A TRANSCRIPTOMIC BIOMARKER TO QUANTIFY SYSTEMIC INFLAMMATION IN SEPSIS – A PROSPECTIVE MULTICENTER PHASE II DIAGNOSTIC STUDY

Michael Bauer MD, Evangelos J. Giamarellos-Bourboulis MD, Andreas Kortgen MD, Eva Möller PhD, Karen Felsmann PhD, Jean Marc Cavaillon DrSc, Orlando Guntinas-Lichius MD, Olivier Rutschmann MD, Andriy Ruryk PhD, Matthias Kohl PhD, Britta Wlotzka PhD, Stefan Rußwurm MD, John C. Marshall MD, Konrad Reinhart MD

Supplementary Appendix

Table of Contents:

Table S1 Demographic characteristics of patients with extreme selected phenotypes enrolled in the training set.

	Systemic inflammation (n= 53)	**Severe sepsis/ Septic shock (n= 43)**	**p**
Male/female (n)	33/19	31/12	0.511*
Age (yrs, mean ± SD)	56.1 ± 20.0	67.0 ± 11.2	0.004**
Number of organ dysfunctions (median, IQR)	3.0 (1.4)	3.2 (1)	0.253*
SOFA score (mean ± SD)	4.7 ± 3.90	10.8 ± 2.79	<0.0001**
Underlying condition (n, %)			<0.0001
CPB	35 (66)	0	
Multiple traumas	18 (34)	0	
Peritonitis	0	18 (41.9)	
Ventilator-associated pneumonia	0	25 (58.1)	
White blood cells (/mm^3, mean ± SD)	12025 ± 5825.0	12850 ± 5650.0	0.723**
CRP (mg/l, median, IQR)	48.3 (67.0)	204.0 (170.0)	<0.0001***
PCT (ng/ml, median, IQR)	0.56 (6.48)	5.12 (24.62)	<0.0001***
Mortality (n, %)	6 (11.3)	23 (53.5)	<0.0001*

*by Chi-square test;
**by Student's "t-test"
***by Mann-Whitney U-test

Abbreviations CPB: cardio-pulmonary bypass; CRP: C-reactive protein; IQR: inter-quartile range; PCT: procalcitonin; SOFA: sequential organ failure assessment score

Table S2 Demographic and clinical characteristics of patients enrolled in the verification set.

	Healthy controls	**Low grade systemic inflammation**	**High grade systemic Inflammation**
Male/female (n)	5/5	13/24	17/8
Age (yrs, mean ± SD)	39.3 ± 13.3	46.7 ± 17.1	69.7 ± 13.4[a]
Cause of infection/ inflammation (n, %)	0	Post-operative systemic inflammation (n= 13) Chronic tonsillitis (n= 8) Peritonsillar abscess (n= 7) UTI (n= 5) Acute oedematous pancreatitis (n= 4)	VAP (n =11) Primary bacteremia (n= 6) ABSSTI (n= 6) PSP (n= 2)
Isolated microorganisms (n)	0	*S. pyogenes* (n= 7) *E. coli* (n= 5)	*S.aureus* (n= 6) *E.faecalis* (n= 2) *E. coli* (n= 8) Other Gram(-) (n= 9)
SOFA score (mean ± SD)	0	4.5 ± 3.50	8.9 ± 3.27[a]
CRP (mg/l, median-IQR)	<5	19.8 (106.1)	232.0 (143.0)[b]
PCT (ng/ml, median-IQR)	<0.06	0.06 (0.18)	2.25 (17.85)[b]
Death (n, %)	0 (0)	2 (4.3)	6 (24.0)[c]

[a]p<0.0001 versus the low grade systemic inflammation group by the Student's "t-test"; [b]p<0.0001 versus the low grade systemic inflammation group by the Mann-Whitney U test; [c]p: 0.018 by the Fisher's exact test

Abbreviations BSI: bloodstream infection; IQR: interquartile range; PSP: primary spontaneous peritonitis; SIRS: systemic inflammatory response syndrome; VAP: ventilator-associated pneumonia; UTI: urinary tract infection; ABSSTI: acute bacterial skin and soft tissue infection.

