCs have been cryopreserved until use. Experiments always included samples from healthy controls compared to hip-fracture patients (all timepoints being performed simultaneously). 24 clinical parameters as well as 33 biological factors were registered. From an immunological point of view, both cellular and plasmatic components were assessed (n=79). We analyzed our data exhaustively using a regularized logistic regression model with lasso penalty. Among the parameters collected for each individual, only 21 were significant in a cross validation of variables by bootstrapping analysis (Figure 1A), comparing among hip fracture patients, the individuals with good clinical output (survival; n=46) vs the ones with bad outcome (death; n=10). Only 2 variables were outstanding (occurring in 2/3 of the sparse models) and therefore kept for the following analysis: the percentage of CD16+CD56dimNKG2C+ NK cells (obtained according to the gating strategy depicted in Figure 4) and neopterin concentration (nmol/l). In a Partial Least Square Discriminant analysis (PLS-DA) these two variables enable us to discriminate between the two subgroups (Figure 1B). Indeed, the primary driver of the first PLS component (PLS-C1) which explains 41.2% of the variance of the dataset, is the sum of the plasmatic neopterin concentration and the % of CD16+CD56dimNKG2C+ NK cells. Employing the loading scores obtained from the PLS-DA each parameter contributes equally (Figure 1C). Contrarily, the second PLS component (PLS-C2) only explains 0.2% of the variance and can therefore practically be neglected. From this analysis, we derived a survival score equal to the sum of the % of CD16+CD56dimNKG2C+ NK cells and neopterin concentration. Employing an appropriate cut-off value this algorithm discriminates clearly between survival and death (Figure 1D; $p < 0.0001$). We identified a cut-off value of 24.7 giving a test with 100% sensitivity and 70% specificity. Alternatively a cut-off value of 31.2 gave a test with 80% sensitivity and 84% specificity. (Figure 1E ROC CURVE)

Logistic regression models of death occurrence with each variable individually show that mortality is best predicted by neopterin plasma levels (Figure 1E; $AUC_{\text{neopterin}} = 0.859$ vs $AUC_{\%CD16+CD56dimNKG2C+NK \text{ cells}} = 0.774$). Moreover, when comparing the ROC curves in Figure 1E, the combination of the 2 variables do not show a clear improvement of the prediction model compared to neopterin alone ($AUC_{\text{combined}} = 0.886$). For these reasons, we choose to focus on the level of neopterin reached by the patients at their arrival to hospital.

As shown in Figure 2A, the concentration of neopterin at day 0 was similar between the control group of healthy elderly and the hip-fracture patients who survive post-fracture.

Importantly, statistical differences were observed when comparing hip-fracture patients who died within the first year post-fracture with either control individuals ($p=0.0009$) or with survivors ($p=0.0004$). A cut-off value was set at 20 nmol/l, which corresponds to the upper 95% confidence interval of the mean of the neopterin concentration obtained from the cohort of control elderly included in our study ($n=42$). This threshold enables us to look at the distribution of the number of patients within each clinical subgroup (Figure 2B). According to the level of neopterin measured in each patient at their arrival to hospital (high level > 20 nmol/l vs low level < 20 nmol/l), the probability of survival (within the year of followup) was statistically significant (Figure 3A; $p = 0.0005$). The test has 80% sensitivity and 74% specificity. Moreover, we could define a negative correlation between neopterin concentration at D0 and the number of days of survival after fracture ($p=0.039$, $r = -0.67$; Figure 3B).

In conclusion, these data demonstrate that neopterin level is a predictive marker of mortality in elderly population suffering from an acute clinical event, i.e. hip fracture in our context. The concentration measured in the patients at their arrival to hospital is the best predictive marker observed in our experiments and correlated negatively with the time of survival after hip fracture surgery. Predictive capacity may be slightly improved by including another marker, the percentage of CD16+CD56dimNKG2C+ NK cells. Noteworthy, adding a known clinical indicator of fitness (time to walk post-surgery) as a third variable further improves the predictive capacity (combined AUC=0.951).

