

(51) International Patent Classification:
C12Q 1/68 (2006.01) *G01N 33/50* (2006.01)(21) International Application Number:
PCT/EP2010/005809(22) International Filing Date:
22 September 2010 (22.09.2010)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
09012160.9 24 September 2009 (24.09.2009) EP

(71) Applicants (for all designated States except US): LUDWIG-MAXIMILIANS-UNIVERSITÄT MÜNCHEN [DE/DE]; Geschwister-Scholl-Platz 1, 80539 München (DE). RUPRECHT-KARLS-UNIVERSITÄT HEIDELBERG [DE/DE]; Grabengasse 1, 69117 Heidelberg (DE).

(72) Inventors; and

(75) Inventors/Applicants (for US only): BIBERTHALER, Peter [DE/DE]; Preysingerstr. 23, 82131 Gauting (DE). BOGNER, Viktoria [DE/DE]; Schulzstr. 11b, 85579 Neubiberg (DE). GIESE, Thomas [DE/DE]; Bluetenweg 7, 69198 Schriesheim (DE).

(74) Agents: ENGELHARD, Markus et al.; Boehmert & Boehmert, Pettenkoferstrasse 20-22, 80336 München (DE).

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— with international search report (Art. 21(3))

— with sequence listing part of description (Rule 5.2(a))

(54) Title: A COMBINATION OF MARKERS FOR PREDICTING THE MORTALITY RISK IN A POLYTRAUMATIZED PATIENT

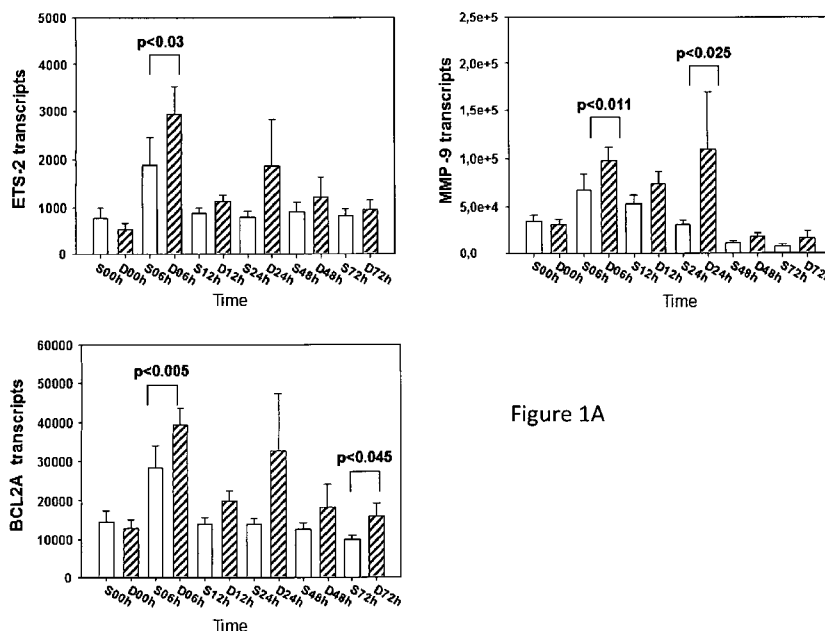


Figure 1A

(57) Abstract: The present invention relates to a combination of markers selected from BCL2A1, MMP-9 and ETS-2 for use in predicting the mortality risk in a patient suffering from polytrauma. The present invention also relates to a kit for predicting the mortality risk in such patient, and to a method of predicting the mortality risk in such patient. Furthermore, the present invention relates to a nucleic acid composition, to a matrix on which such nucleic acid composition has been immobilized. Furthermore, the present invention relates to uses of the aforementioned kit, of the nucleic acid composition and of the matrix.

A combination of markers for predicting the mortality risk in a polytraumatized patient

The present invention relates to a combination of markers for use in predicting the mortality risk in a patient suffering from polytrauma. The present invention also relates to a kit for predicting the mortality risk in such patient, and to a method of predicting the mortality risk in such patient. Furthermore, the present invention relates to a nucleic acid composition, to a matrix on which such nucleic acid composition has been immobilized. Furthermore, the present invention relates to uses of the aforementioned kit, of the nucleic acid composition and of the matrix.

Patients after multiple, severe trauma suffer from posttraumatic immune system destabilization and subsequent multiple organ dysfunction. This closely influences the patients' further clinical development. The Federal Bureau of Statistics for Germany indicates 88382 severely injured casualties in the year 2002 in Germany. The average age of these is approximately 40 years which means that these patients are in the midst of their working career. Multiply and severely traumatized patients who survived the initial posttraumatic resuscitation period suffer from substantial destabilization of their immune system. This phenomenon has been termed "systemic inflammatory response syndrome" (SIRS), which may lead to multiple organ dysfunction (and possibly failure) and thereby clearly impacts morbidity and mortality after multiple injury. Although these pathological immune reactions affect both components, namely the adaptive and innate immune system, the latter seems to play a superior role in the development of subsequent posttraumatic SIRS and posttraumatic multiple organ failure syndrome.

The admission into hospital and subsequent surgical management of polytraumatized patients involves a complex and prioritized approach. The central question in such management is which invasive method should be used in order to address each specific problem of the patient. On the one hand, a swift and thorough treatment of the individual injury needs to be secured, on the other hand, a significant deterioration of the general situation of the patient through further trauma, this time caused by surgical procedures, needs to be avoided. The determination of the therapeutic strategy and of the chances of success is based on a prediction of the extent of inflammation in the body. Clinical parameters could, so far, only be in-

sufficiently used to make such predictions, since the initial intercellular mechanisms in human immune cells of polytraumatized patients are only poorly understood.

In the past, various clinical parameters have been used for making predictions of the clinical outcome of a patient. One example is interleukin-6 which has clinically been used for such purpose. However, all of these parameters, including interleukin-6 are not specific for polytraumatized patients and are subject to many other influences.

In previous genome-wide mRNA expression studies, the present inventors identified 763 genes which appear to be differentially expressed in polytraumatic patients. (Biberthaler et al., Shock, Vol. 24, No. 1, pp. 11-19, 2005; Bogner et al., Langenbecks Arch. Surg., 2007, 392: pp. 255-265). However, the teachings of these preliminary results remain very unspecific and do not point into a particular direction with respect to individual markers or specific combinations of markers that might be suitable for predicting the clinical outcome. Moreover, no teachings or suggestions are made with respect to markers that would be particularly suitable for predicting the outcome already at an early stage, e.g. a few hours, after onset of trauma. Therefore, there remains a need for a method that would allow to predict the clinical outcome of polytraumatized patients at an early stage.

Accordingly, it was an object of the present invention to provide for markers that allow to predict the clinical outcome of polytraumatized patients at a very early stage after onset of the polytrauma. It was also an object of the present invention to provide for markers which can be swiftly and easily determined in polytraumatized patients.

All these objects are solved by the use of a combination of markers, said markers being selected from the group comprising BCL2A, MMP-9 and ETS-2, for predicting the mortality risk in a patient suffering from polytrauma.

In one embodiment said combination is one of the following combinations:

- a) BCL2A and MMP-9
- b) BCL2A and ETS-2
- c) MMP-9 and ETS-2
- d) BCL2A, MMP-9 and ETS-2.

In one embodiment

- BCL2A is encoded by or is a nucleic acid, said nucleic acid being represented by SEQ ID NO: 1,
- MMP-9 is encoded by or is a nucleic acid, said nucleic acid being represented by SEQ ID NO: 2, and
- ETS-2 is encoded by or is a nucleic acid, said nucleic acid being represented by SEQ ID NO: 3.

In one embodiment said use comprises the quantitative determination of expression levels of said combination of markers in a body fluid of said patient.

In one embodiment said body fluid is blood, urine, lymphatic fluid, saliva or spinal fluid.

In one embodiment said use comprises the quantitative determination of expression levels of said combination of markers in leukocytes, preferably granulocytes, more preferably neutrophil granulocytes of said patient.

In one embodiment elevated expression levels of said combination of markers are indicative of an increased mortality risk.

In one embodiment said quantitative determination of expression levels is performed on samples of body fluid of said patient, said samples having been withdrawn from said patient at 60 – 120 minutes after onset of said polytrauma, and/or thereafter at 2 – 3 h and/or 3 – 4 h and/or 4 – 5 h and/or 5- 6 h and/or 6 – 7 h and/or 7 – 8 h and/or 8 – 9 h and/or 9 – 10 h and/or 10 – 11 h and/or 11 – 12 h after onset of said polytrauma. Additionally, said quantitative determination of expression levels may also be performed on samples of body fluid of said patient, said samples having been withdrawn at the onset of said polytrauma, or shortly thereafter. This may also sometimes herein be referred to as “0h”.

Additionally, in one embodiment, samples are withdrawn from said patient at 5-7 h and/or 11-13 h and/or 23-25 h and/or 47-49 h and/or 71-73 h after onset of said polytrauma. In a preferred embodiment, samples are withdrawn from said patient at 6 h and/or 12 h and/or 24 h and/or 48 h and/or 72 h after onset of said polytrauma.

In one embodiment said determination of expression levels of said combination of markers occurs by detecting mRNA transcripts and/or proteins of said markers.