Table S3 Demographic characteristics of the German cohort of the confirmation set of the genomic score

	Systemic inflammation (n= 56)	**Severe sepsis/ septic shock (n= 140)**	**p**
Male/female (n, %)	41 (73.2)/ 15 (26.8)	101 (72.1)/ 39 (27.9)	0.515*
Age (yrs, mean ± SD)	56.7 ± 15.7	64.9 ± 13.8	<0.0001**
Median time (range) from ICU admission to study enrolment (days)	1.5 (1-14)	1 (1-75)	0.023
White blood cells (/mm3, mean ± SD)	12977 ± 7566.6	17640 ± 11019.3	0.004**
APACHE II score (mean ± SD)	9.3 ± 3.80	18.1 ± 6.60	<0.0001**
SOFA score (mean ± SD)	3.8 ± 2.69	8.7 ± 3.32	<0.0001**
Underlying infection (n, %)			<0.0001*
Ventilator-associated pneumonia	0	46 (33.8)	
Peritonitis	0	37 (26.4)	
Skin and skin-structure infections	0	24 (17.1)	
Primary bacteremia	0	17 (12.1)	
Endocarditis	0	9 (6.4)	
Community-acquired pneumonia	0	3 (2.1)	
Acute pyelonephritis	0	4 (2.9)	
Isolated pathogen (n, %)			<0.0001*
None	56 (100)	51 (36.4)	
Enterococcus faecalis	0	17 (12.1)	
Candida albicans	0	15 (10.7)	
Staphylococcus aureus	0	14 (10.0)	
Escherichia coli	0	12 (8.5)	
Enterococcus faecium	0	8 (5.7)	
Pseudomonas aeruginosa	0	8 (5.7)	
Others	0	15 (10.7)	
Co-existing disorders/reason for ICU admission (n, %)			
Colectomy for colon Ca	4 (7.1)	14 (10.0)	0.531*
Pancreatectomy for pancreas Ca	17 (30.3)	12 (8.6)	<0.0001
Oesophagectomy for oesophagus Ca	13 (23.2)	4 (2.9)	<0.0001*
Aortic aneurysm repair	1 (1.8)	5 (3.6)	0.512*
Necrotizing fasciitis	0 (0)	3 (2.1)	0.269*
Intraabdominal abscess	0 (0)	4 (2.9)	0.201*
Femur fracture	1 (1.8)	16 (11.4)	0.030*
Multiple injuries	8 (14.3)	12 (8.6)	0.232*
Acute endocarditis	2 (3.6)	10 (7.1)	0.346*
Type 2 diabetes mellitus	6 (10.7)	28 (20.0)	0.146*

Uncompensated liver cirrhosis	2 (3.6)	11 (7.8)	0.276*
Dilated cardiomyopathy	0 (0)	3 (2.1)	0.269*
Aortic insufficiency	0 (0)	5 (3.6)	0.151*
Others	6 (14.3)	11 (7.9)	
Total days of ICU stay (median, range)	2 (1-26)	11 (1-97)	<0.0001***
Total days in hospital (median, range)	20 (8-94)	32 (1-123)	<0.0001***
Mortality on day 28 (n, %)	0 (0)	24 (17.1)	<0.0001*
Mortality on day 100 (n, %)	2 (3.6)	43 (30.7)	<0.0001*

*by Chi-square test;
**by Student's "t-test"
***by Mann-Whitney U test
Abbreviations APACHE: acute physiology and chronic health evaluation score; Ca: carcinoma; CRP: C-reactive protein; IQR: inter-quartile range; PCT: procalcitonin; SOFA: sequential organ failure assessment score

Table S4 Demographic characteristics of the Greek cohort of the confirmation set of the genomic score

		p compared to patients with severe sepsis/septic shock of the German cohort
Male/female (n, %)	26 (52.0)/24(48.0)	0.009
Age (yrs, mean ± SD)	65.9 ± 18.2	0.589
White blood cells (/mm^3, mean ± SD)	16,616 ± 9,392.7	<0.0001
APACHE II score (mean ± SD)	16.5 ± 7.83	0.191
SOFA score (mean ± SD)	6.70 ± 3.83	<0.0001
Underlying infection (n, %)		
Community-acquired pneumonia	15 (30.0)	<0.0001
Acute cholangitis	11 (22.0)	<0.0001
Ventilator-associated pneumonia	10 (20.0)	0.008
Acute pyelonephritis	8 (16.0)	<0.0001
Primary bacteremia	6 (12.0)	0.978
Isolated pathogen (n, %)		
None	25 (50.0)	
Klebsiella pneumoniae	8 (16.0)	<0.0001
Pseudomonas aeruginosa	5 (10.0)	0.302
Escherichia coli	4 (8.0)	0.900
Acinetobacter baumannii	3 (6.0)	0.003
Staphylococcus aureus	3 (6.0)	0.272
Candida albicans	2 (4.0)	0.153
Co-existing disorders (n, %)		
Type 2 diabetes mellitus	18 (36.0)	0.020
Chronic heart failure	9 (18.0)	0.008
Chronic obstructive pulmonary disorder	7 (14.0)	<0.0001
Solid tumor malignancy	7 (14.0)	0.010
Total days of ICU stay (median, range)	11 (4-57)	<0.0001
Total days in hospital (median, range)	13 (6-64)	0.659
Mortality on day 28 (n, %)	15 (30.0)	0.053
Mortality on day 100 (n, %)	19 (38.0)	0.345