REFERENCES:

Throughout this application, various references describe the state of the art to which this invention pertains. The disclosures of these references are hereby incorporated by
5 reference into the present disclosure.

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- 5 5 Hoffmann, G., Wirleitner, B. and Fuchs, D., Potential role of immune system activation-associated production of neopterin derivatives in humans. *Inflamm Res*
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CLAIMS:

- 5 1. A method for predicting the survival time in a subject suffering from an acute event comprising the following steps: i) determining neopterin level in a biological sample obtained from said subject; ii) comparing said level with its predetermined reference value and iii) concluding that the subject will have a short survival time when the level of neopterin is higher than its predetermined reference value or concluding that the subject will have a long survival time when the level of neopterin is lower than its predetermined reference value.
- 10 2. The method according to claim 1, wherein the subject is an elderly subject.
3. The method according to claim 1, wherein the acute event is selected from the group consisting of: hip fracture, infectious diseases (e.g viral infections, bacterial
15 infections, parasitic infections), autoimmune diseases or transplantation.
4. The method according to claim 1, wherein the biological sample is blood sample.
5. The method according to claims 1 or 4, wherein the blood sample is fresh or cryo-
20 preserved at -80°C.
6. The method according to claim 1, wherein the determination of neopterin level is performed in dark conditions.
- 25 7. A method for predicting the risk of having mortality of the subject as identified having a short time survival according to claim 1, comprising the following steps: i) determining neopterin level in a biological sample obtained from said subject; ii) comparing said level with its reference standard curve and iii) concluding that the subject is at risk of early death when the level of neopterin is higher than its
30 reference standard curve or concluding that the subject is not at risk of having a mortality when the level of neopterin is lower than its reference standard curve.
8. The method according to claims 1 or 7 comprising a further step of quantifying the percentage of CD16+CD56dimNKG2C+ NK cells in a biological sample obtained

from said subject and concluding that the subject will have a short survival time when the sum of the level of neopterin and the percentage of CD16+CD56dimNKG2C+ NK cells is higher than a predetermined reference value or concluding that the subject will have a long survival time when the sum of the level of neopterin and the percentage of CD16+CD56dimNKG2C+ NK cells is lower than a predetermined reference score.

9. The method according to claim 8, wherein the percentage of CD16+CD56dimNKG2C+ NK cells is quantified by flow cytometry.

10. A method for determining whether a subject will achieve a response to a treatment comprising: i) determining neopterin level in a biological sample obtained from said subject; ii) comparing said level with its predetermined reference value and iii) concluding that the subject will achieve a response to the treatment when the level of neopterin is higher than its predetermined reference value or concluding that the subject will not achieve a response to the treatment when the level of neopterin is lower than its predetermined reference value.

11. The method according to claim 11, wherein the treatment is selected from the group consisting of: antiviral, antibacterial, antiparasite, anti-inflammatory, immunomodulatory or adoptive immunotherapy treatment.

12. The method according to claim 11 is suitable for determining whether a subject will achieve a response to a vaccination.