The objects of the present invention are also solved by a kit for predicting the mortality risk in a patient suffering from polytrauma, said kit comprising:
means to perform a quantitative determination of expression levels of a combination of markers, said combination of markers being as defined above, said quantitative determination of expression levels being as defined above.

In one embodiment said means are a set of primers for each marker to be determined, respectively, or a composition of antibodies, each antibody being specific for a marker to be determined.

The primers are used for performing nucleic acid amplification reactions, such as PCR, and can be designed without difficulties by someone skilled in the art, based on the sequence(s) of the respective marker. More specifically, the design of the respective primers may, as an example, be performed with respect to the following amplicon regions (nucleotide positions with reference to sequence in sequence listing of this application):

BCL2-A	nucleotide positions	509-746,
MMP-9	nucleotide positions	1984-2159,
ETS-2	nucleotide positions	1157-1454,
actin beta	nucleotide positions	215-543,
cyclophilin B	nucleotide positions	286-538.

Other amplicon regions are also possible and can be determined by someone skilled in the art, based on the full-length sequence of the respective marker, as shown in the enclosed sequence listing. Such primers may also be obtained commercially and have, e.g., in the present case, been obtained from SEARCH-LC GmbH, Heidelberg. These commercially obtained primers were based on the afore-mentioned amplicon regions.

In one embodiment said kit further comprises reagents to perform nucleic acid amplification, and means to detect and quantitate amplified reaction products.

In one embodiment said kit further comprises reagents to perform an ELISA-assay, to detect and quantitatively determine expression levels of said combination of markers.

The objects of the present invention are also solved by a nucleic acid composition consisting of

- a) two or three different nucleic acids, said nucleic acids coding for BCL2A, MMP-9, and ETS-2, respectively, and/or
- b) the complementary counterparts thereof, and/or
- c) partial sequences of a) and/or b), said partial sequences consisting of 15 contiguous nucleotides or more.

In one embodiment said nucleic acids coding for BCL2A, MMP-9, and ETS-2, respectively, are represented by SEQ ID NO: 1-3, respectively.

The afore-mentioned nucleic acid composition may be used in different applications. For example, if the nucleic acid composition consists of partial sequences of the nucleic acids coding for BCL2A, MMP-9 and ETS-2 (a), and partial sequences of the complementary counterparts of said coding nucleic acids (b), these partial sequences may be acting as primers in a nucleic acid amplification reaction. In another embodiment, the nucleic acid composition may be simply used as probes, either in solution or attached to some matrix. In this instance, such probes may comprise the coding sequences in accordance with a) and/or complementary counterparts thereof, and/or partial sequences of a) and/or b), which partial sequences may act as specific probes. In order for these to act as specific probes or primers, they must be of a sufficient length in order to achieve specific hybridization. In many instances, this can be achieved by sequence stretches of 12 nucleotides or more, such as 13, or 14, or 15, or 16, or 17, or 18, or 19, or 20, or 21, or 22, or 23, or 24, or 25, or 26 nucleotides.

The objects of the present invention are also solved by a solid matrix having a surface on which a nucleic acid composition as defined above has been immobilized.

In one embodiment said matrix is a substrate having a planar surface on which said nucleic acid composition has been immobilized.

It should be noted that in the afore-mentioned matrix-embodiments, i.e. those embodiments, wherein the nucleic acid composition as defined above has been immobilized on the surface of a matrix, there may be additional nucleic acids immobilized on said matrix, with the proviso that said matrix is not a genome-wide array of nucleic acid probes. In one embodiment of said matrix, said additional nucleic acids which may be immobilized on the surface of said matrix are not a complete collection of nucleic acid probes representing the entire genome of an organism, such as a human. In one embodiment of said matrix, said nucleic acids which may be immobilized on a surface of said matrix are selected from a group of genes consisting of housekeeping genes and partial sequences thereof. Such housekeeping genes are constitutively expressed genes, and are exemplified by beta actin and cyclophilin B. Other examples can be found in Trends in Genetics 19, 362-365 (2003). Whenever in such matrix embodiments housekeeping genes or partial sequences thereof (or the respective complementary counterparts thereof) are immobilized on a surface of the matrix in addition to the afore-mentioned nucleic acid composition, the nucleic acid matrix still does not have probes for the entire genome of an organism immobilized on its surface.

In one embodiment said matrix is a set of beads suitable for use in a high-throughput assay, said beads having surfaces on which said nucleic acid composition as defined above has been immobilized.

Similar remarks apply to this matrix-embodiment of a set of beads, with respect to the possibility of having additionally housekeeping genes or probes therefore immobilized on the surfaces of said beads. Again, altogether, the probes immobilized on the surfaces of the beads do not represent probes for the entire genome of an organism.

The objects of the present invention are also solved by the use of the kit according to the present invention, or of the composition according to the present invention, or of the matrix according to the present invention, for predicting the mortality risk in a patient suffering from polytrauma, wherein said use comprises the quantitative determination of a combination of markers, as defined above, or of said nucleic acid composition as defined above, wherein elevated expression levels of said combination of markers or of said nucleic acid composition are indicative of an increased mortality risk.

The objects of the present invention are also solved by a method of predicting the mortality risk in a patient suffering from polytrauma, said method comprising:
quantitatively determining the expression levels of at least two markers selected from the group comprising BCL2A, MMP-9, ETS-2 in a body fluid of said patient,
wherein elevated expression levels of said at least two markers are indicative of an increased mortality risk.

The term “polytrauma”, as used herein, is meant to refer to a state of a patient who has suffered from multiple injuries, with at least one injury or a combination of injuries being life threatening.

The terms “BCL2A”, “MMP-9” and “ETS-2”, as used herein, refer to the respective genes on the nucleic acid level (DNA or RNA) and on the protein level. Therefore, if e.g. BCL2A is used for predicting the mortality risk in a patient, this means that such use may occur on the nucleic acid level, for example by determining the level of mRNA transcripts, or it may occur on the protein level, by determining the level of BCL2A-protein. The same applies to MMP-9 and ETS-2.

In one embodiment, said elevated expression levels indicative of an increased mortality risk are BCL2A-expression levels which are 1.3-fold to 3-fold of the BCL2A-expression levels of a polytraumatized patient who survives said polytrauma; and/or said elevated expression levels are MMP-9-expression levels which are 1.3-fold to 4-fold of the MMP-9-expression levels of a polytraumatized patient who survives said polytrauma; and/or said elevated expression levels are ETS-2-expression levels which are 1.1-fold to 3-fold of the ETS-2-expression levels of a polytraumatized patient who survives said polytrauma. In one embodiment, said elevated levels are determined by PCR.

In another embodiment, said elevated expression levels indicative of an increased mortality risk are BCL2-A expression levels which, at 5-7 h after onset of said polytrauma in said patient, are at least 2.5-fold the BCL2-A expression levels at the onset of said polytrauma in said patient; and/or said elevated expression levels indicative of an increased mortality risk are MMP-9 expression levels which, at 5-7 h after onset of said polytrauma in said patient, are at least 2.5-fold the MMP-9 expression levels at the onset of said polytrauma in said patient; and/or said elevated expression levels indicative of an increased mortality risk are ETS-2 ex-

pression levels which, at 5-7 h after onset of said polytrauma in said patient, are at least 5-fold the ETS-2 expression levels at the onset of said polytrauma in said patient.

Preferably said expression levels are determined by PCR. "at the onset of said polytrauma", as used herein, is meant to refer to the time point "0 h".

A "quantitative determination of expression levels", as used herein, is performed by measuring the levels of transcripts of the respective markers and/or by measuring the levels of expressed protein of the respective marker. Such quantitative determination is performed on samples which have been withdrawn from the patients already at an early stage. Prior to such quantitative determination, the respective sample may have to be processed such as further purified, concentrated, etc. In a preferred embodiment, such quantitative determination is performed on a body fluid of said patient, preferably on blood. In a more specific embodiment, the quantitative determination is performed on individual cell populations within said body-fluid, such as leukocytes, preferably granulocytes, preferably neutrophils.

The present inventors have surprisingly found that BCL2A and MMP-9 and ETS-2 show a highly significant correlation of their expression with the later clinical outcome of the respective patient. In other words, if these markers are expressed at an elevated level within a few hours after onset of trauma (typically 6 hours), this elevated expression correlates with a higher mortality of the respective patient than in a polytraumatized reference patient whose expression levels are not elevated at the same time. It is of vital importance that, for these markers, the elevated expression can already be determined very early, e.g. 6 hours after onset of trauma. Accordingly, these markers or their combination are particularly suitable for making a very early prognosis, namely at a point in time which is of extreme importance for planning the further therapy of the patient. The expression data are statistically correlated with the mortality of the patient using Receiver Operating Curves (ROC). For the entire data set, sensitivity and specificity of the individual marker are calculated with respect to its diagnostic potential for the clinical event. The 1-specificity value is plotted on the x-axis and the sensitivity value on the y-axis, and the individual data points are connected, thus forming a curve. The integral of this curve corresponds to the area under the curve (AUC), which is considered to be a significant discriminating tool as of values of 0.7 (or greater). For BCL2A, the ROC-analysis yields an error probability of $p < 0.0001$, already at a time point of 6 h after onset of the polytrauma (AUC: 78 %, 95 % confidence interval: 0.63 – 0.93). With respect to MMP-9 at the time point of 6 h after onset of polytrauma, the error probability is at $p < 0.001$ (AUC: 75

%, 95 %-confidence interval: 0.58 – 0.93). With respect to ETS-2 at the time point of 6 h after onset of polytrauma, the error probability is $p < 0.016$ (AUC: 71 %, 95 %-confidence interval: 0.52 – 0.90) BCL2A, MMP-9 and ETS-2 have, so far, not been used as parameters for prognosis of clinical outcome in polytraumatized patients.