Abbreviations :APACHE: acute physiology and chronic health evaluation score; SOFA: sequential organ failure assessment score

Table S5: Classification characteristics of PCT and CRP regarding the state of infection (Definitive/possible/probable infection vs. No infection) for the German cohort of the confirmation set.

	PCT	**CRP**
AUC (95%-CI)	76.5% (67.2% - 85.7%)	82.6% (76.2% - 89.1%)
Threshold for Sensitivity of 80%	**0.78 ng/ml**	**110 mg/l**
Sensitivity (95%-CI):	80% (71.5 %-86.1%)	80% (72.2%-85.9%)
Specificity (95%-CI)	55% (36.0% - 73.0%)	67% (52.3% - 79.0%)
PPV (95%-CI)	89% (81.1% - 93.6%)	87% (79.2% - 91.6%)
NPV (95%-CI)	38% (24.0% - 54.3%)	56% (43.3% - 68.1%)

In concordance to Table 2 the classification thresholds were adjusted for the sensitivity of 80%, thus making the results comparable

Figure S1:

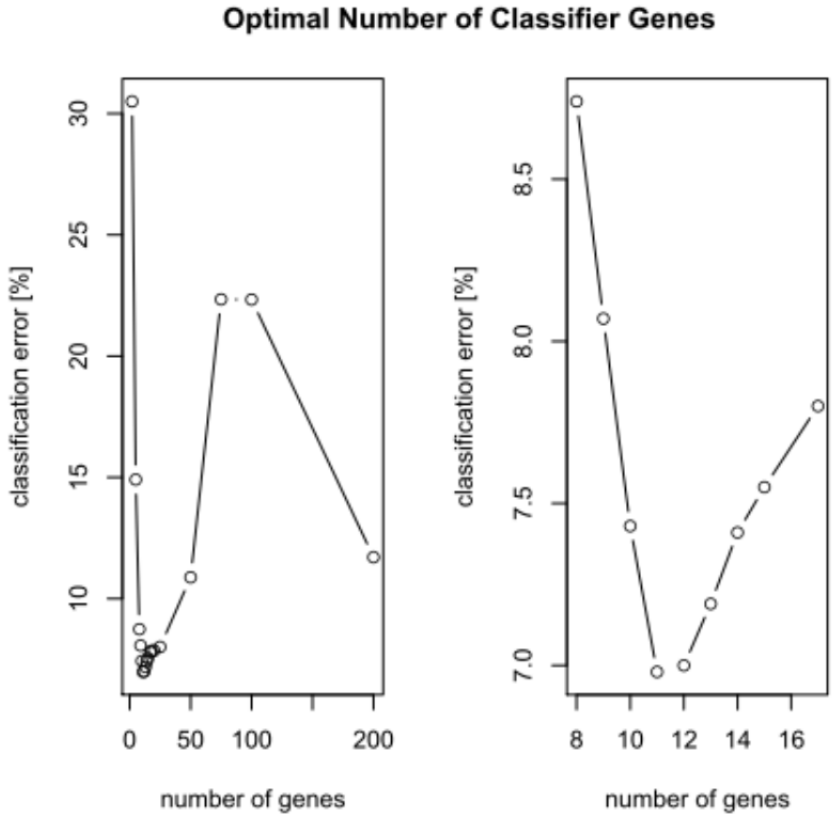

Figure S1: Mean classification error of the training set depending on the number of genes used for the classification. Obviously the classification error decreased with the number of markers, especially with 5 markers and more. However the classification became worse when too many markers were used. Best result were obtained with 10 to 20 gene markers.