A

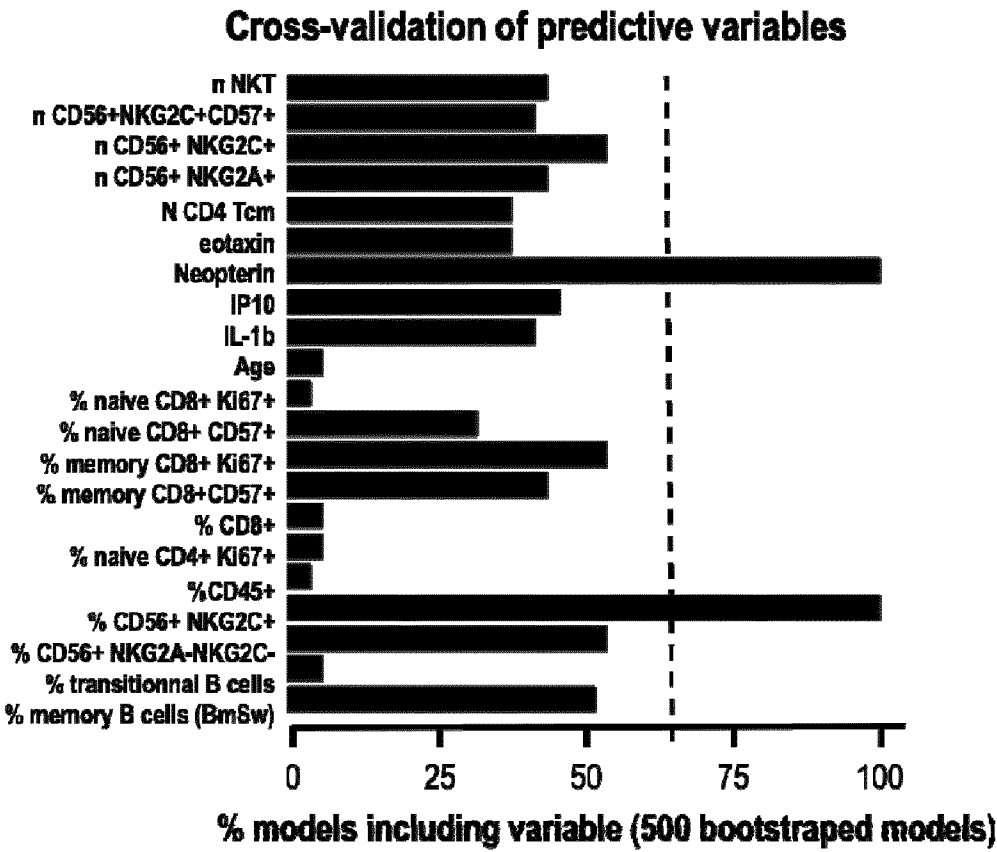


Figure 1A

B

**Partial Least Square – Discriminant analysis
(2 variables)**

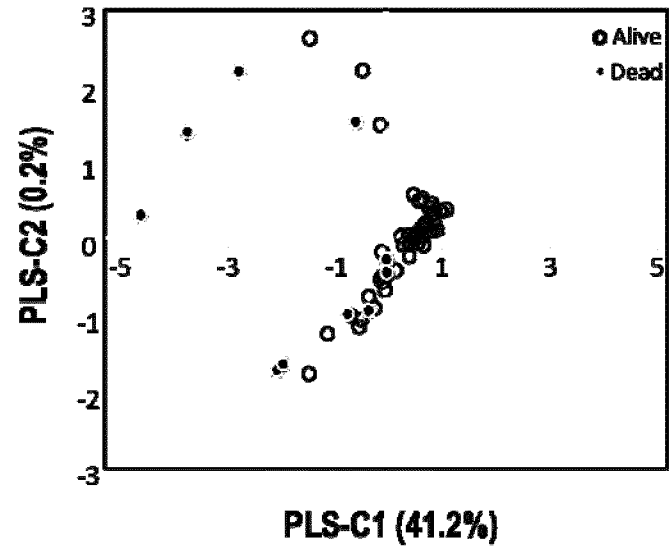


Figure 1B

2/6

C.