In accordance with the present invention, for the markers BCL2A, MMP-9 and ETS-2, the expression levels of these markers are quantitatively determined. Such determination is typically performed on a sample of body fluid of the patient, which has been withdrawn early after onset of polytrauma, typically not more than 90 minutes and in repeated intervals thereafter, e.g. 2h, 3h, 4h, 5h, 6h, 7h, 8h, ... and so forth ..., 72 h, ...96 h and so forth. Additionally, a determination may also be performed, where possible, on a sample of body fluid of the patient which has been withdrawn as early as possible after onset of polytrauma in said patient, e.g. a few minutes after onset, such as 5 minutes or 10 minutes. This is also meant to be included by the term “a sample withdrawn at 0 h”.

A person skilled in the art knows how to quantitatively determine expression levels. For example, this may be done by performing quantitative real-time PCR using primer sets which are specific for the individual markers. Alternatively, ELISAs or western blots may be performed for such quantitative determinations.

The samples on which this quantitative determination is performed are typically body fluids from a patient, more typically blood. In one embodiment, such blood is subjected to further purification steps before a quantitative determination is performed. Such purification steps typically are directed at getting rid of impurities or cellular components which are of no relevance for the quantitative determination. They may also be directed at the isolation of specific cell populations which are particularly suitable for a quantitative determination of the respective markers, such as neutrophil granulocytes. Whilst in the specific description, hereafter, an example is given wherein neutrophils are used for such quantitative determination, the present invention is, by no means, limited to quantitative determination in such neutrophils. For example, a quantitative determination may also be performed in blood serum, whole blood, leukocytes, granulocytes, monocytes and liquor.

Typically, the quantitative determination of the expression levels of the respective markers includes a detection step. A person skilled in the art knows how to perform such detection

step, and preferred means involve the use of fluorescent dyes which may be used to detect amplified expression products, if for example real-time PCR is used, or which may be used to detect antibody-marker-complexes, if ELISAs or western blots are used. A typical example of such a fluorescent dye is SYBR green, which can be used in real-time PCR protocols.

Another possibility for quantitatively determining expression levels is the use of an array in which probes specific for the respective markers are immobilized on a matrix, such as a substrate having a surface, or beads on the surface of which the respective probes are immobilized. It should be noted that, in accordance with the present invention, an array of such marker-specific probes does not comprise probes for the entire genome. Consequently, a typical array in accordance with the present invention comprises a nucleic acid composition consisting of two or three different nucleic acids only, which are specific for the individual markers. The nucleic acid composition does not comprise further different nucleic acids, with the exception of one to ten reference nucleic acids as a control. Such controls may e.g. be house-keeping genes. The term “specific for an individual marker”, when used herein in connection with a nucleic acid or a nucleic acid probe, is meant to refer to a nucleic acid which has a nucleic acid sequence that is specific for such marker, or which has a nucleic acid sequence that hybridizes under stringent conditions to the nucleic acid sequence of said marker. The term “two or three different nucleic acids” is meant to refer to two or three nucleic acids having sequences which differ from each other.

The markers and combination of markers in accordance with the present invention have the unprecedented advantage of being highly and significantly predictive for the mortality and clinical outcome of a polytraumatized patient, especially at an early time after onset of said trauma, for example already at 6 h after onset of said trauma. This is unprecedented and was nowhere suggested or described in the prior art before. It opens up a new perspective for treatment and treatment management of polytraumatized persons at a very early stage of their multiple injuries and thus greatly improves prospects of treatment for such patients.

Moreover, reference is made to the enclosed sequence listing, wherein
SEQ ID NO:1 represents the sequence for BLC2A,
SEQ ID NO:2 represents the sequence for MMP-9,
SEQ ID NO:3 represents the sequence for ETS-2.

SEQ ID NO: 4 represents the sequence for actin beta, and
SEQ ID NO: 5 represents the sequence for cyclophilin B.

Moreover, reference is made to the following figures, wherein

Figure 1: *shows outcome related differential gene expression*

These plots visualize the differential gene expression of (a) ETS-2, BCL2A, and MMP-9, and b) JUN and FOS, depending on the patients' clinical outcome. JUN and FOS exemplify markers not showing significant expression differences. The white bar charts depict the mean ($\pm$ SEM) gene transcripts in patients with favourable outcome (n=28) as compared to expression levels of the respective gene in patients who deceased (n=12). Significant differences are marked by the respecting p-values calculated by Mann-Whitney-Rank Sum Tests.

Figure 2: *shows outcome classifying ROCs:*

This graph shows the potential of outcome classification using a) BCL2A, b) ETS-2, c) MMP-9, d) JUN and e) FOS transcript measurement in the posttraumatic period (0-72h) assessed by receiver operating curves (ROC).

Figure 3: *shows a table outlining the mean copy numbers as determined by PCR of BCL2A, MMP-9, ETS-2, JUN and FOS:*

The tables shows the mean copy numbers of BCL2-A, MMP-9 and ETS-2 in samples of blood withdrawn at 0 h, 6 h, 12 h, 24 h, 48 h and 72 h after onset of polytrauma; the values are classified according to clinical outcome (deceased = patients who died; survived = patients who survived). Copy numbers were normalized using the ratios of housekeeping genes actin and cyclophilin B (CPB). Also shown is the ratio of mean copy numbers in deceased patients to mean copy numbers in surviving patients at the individual time points. Also shown is the ratio of the mean at time point x to the mean at time point 0h.

Moreover, reference is made to the following examples which are given to illustrate, not to limit the present invention:

Examples

Example 1

Patients and Methods:

This investigation was conducted in our level 1 trauma center strictly according to the Good Clinical Practice Guidelines. Ethical innocuousness was certified by the local institutional review board (reference number: 012/00). Patients of full age suffering from blunt multiple injuries [Injury Severity Score (ISS) of >16 points (9)] who were admitted to our emergency department within 90 minutes after trauma were included into the study. Consent form of study participation was obtained from the patients or a legal representative. Patients who departed within the first 24h after trauma were excluded from the study. Patients' treatment was performed according to standard of care and was not affected by study participation. The patient collective was retrospectively classified into opposed clinical groups according to their injury severity (ISS), injury pattern concerning the presence of traumatic brain injury (positive initial CCT scan), presence of massive red blood cell substitution unit and finally according to their ultimate clinical outcome (90-day survival). Application of more than 10 RBC units/24h has been defined as the presence of massive RBC transfusion (10).

Blood sampling and neutrophil isolation

Blood samples were drawn on admission of the patient – but purposefully not later than 90 minutes after trauma – and below at 6h, 12h, 24h, 48h and 72h. Sampling time points are strictly standardized to the traumatic event. After three rounds of red blood cell lysis, the samples were centrifuged at 450 x g. The resulting cell pellets were resuspended in PBS/EDTA buffer. 200µl anti-CD15 antibody coated micromagnetic beads (Miltenyi Biotech, Auburn, CA, USA) were added to the 1×10^8 cells diluted in 800 µl PBS/EDTA buffer for 15 minutes at 4° C. After pelleting and dilution, the cells were added to a pre-equalized high gradient magnet separation column using the magnetic cell separator Mini-MACS™ system (Miltenyi Biotech). The cell suspension was lysed in 2 ml of RLT buffer (Qiagen, Hilden, Germany) containing 0.1% v/v β-mercaptoethanol and stored at –80° C. Total RNA preparation from lysed neutrophils was done by the RNeasy Midi Kit™ according to the supplier's recommendations (Qiagen). DNA was removed by on-column DNA-se digestion using the MiniElute™ Clean Up Kit (Qiagen).

cDNA Synthesis and Quantitative Real-Time PCR

mRNA preparation was performed using the RNeasy Midi Kit (Qiagen, Hilden). cDNA was prepared with a first-strand cDNA synthesis kit; and QRT-PCR was performed with the Light Cycler Fast Start DNA SYBR Green kit, all according to the manufacturer's instructions (Roche, Mannheim). All primers, including such of the housekeeping genes were derived from Search-LC (Heidelberg, Germany). Primers were based on the following amplicon regions of the respective full-length sequence, as shown in the sequence listing:

BCL2-A: nucleotide positions 509-746

MMP-9: nucleotide positions 1984-2159

ETS-2: nucleotide positions 1157-1454.

actin beta: nucleotide positions 215-543

cyclophilin B: nucleotide positions 286-538.

Detected transcript amounts were normalized using the ratios of housekeeping genes actin and cyclophilin B (CPB).