Figure S2:

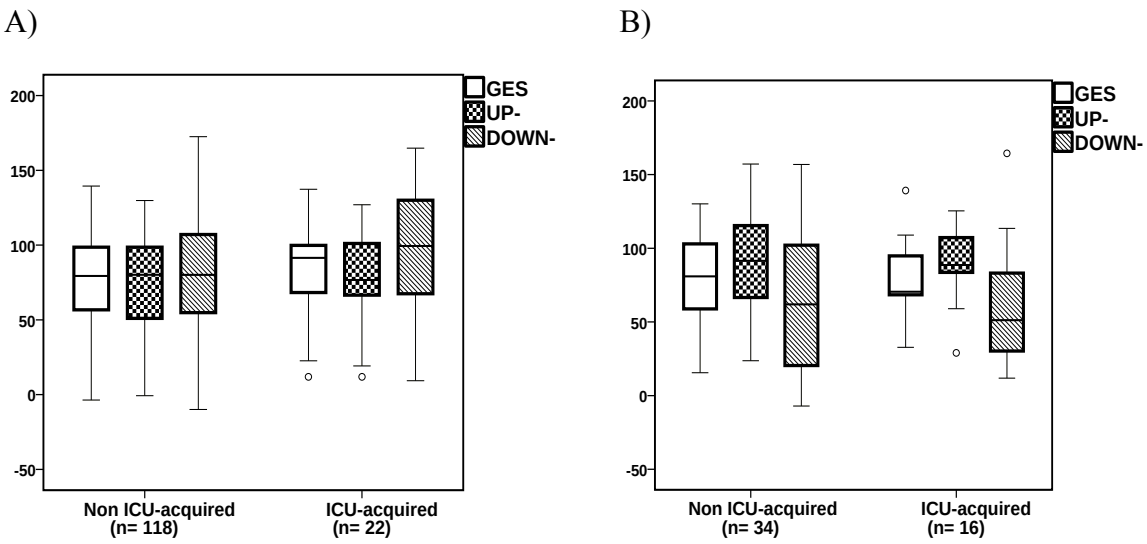

Figure S2: Genomic score (GES), UP- and DOWN-score in relation to the presence of an intensive care unit (ICU)-acquired infection. Patients with possible/probable and definitive infection were subgrouped into those admitted with infection in the ICU (non ICU-acquired) and into those with infection developing at least three days post-ICU admission (ICU-acquired). Results are provided separately for the German cohort (A) and for the Greek cohort (B). The numbers of patients without ICU-acquired and with ICU-acquired infections in each cohort are provided. No significant differences for GES or its components were found between non ICU-acquired and ICU-acquired infections in the two studied cohorts.

Figure S3:

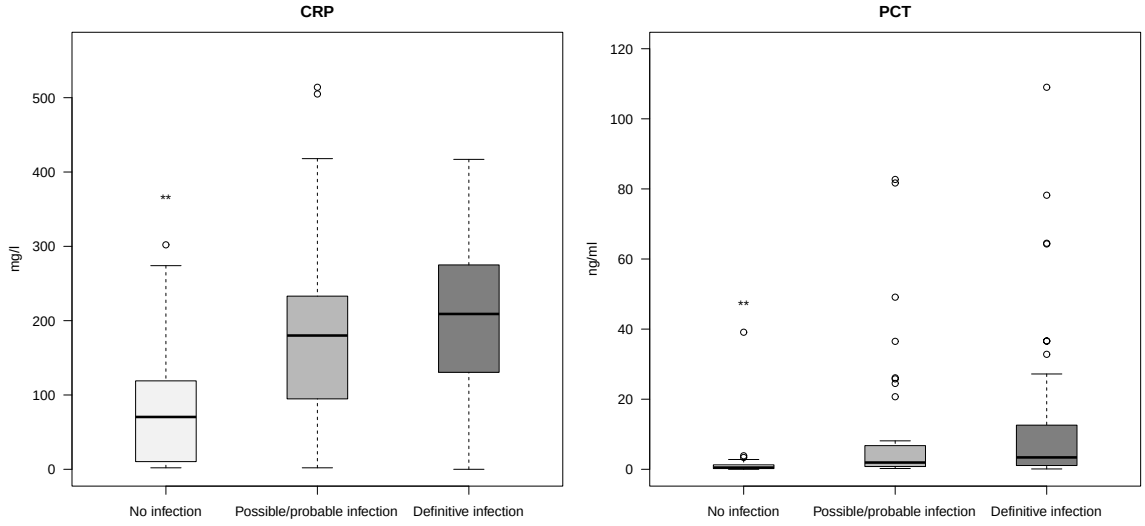

Figure S3: CRP and PCT depending on the classification of state of infection for the German cohort of the confirmation set.