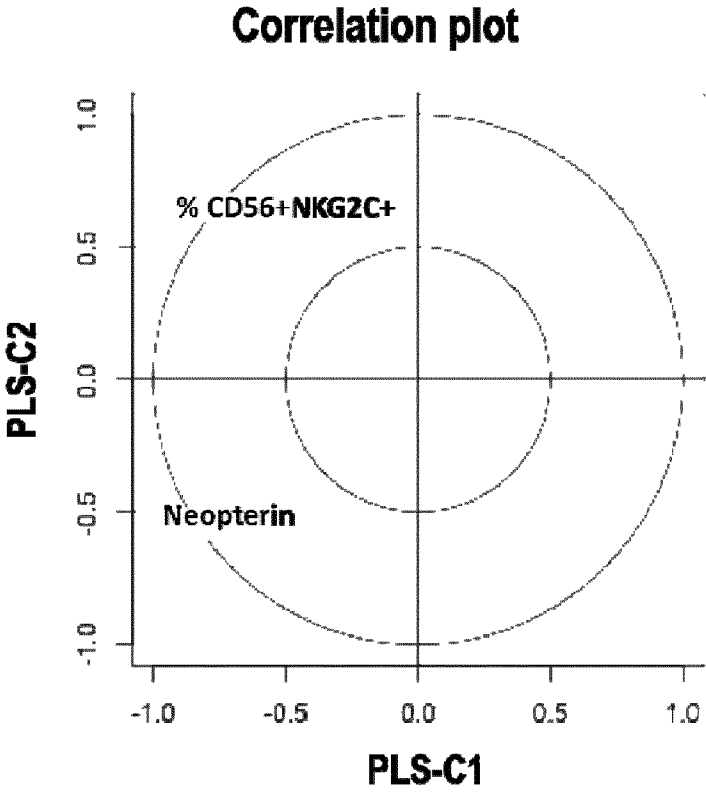


Figure 1C

D.

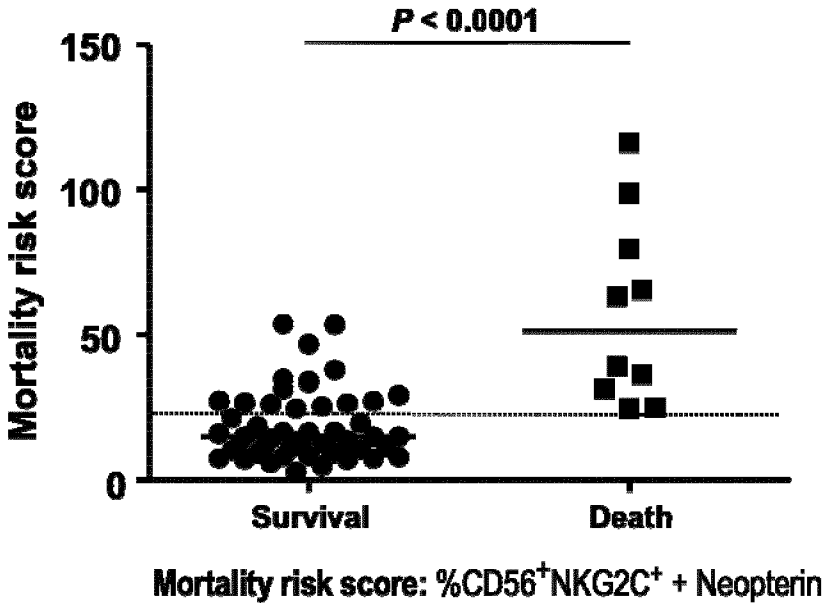


Figure 1D

E.