Statistical Analyses

Analyses of expression comparison between the clinical groups have been performed by Mann-Whitney-Rank Sum Test for non-normal variables and t-tests for normal variables (SigmaStat®, SPSS. Inc.). Visualization of the data was performed by Sigma Plot 8.0 (SPSS. Inc.). Data are given as mean $\pm$ SEM. The outcome and massive transfusion classifying values of the different genes were assessed by Receiver Operating Curves (ROC; Analyse IT, Windows). ROC curve results are given as p-values, area under the curve (AUC) and 95% confidence interval.

Example 2**Results:****Patients and Methods:**

40 patients (28 males and 12 females) fulfilled the entry criteria and were included into the study. Patients' age ranged from 18 to 71 years. Injury Severity Score in the entire study collective counted 36 ± 14 points, mean New Injury Severity Score (NISS) 41 ± 13 points (mean $\pm$ SD). The injury pattern of 15 patients included severe traumatic brain injury (intracra-

nial lesion in the initial CCT scan). 28 of the collective survived the trauma, whereas 12 had an unfavourable outcome. Thereof, 6 patients deceased because of severe traumatic brain injury and 6 patients died of multiple organ failure in the further posttraumatic clinical course. Mean ISS ($\pm$ SD) in the patients who survived the traumatic event was 36 ± 13 points (NISS 39 ± 12), in those who deceased 37 ± 17 points (NISS 46 ± 14). During the observation period, all patients developed clinical signs of multiple organ failure (MOF score ≥ 4 points). 21 patients required massive RBC substitution and were resuscitated with $25,6\pm 10$ RBC units in contrast to the 19 patients who received less, namely $5,0\pm 3$ RBC units (mean $\pm$ SD). 11 patients in the massive transfusion group deceased.

Differential Gene Expression depending on clinical outcome

Patients who did not survive the traumatic event (90-day survival) exhibit significant higher BCL2A levels at all blood sampling time points. Significant differences can be observed already at 6h after trauma in comparison to patients with a favourable outcome $p<0.005$, ROC curve (data given as p, AUC and 95% confidence interval): $p<0.0001$; 0,78; 0,63-0,93. Also at 72h BCL2A expression changes are significantly higher in deceased patients $p<0.045$.

Neutrophil MMP-9 expression exhibits significant differences in patients who decease as compared to those who survive at 6h: $p<0.011$, ROC curve $p<0.001$; 0,75; 0,58-0,93 and at 24h post trauma: $p<0.025$, ROC curve $p<0.011$; 0,72; 0,53-0,92.

ETS-2 expression also shows higher expression at 6h after trauma in patients with unfavourable outcome $p<0.03$, ROC: $p<0.01$, 0,71; 0,51-0,90. The outcome related plots are visualized in **figure 1A**, the respecting ROC curves in **figure 2**.

Figure 1B shows two examples of markers (JUN and FOS) which do not show significant differences in expression depending on clinical outcome, thus illustrating the suitability and superiority of BCL2A, MMP-9 and ETS-2 for predicting clinical outcome, in comparison to markers like JUN and FOS.

Altogether, the present invention has identified new combinations of markers consisting of at least two from BCL2 A, MMP-9, and ETS-2, which are differentially upregulated and over-expressed very early after the onset of polytrauma in such patients with a fatal clinical outcome (non-survival within 90 days after polytrauma). This means that use of these markers and combinations thereof enables new approaches for treatment of such patients who are at a

higher mortality risk, in choosing less invasive treatments, where possible, to avoid additional risks posed by surgical measures.

Treatment of such patients has so far mainly relied upon the experience of the treating physician. Decisions on specific courses of treatments can now be taken based on the present invention at a very early stage after the patients have been admitted.

Literature:

- (1) Mannick JA, Rodrick ML, Lederer JA. The immunologic response to injury. *J Am Coll Surg* 2001; 193(3):237-244.
- (2) Baue AE, Durham R, Faist E. Systemic inflammatory response syndrome (SIRS), multiple organ dysfunction syndrome (MODS), multiple organ failure (MOF): are we winning the battle? *Shock* 1998; 10(2):79-89.
- (3) Angele MK, Faist E. Clinical review: immunodepression in the surgical patient and increased susceptibility to infection. *Crit Care* 2002; 6(4):298-305.
- (4) Cioffi WG, Burleson DG, Pruitt BAJ. Leukocyte responses to injury. *Arch Surg* 1993; 128(11):1260-1267.
- (5) Oberholzer A, Oberholzer C, Moldawer LL. Sepsis syndromes: understanding the role of innate and acquired immunity. *Shock* 2001; 16(2):83-96.
- (6) Biberthaler P, Bogner V, Baker HV, Lopez MC, Neth P, Kanz KG et al. Genome-wide monocytic mRNA expression in polytrauma patients for identification of clinical outcome. *Shock* 2005; 24(1):11-19.
- (7) Matsuda N, Hattori Y. - Systemic inflammatory response syndrome (SIRS): molecular pathophysiology and gene therapy.(3):-98.
- (8) Xing L, Remick DG. - Relative cytokine and cytokine inhibitor production by mononuclear cells and neutrophils.(1):-6.
- (9) Committee on Injury Scaling (1990). Abbreviated Injury Scale 1990 Revision. Des Plaines, Illinois: Association of the Advancement of Automobile Medicine, 2002.
- (10) Como JJ, Dutton RP, Scalea TM, Edelman BB, Hess JR. Blood transfusion rates in the care of acute trauma. *Transfusion* 2004; 44(6):809-813.
- (11) Foletta VC, Segal DH, Cohen DR. - Transcriptional regulation in the immune system: all roads lead to AP-1.(2):-52.
- (12) Yao YM, Redl H, Bahrami S, Schlag G. - The inflammatory basis of trauma/shock-associated multiple organ failure.(5):-10.

- (13) Pillay J, Hietbrink F, Koenderman L, Leenen LP. - The systemic inflammatory response induced by trauma is reflected by multiple phenotypes of blood neutrophils.(12):-72.
- (14) Kreth S, Ledderose C, Kaufmann I, Groeger G, Thiel M. - Differential expression of 5'-UTR splice variants of the adenosine A2A receptor gene in human granulocytes: identification, characterization, and functional impact on activation.(9):-86.
- (15) Dunne JR, Malone DL, Tracy JK, Napolitano LM. Allogenic blood transfusion in the first 24 hours after trauma is associated with increased systemic inflammatory response syndrome (SIRS) and death. Surg Infect (Larchmt) 2004; 5(4):395-404.
- (16) Malone DL, Dunne J, Tracy JK, Putnam AT, Scalea TM, Napolitano LM. Blood transfusion, independent of shock severity, is associated with worse outcome in trauma. J Trauma 2003; 54(5):898-905.
- (17) Villunger A, Scott C, Bouillet P, Strasser A. - Essential role for the BH3-only protein Bim but redundant roles for Bax, Bcl-2, and Bcl-w in the control of granulocyte survival.(6):-400.
- (18) Villunger A, O'Reilly LA, Holler N, Adams J, Strasser A. - Fas ligand, Bcl-2, granulocyte colony-stimulating factor, and p38 mitogen-activated protein kinase: Regulators of distinct cell death and survival pathways in granulocytes.(5):-58.
- (19) Iwai K, Miyawaki T, Takizawa T, Konno A, Ohta K, Yachie A et al. - Differential expression of bcl-2 and susceptibility to anti-Fas-mediated cell death in peripheral blood lymphocytes, monocytes, and neutrophils.(4):-8.
- (20) Maier M, Wutzler S, Bauer M, Trendafilov P, Henrich D, Marzi I. - ALTERED GENE EXPRESSION PATTERNS IN DENDRITIC CELLS AFTER SEVERE TRAUMA: IMPLICATIONS FOR SYSTEMIC INFLAMMATION AND ORGAN INJURY.:Shock.
- (21) Chow AK, Cena J, Schulz R. - Acute actions and novel targets of matrix metalloproteinases in the heart and vasculature.(2):-205.
- (22) Oettgen P. - Regulation of vascular inflammation and remodeling by ETS factors.(11):-66.
- (23) Wei G, Guo J, Doseff AI, Kusewitt DF, Man AK, Oshima RG et al. - Activated Ets2 is required for persistent inflammatory responses in the motheaten viable model.(2):-9.
- (24) Hildebrand F, Pape HC, Krettek C. [The importance of cytokines in the posttraumatic inflammatory reaction]. Unfallchirurg 2005; 108(10):793-803.
- (25) Liener UC, Bruckner UB, Knoferl MW, Steinbach G, Kinzl L, Gebhard F. - Chemokine activation within 24 hours after blunt accident trauma.(3):-72.
- (26) Ni CN, Redmond HP. - Cell response to surgery.(11):-40.
- (27) Gardner J, Ghorpade A. - Tissue inhibitor of metalloproteinase (TIMP)-1: the TIMPed balance of matrix metalloproteinases in the central nervous system.(6):-6.

- (28) von Gertten C, Holmin S, Mathiesen T, Nordqvist AC. - Increases in matrix metalloproteinase-9 and tissue inhibitor of matrix metalloproteinase-1 mRNA after cerebral contusion and depolarisation.(6):-10.
- (29) Wang X, Jung J, Asahi M, Chwang W, Russo L, Moskowitz MA et al. - Effects of matrix metalloproteinase-9 gene knock-out on morphological and motor outcomes after traumatic brain injury.(18):-42.

Claims

1. Use of a combination of markers, said markers being selected from the group comprising BCL2A, MMP-9 and ETS-2, for predicting the mortality risk in a patient suffering from polytrauma.
2. Use according to claim 1, wherein said combination is one of the following combinations:
 - a) BCL2A and MMP-9
 - b) BCL2A and ETS-2
 - c) MMP-9 and ETS-2
 - d) BCL2A, MMP-9 and ETS-2
3. Use according to any of claims 1-2, wherein
 - BCL2A is encoded by or is a nucleic acid, said nucleic acid being represented by SEQ ID NO: 1,
 - MMP-9 is encoded by or is a nucleic acid, said nucleic acid being represented by SEQ ID NO: 2, and
 - ETS-2 is encoded by or is a nucleic acid, said nucleic acid being represented by SEQ ID NO: 3.
4. Use according to any of claims 1-3, wherein said use comprises the quantitative determination of expression levels of said combination of markers in a body fluid of said patient.
5. Use according to claim 4, wherein said body fluid is blood, urine, lymphatic fluid, saliva or spinal fluid.

6. Use according to any of claims 4-5, wherein said use comprises the quantitative determination of expression levels of said combination of markers in leukocytes, preferably granulocytes, more preferably neutrophil granulocytes of said patient.
7. Use according to any of claims 4-6, wherein elevated expression levels of said combination of markers are indicative of an increased mortality risk.
8. Use according to any of claims 4-7, wherein said quantitative determination of expression levels is performed on samples of body fluid of said patient, said samples having been withdrawn from said patient at 60 – 120 minutes after onset of said polytrauma, and/or thereafter at 2h – 3h and/or 3h – 4h and/or 4h – 5h and/or 5h – 6h and/or 6h – 7h and/or 7h – 8h and/or 8h – 9h and/or 9h-10h and/or 10h – 11h and/or 11h – 12h after onset of said polytrauma..
9. Use according to any of claims 4-8, wherein said determination of expression levels of said combination of markers occurs by detecting mRNA transcripts and/or proteins of said markers.
10. A kit for predicting the mortality risk in a patient suffering from polytrauma, said kit comprising:
means to perform a quantitative determination of expression levels of a combination of markers, said combination of markers being as defined in any of claims 1-3, said quantitative determination of expression levels being as defined in any of claims 3-9.
11. Kit according to claim 10, wherein said means are a set of primers for each marker to be determined, respectively, or a composition of antibodies, each antibody being specific for a marker to be determined.
12. A nucleic acid composition consisting of
 - a) two or three different nucleic acids, said nucleic acids coding for BCL2A, MMP-9, and ETS-2, respectively, and/or
 - b) the complementary counterparts thereof, and/or
 - c) partial sequences of a) or b), said partial sequences consisting of 15 contiguous nucleotides or more, and/or

- d) partial sequences of a) and b), said partial sequences consisting of 15 contiguous nucleotides or more.
13. Nucleic acid composition according to claim 12, wherein said nucleic acids coding for BCL2A, MMP-9, and ETS-2, respectively, are represented by SEQ ID NO: 1-3, respectively.
 14. A solid matrix having a surface on which a nucleic acid composition as defined in any of claims 12-13 has been immobilized.

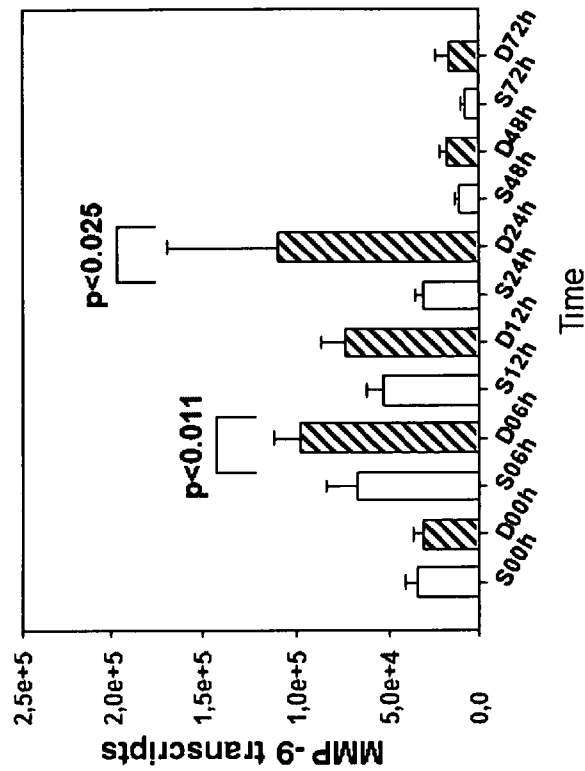
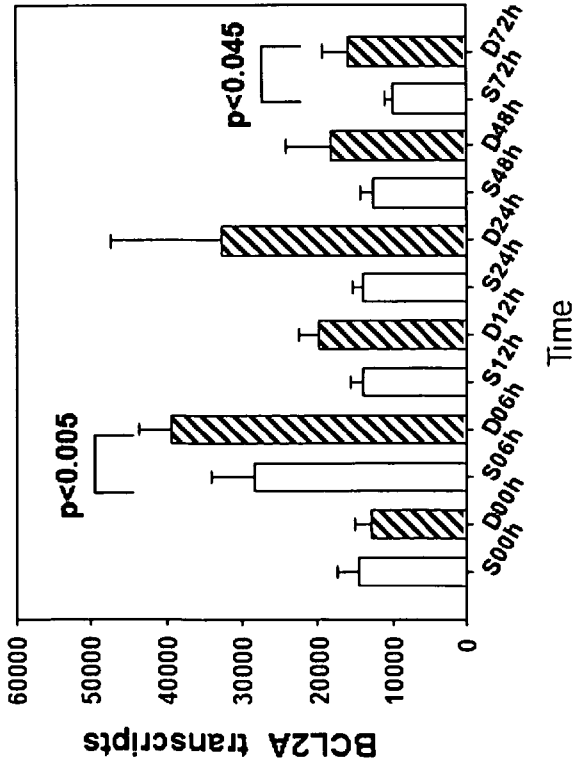
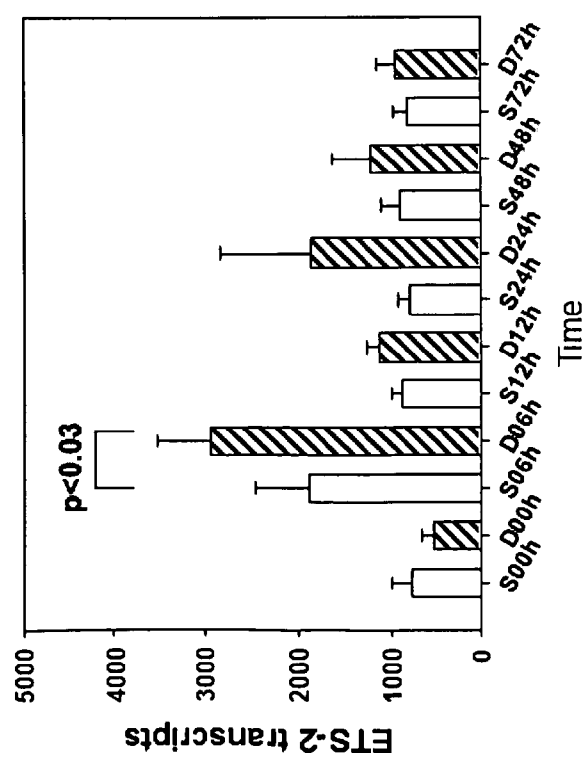