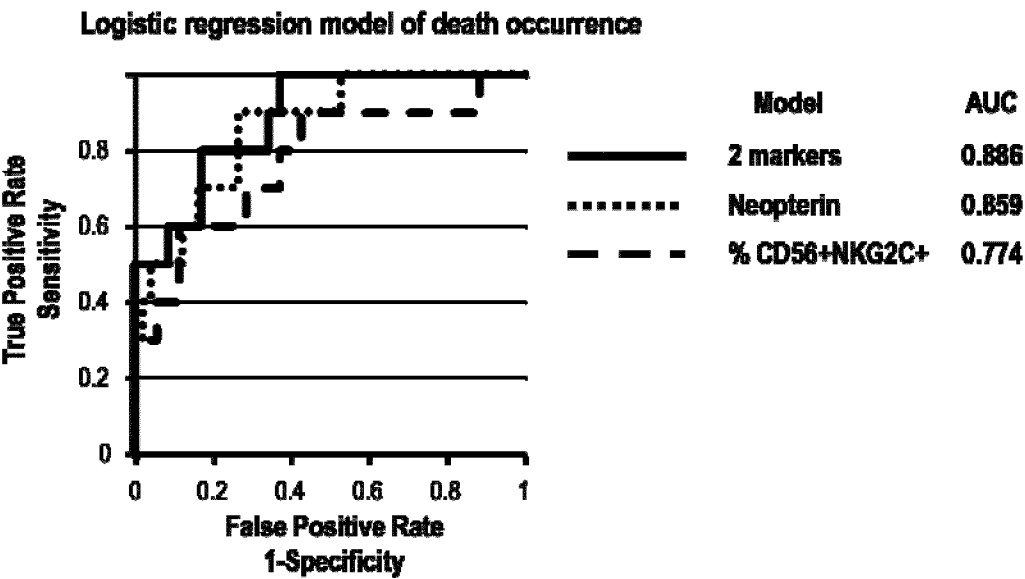


Figure 1E

A

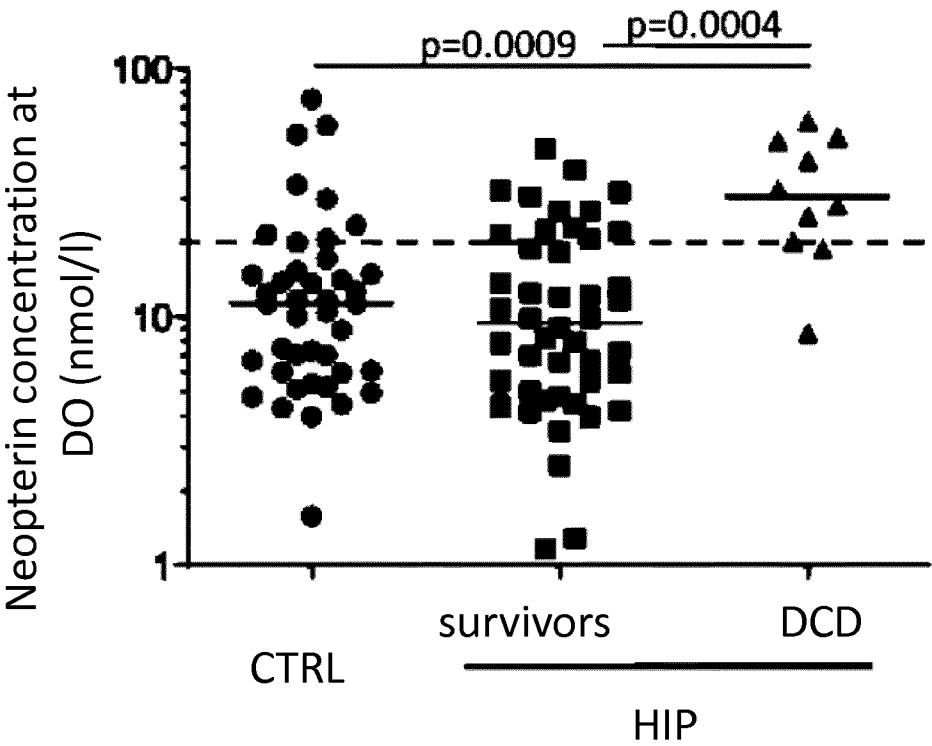


Figure 2A

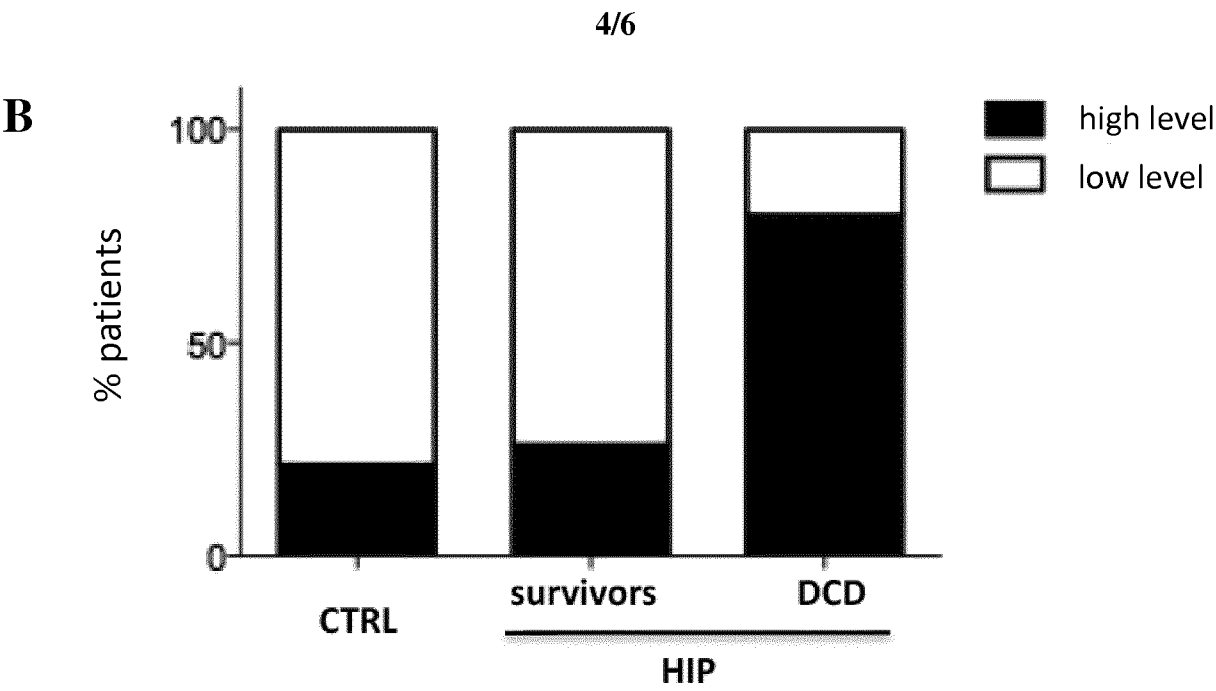


Figure 2B

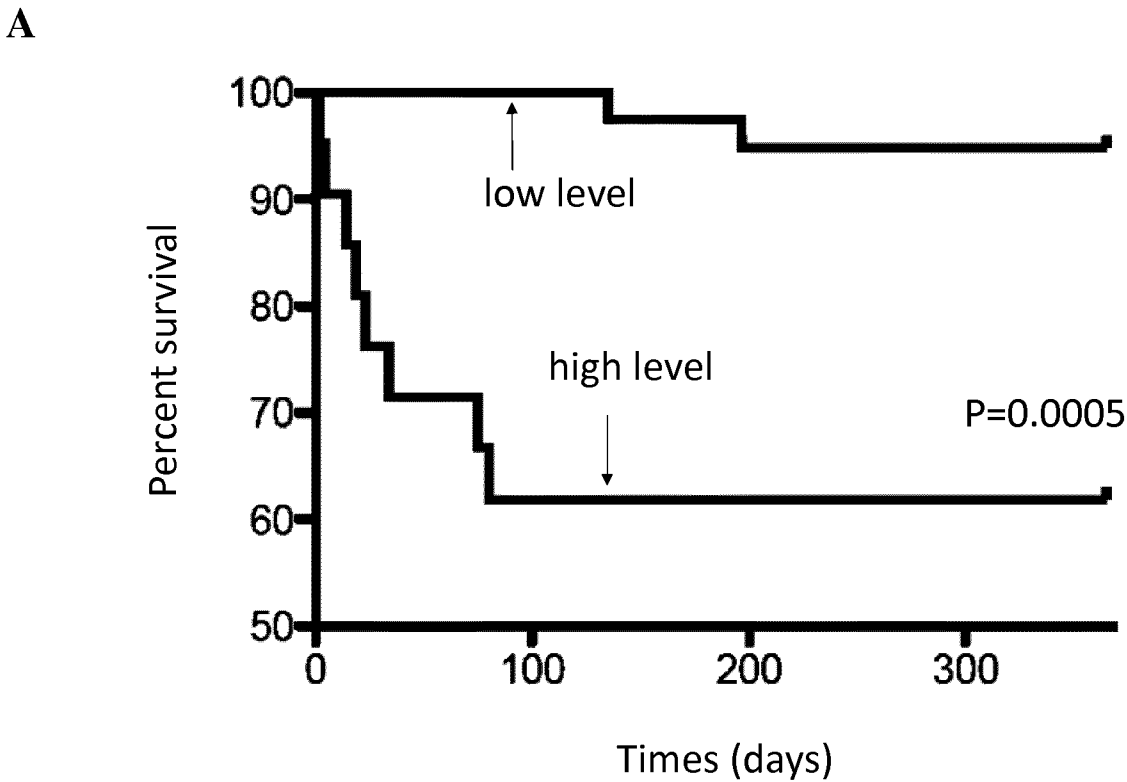


Figure 3A

5/6

B

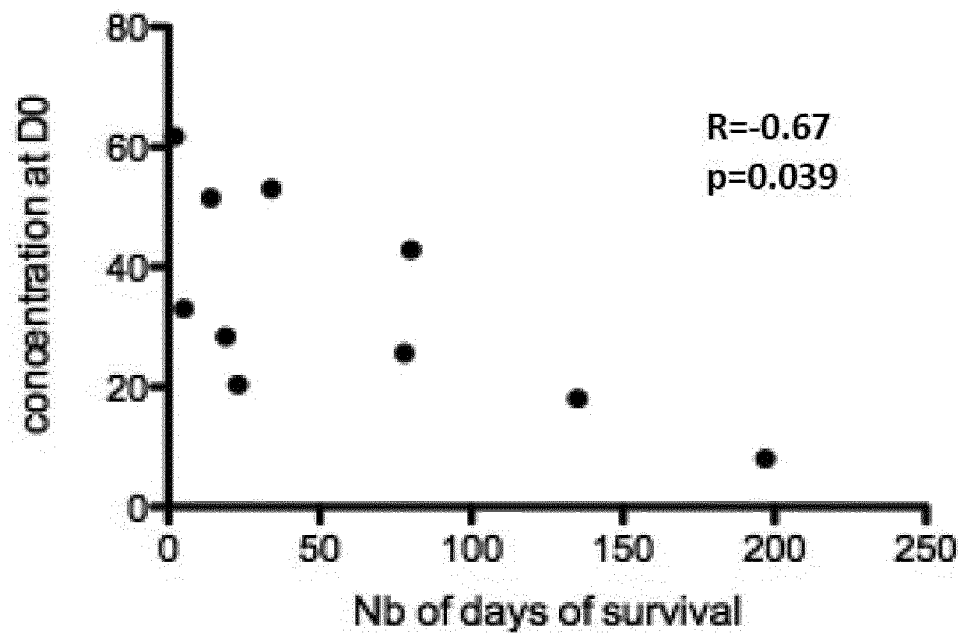


Figure 3B

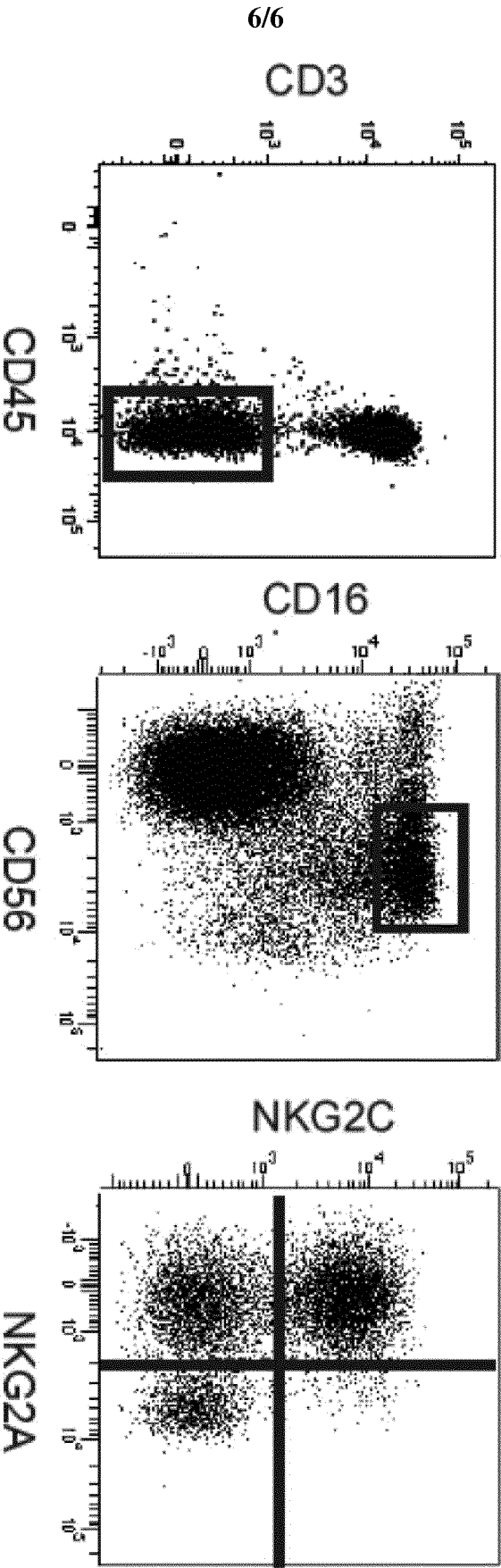


Figure 4

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2017/072309A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N33/53
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, WPI Data, EMBASE, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GILBERT REIBNEGGER ET AL: "POSTTRANSPLANT NEOPTERIN EXCRETION IN RENAL ALLOGRAFT RECIPIENTS-A RELIABLE DIAGNOSTIC AID FOR ACUTE REJECTION AND A PREDICTIVE MARKER OF LONG-TERM GRAFT SURVIVAL", TRANSPLANTATION, vol. 52, no. 1, 1 July 1991 (1991-07-01), pages 58-63, XP055332494, GB ISSN: 0041-1337, DOI: 10.1097/00007890-199107000-00012 abstract page 58, line 59 ----- -/--	1-3,6,7