Figure 1A



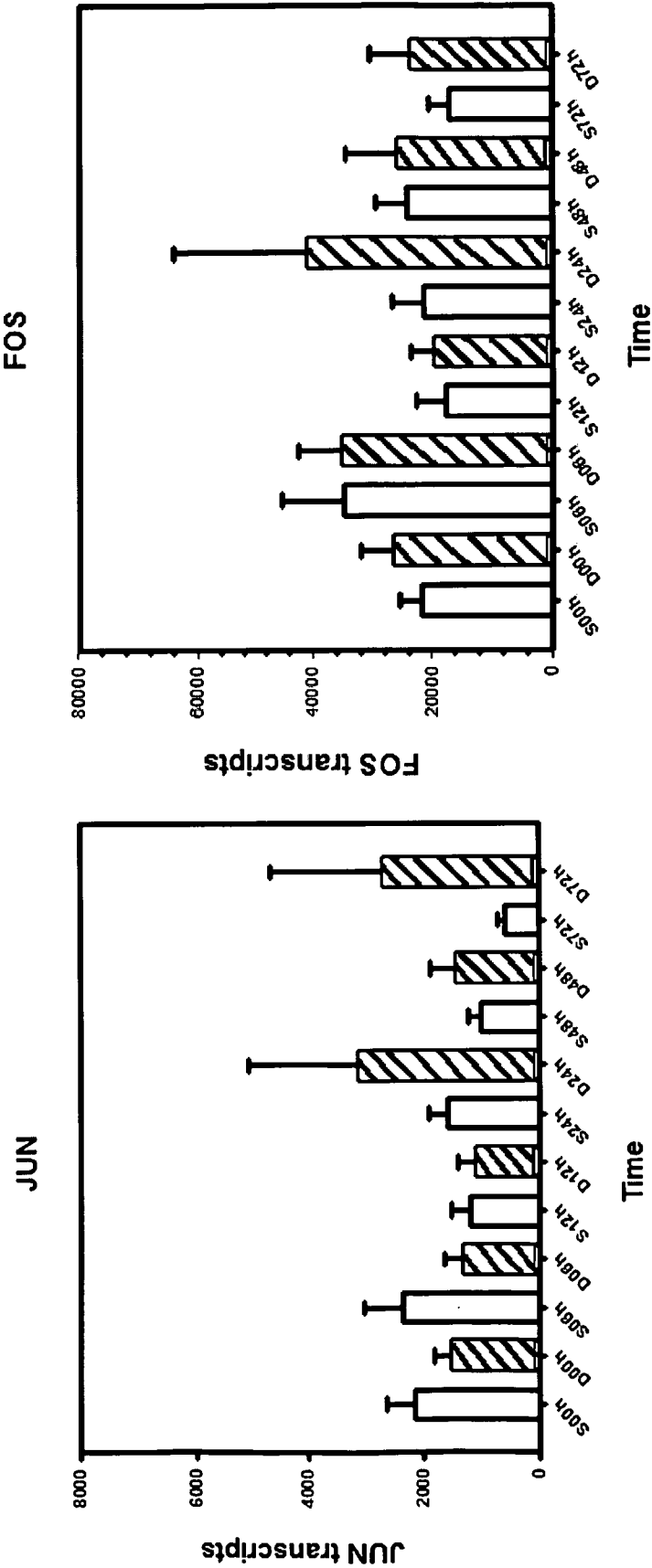


Figure 1B

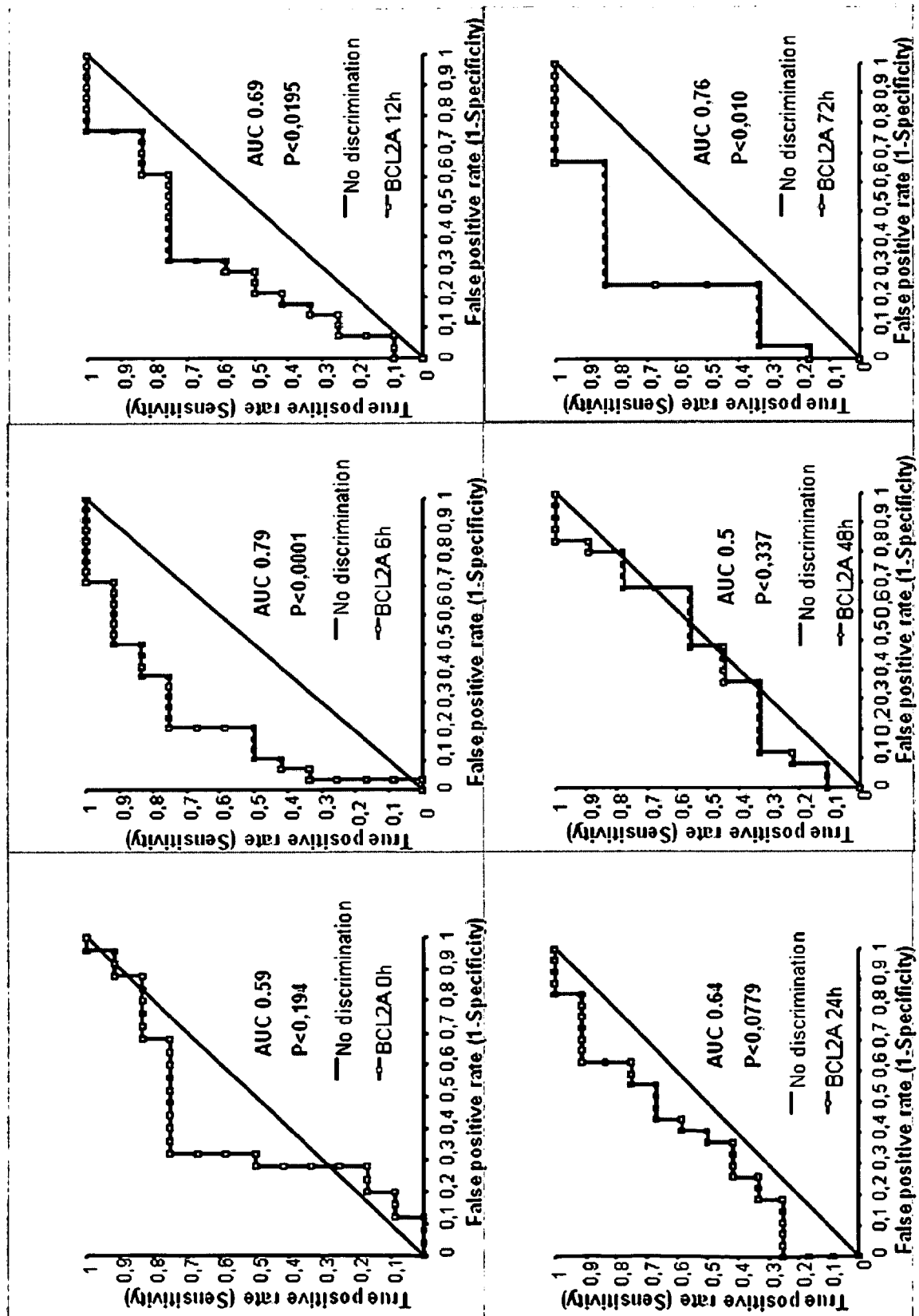


Figure 2A

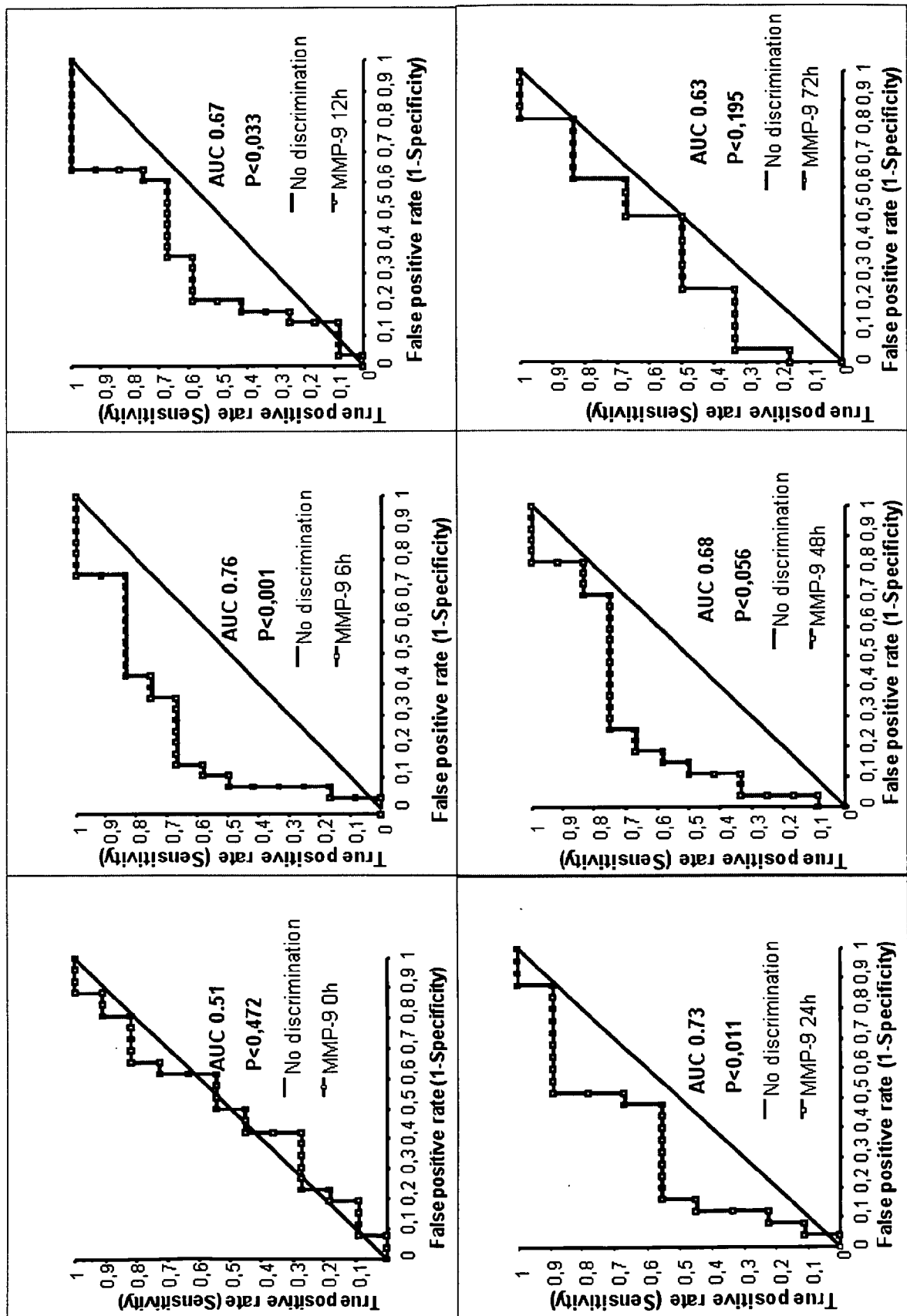


Figure 2B

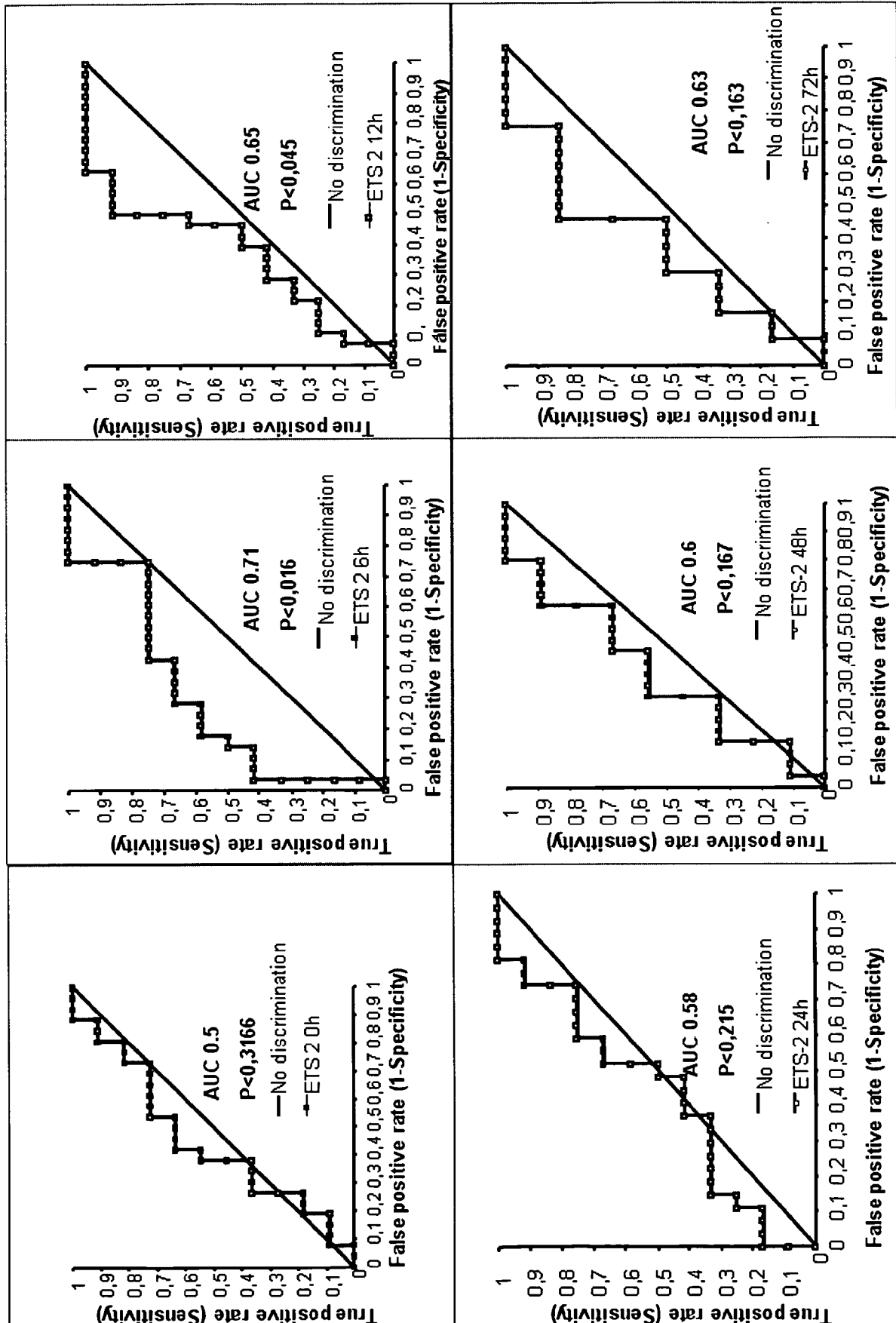


Figure 2C

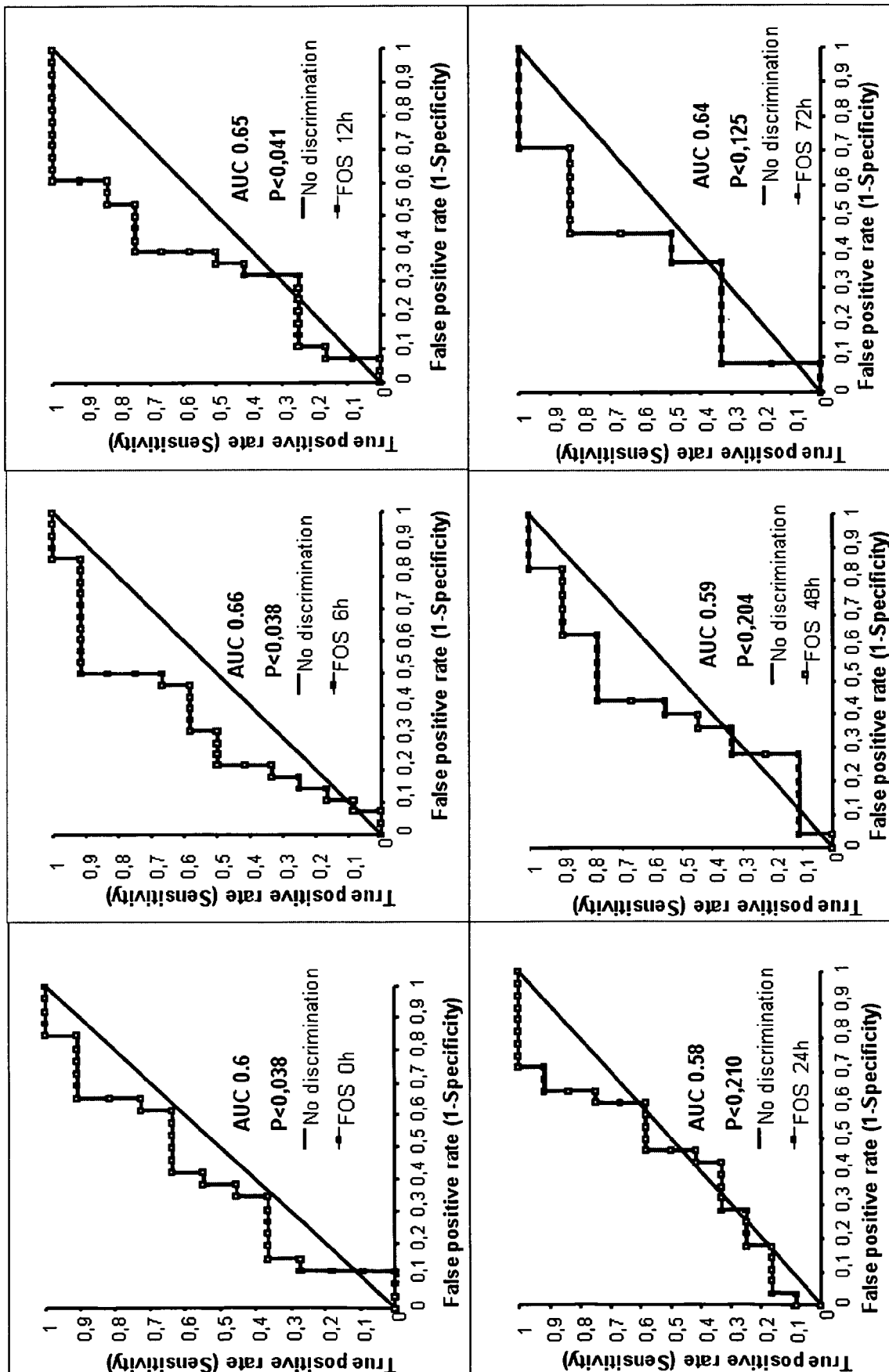


Figure 2D

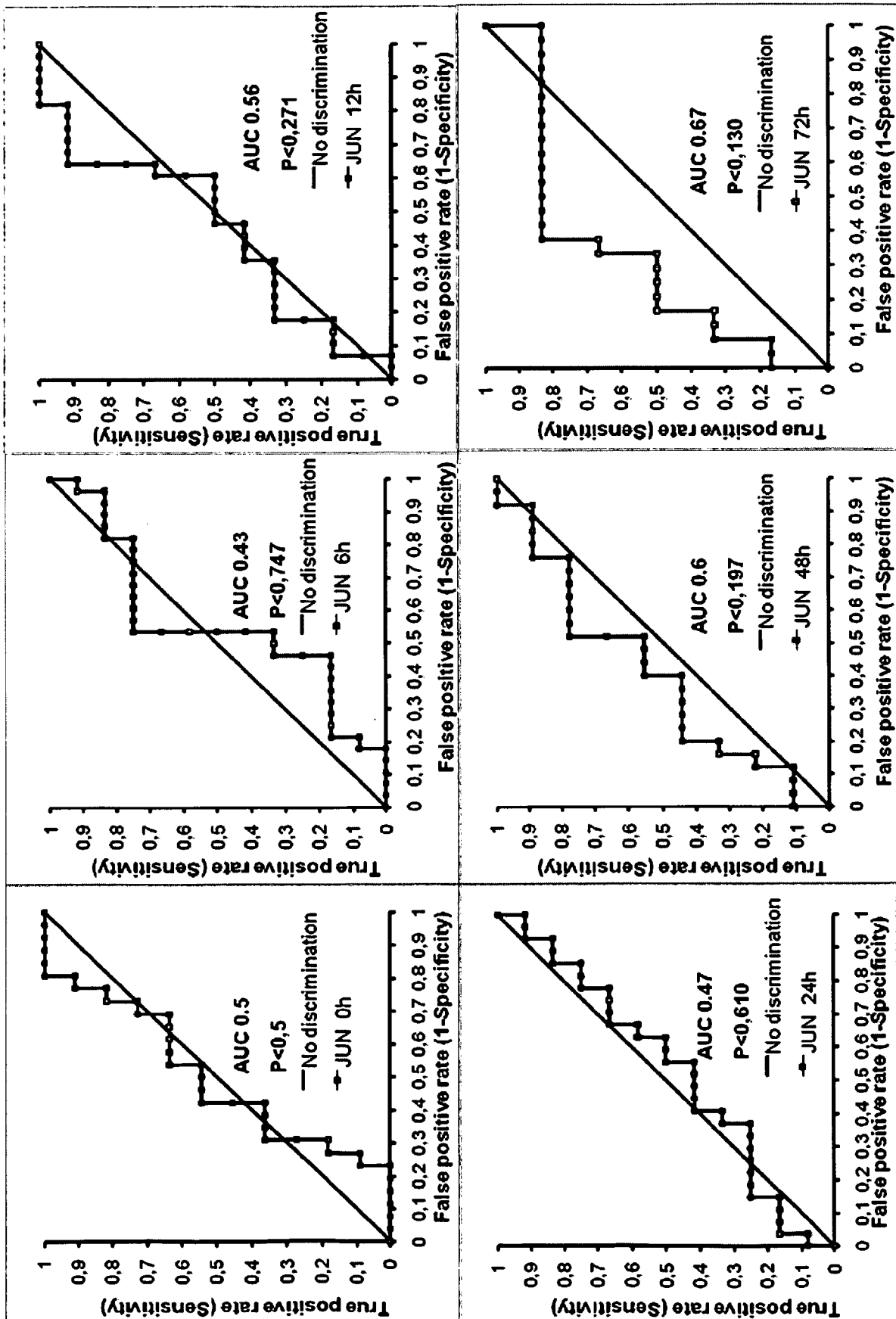


Figure 2E

Table: mean copy number as determined by PCR of BCL2A, MMP-9 and ETS-2 in samples withdrawn at 0h, 6h, 12h, 24h, 48h and 72h after onset of polytrauma

							survived		deceased	
	Time Point	Mean Survived	SEM	Mean deceased	SEM	ratio of mean deceased: mean survived	ratio mean at time point: mean at 0h			
BCL2A	0h	14487	2912	12915	2009	0.89	1	1	1	
	6h	28485	5398	39430	4075	1.38	1.97	1.97	3.05	
	12h	13921	1573	19727	2874	1.42	0.96	0.96	1.53	
	24h	13830	1447	32549	14733	2.35	0.95	0.95	2.52	
	48h	12507	1650	18339	5754	1.47	0.86	0.86	1.42	
	72h	9846	1188	15893	3359	1.61	0.68	0.68	1.23	
MMP-9	Time Point	Mean Survived	SEM	Mean deceased	SEM					
	0h	33923	6498	30815	6069	0.91	1	1	1	
	6h	67379	15981	97958	14040	1.45	1.99	1.99	3.18	
	12h	53346	8202	74025	12245	1.39	1.57	1.57	2.40	
	24h	30425	4489	109875	59545	3.61	0.896	0.896	3.57	
	48h	11672	2254	18201	4380	1.56	0.34	0.34	0.59	
	72h	8240	1558	16789	7276	2.04	0.24	0.24	0.54	
ETS-2	Time Point	Mean Survived	SEM	Mean deceased	SEM					
	0h	763	219	534	122	0.70	1	1	1	
	6h	1899	564	2930	610	1.54	2.49	2.49	5.49	
	12h	878	117	1115	148	1.27	1.15	1.15	2.09	
	24h	801	118	1861	978	2.32	1.05	1.05	3.49	
	48h	908	185	1219	412	1.34	1.19	1.19	2.28	
	72h	825	150	955	190	1.16	1.08	1.08	1.79	

Figure 3

JUN										survived	deceased
	Time Point	Mean Survived	SEM	Mean deceased	SEM	ratio of mean deceased: mean survived	ratio mean at time point: mean at 0h				
	0h	2154	468	1506	308	0.699	1	1			
	6h	2360	661	1283	327	0.54	1.1	0.85			
	12h	1161	346	1077	294	0.93	0.54	0.72			
	24h	1559	330	3107	1936	1.99	0.72	2.06			
	48h	999	207	1416	457	1.42	0.46	0.94			
	72h	564	116	2693	1960	4.77	0.26	1.79			
FOS											
	Time Point	Mean Survived	SEM	Mean deceased	SEM						
	0h	21757	3707	26269	5548	1.21	1	1			
	6h	34756	10668	35021	7561	1.01	1.6	1.33			
	12h	17671	5080	19629	3829	1.11	0.81	0.74			
	24h	21292	5218	41002	23096	1.93	0.98	1.56			
	48h	24264	5183	25787	8506	1.06	1.12	0.98			
	72h	16866	3550	23650	6852	1.40	0.78	0.90			

Figure 3 cont'd

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2010/005809

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12Q1/68 G01N33/50
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12Q 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 practical, search terms used)

EPQ-Internal, EMBASE, WPI Data, Sequence Search

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2006/246495 A1 (GARRETT JAMES A [US] ET AL) 2 November 2006 (2006-11-02) page 101, paragraphs 6-7,155,160-164,221,351, 461,473 -----	1-14
A	US 2008/138832 A1 (IVEY RICHARD M [US] ET AL) 12 June 2008 (2008-06-12) claim 92; tables 16-17 -----	1-9
A	LENZ ET AL: "Systemic inflammation after trauma" INJURY, JOHN WRIGHT AND SONS, BRISTOL, GB, vol. 38, no. 12, 6 December 2007 (2007-12-06), pages 1336-1345, XP022376217 ISSN: 0020-1383 the whole document ----- -/-	1-9

☒ 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 document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

27 October 2010

Date of mailing of the international search report

11/11/2010

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

Knudsen, Henrik

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2010/005809

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HARDIMAN G: "MICROARRAY PLATFORMS - COMPARISONS AND CONTRASTS" PHARMACOGENOMICS, ASHLEY PUBLICATIONS, GB, vol. 5, no. 5, 1 January 2004 (2004-01-01) , pages 487-502, XP009079995 ISSN: 1462-2416 table 2	10-14