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)

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"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

26 September 2017

Date of mailing of the international search report

06/10/2017

Name and mailing address of the ISA/

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

Authorized officer

Moreno de Vega, C

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2017/072309

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	K. E. ROGSTAD ET AL: "Urinary neopterin as a prognostic index of survival time in HIV-1 infection", INTERNATIONAL JOURNAL OF STD AND AIDS., vol. 9, no. 6, 1 June 1998 (1998-06-01), pages 326-329, XP055331975, GB ISSN: 0956-4624, DOI: 10.1258/0956462981922340 the whole document	1-3,6,7
X	----- Rupert Prommegger ET AL: "Neopterin: a prognostic variable in operations for lung cancer", The Annals of Thoracic Surgery, 1 December 2000 (2000-12-01), pages 1861-1864, XP55332489, United States ISSN: 0003-4975, DOI: 10.1016/S0003-4975(00)01840-3 Retrieved from the Internet: URL:http://ac.els-cdn.com/S0003497500018403/1-s2.0-S0003497500018403-main.pdf?_tid=6b0a32fa-d333-11e6-95c1-00000aacb35f&acdnt=1483613027_c354de92b17ccc6a301afdc416846bf6 [retrieved on 2017-01-05] the whole document	1-3,6,7
X	----- MURR C ET AL: "Neopterin as a marker for immune system activation", CURRENT DRUG METABOLISM, BENTHAM SCIENCE PUBLISHERS, US, vol. 3, no. 2, 1 April 2002 (2002-04-01), pages 175-187, XP008116733, ISSN: 1389-2002, DOI: 10.2174/1389200024605082 the whole document	1-7
A	----- TANAKA J ET AL: "Increased number of CD16<+>CD56<dim> NK cells in peripheral blood mononuclear cells after allogeneic cord blood transplantation", HUMAN IMMUNOLOGY, NEW YORK, NY, US, vol. 70, no. 9, 1 September 2009 (2009-09-01), pages 701-705, XP026494474, ISSN: 0198-8859, DOI: 10.1016/J.HUMIMM.2009.06.002 [retrieved on 2009-06-11] abstract ----- -/--	1-12

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2017/072309

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2012/115244 A1 (ADAMCZYK MACIEJ [US] ET AL) 10 May 2012 (2012-05-10) column 1, paragraph 4 - column 4, paragraph 17; claims 1-11 -----	1-7, 10-12
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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2017/072309

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2012115244 A1	10-05-2012	US 2012115244 A1	10-05-2012
		WO 2012064674 A1	18-05-2012