X	SCHWEITZER B ET AL: "MICROARRAYS TO CHARACTERIZE PROTEIN INTERACTIONS ON A WHOLE-PROTEOME SCALE" PROTEOMICS, WILEY - VCH VERLAG, WEINHEIM, DE, vol. 3, no. 11, 1 November 2003 (2003-11-01), pages 2190-2199, XP009053823 ISSN: 1615-9853 page 2191	10,11
X	KAWABATA D ET AL: "Ameliorative effects of follistatin-related protein/TSC-36/FSTL1 on joint inflammation in a mouse model of arthritis" ARTHRITIS AND RHEUMATISM, LIPPINCOTT, PHILADELPHIA, US, vol. 50, no. 2, 1 February 2004 (2004-02-01), pages 660-668, XP002998883 ISSN: 0004-3591 * abstract	10-14
X	TAKADA YASUNARI ET AL: "Evodiamine abolishes constitutive and inducible NF-kappaB activation by inhibiting IkappaBalpha kinase activation, thereby suppressing NF-kappaB-regulated antiapoptotic and metastatic gene expression, up-regulating apoptosis, and inhibiting invasion." THE JOURNAL OF BIOLOGICAL CHEMISTRY 29 APR 2005, vol. 280, no. 17, 29 April 2005 (2005-04-29), pages 17203-17212, XP002562397 ISSN: 0021-9258 * abstract	10-14
A	WO 2004/087949 A2 (SIRS LAB GMBH [DE]; RUSSWURM STEFAN [DE]; REINHART KONRAD [DE]; SALUZ) 14 October 2004 (2004-10-14) table 2	1-9
	----- -/--	

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2010/005809

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	WO 2009/115478 A2 (SIRS LAB GMBH [DE]; RUSSWURM STEFAN [DE]) 24 September 2009 (2009-09-24) the whole document -----	1-14
T	DATABASE GeneCards [Online] 2009, "BCL2-related protein A1" XP002562398 Database accession no. BCL2A1 the whole document -----	

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2010/005809

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
US 2006246495	A1	02-11-2006	NONE	
US 2008138832	A1	12-06-2008	US 2004096917 A1	20-05-2004
WO 2004087949	A2	14-10-2004	EP 1611255 A2	04-01-2006
			US 2008070235 A1	20-03-2008
WO 2009115478	A2	24-09-2009	DE 102008000715 A1	24-09-2009