**p<0·01 (results of the post-hoc pairwise comparison after Kruskal-Wallis test)

Text S1: Investigational sites:

- Jena University Hospital, Germany
- ATTIKON University Hospital, Greece
- Korinthos General Hospital, Greece
- Alexandra General Hospital, Greece
- Delafontaine Hospital, France
- Jacques Cartier Institute, France
- Saint-Joseph Hospital, France
- Division of Emergency Medicine, Geneva, Switzerland

Text S2: Definitions for sepsis and infections:

Clinical assessment of the sepsis status of each patient upon acquisition of the sample based on the SCCM/ACCP consensus conference criteria.[S1]

Blood stream infection (BSI) was defined as the presence of a Gram-positive or a Gram-negative pathogen or fungus in at least one blood culture with a corresponding PCR amplicon.[S2,S3] BSI was considered primary when no other focus or secondary when another focus of infection was defined.[S4] Patients with catheter-related BSIs with the same pathogen isolated from the lumen of the catheter and from peripheral blood and patients with blood cultures positive for skin commensals were excluded.

Local infection was defined by (i) serial negative blood cultures and corresponding negative PCR,[S2,S3] but by (ii) presence of pathogens in other cultures collected.

Ventilator-associated pneumonia (VAP) was defined as new consolidation on a chest- X-ray in a patient with a clinical pulmonary infection score more than 6 and one pathogen isolated at counts greater than 10^6 cfu/ml in quantitative cultures of tracheobronchial secretions.[S4] Peritonitis was defined as the presence of clinical signs of intrabdominal infection (IAI) in a patient previously operated for colon perforation.[S4] Acute pyelonephritis was defined by the presence of flank pain with pyuria and $>10^5$ cfu/ml of an uropathogen in quantitative urine culture.[S4]

Infection was considered absent if a) serial blood cultures and cultures of urine and of tracheobronchial secretions were negative during the entire hospitalization; and b) serial chest X-rays and chest and abdominal computed tomographies were negative. These patients were assigned into the "no infection" group. Patients were assigned into the "possible/probable infection" group when intensive work-out comprising physical findings, chest X-rays and/or chest and abdominal computed tomographies provided clinical suspicion for infection. If in these patients serial blood and other cultures failed to isolate a pathogen, patients were considered as "possibly infected". Patients were assigned into the "definitively infected" group when both clinical and microbiological findings were positive for the presence of an infection.[S4]

Exclusion criterion immunodeficiency

a) infection by the Human Immunodeficiency-1 virus;

b) neutropenia defined as <1000 neutrophils/mm^3;

c) chronic corticosteroid intake (defined as the systemic intake of more than or equal to 1mg/kg of equivalent prednisone for more than one week); and

d) any other causes of immunodeficiency such as organ transplantation, hematologic malignancies, solid tumor malignancies or chemotherapy.

Training set groups:

Severe sepsis/septic shock group: Patients with organ failure and/or shock of infectious origin should meet all the following criteria: i) high probability of infection, manifested as confirmed post-operative VAP or post-operative peritonitis; and ii) signs of at least two organ dysfunctions and/or shock.

Control group: Patients with systemic inflammation and organ failures should meet all the following criteria: i) presence of at least two of four SIRS criteria;[S1] ii) ICU admission after cardiopulmonary bypass or multiple trauma; iii) minimal probability of infection ; and (iv) at least two organ dysfunctions.

Text S3: Laboratory techniques

Concentrations of CRP in serum were measured by a nephelometric assay (Behring, Marburg, Germany). The lower limit of detection was 5 mg/l. PCT was measured in serum in duplicate by an immuno-time-resolved amplified cryptate technology assay (Kryptor PCT; BRAHMS GmbH, Henningsdorf, Germany) with a functional assay sensitivity of 0.06 ng/ml.

For transcriptomic analysis, PaxGene tubes were used for leukocyte sampling under controlled venous stasis (<30s, 40 torr) and total RNA isolated according to the manufacturer's instructions from the PaxGene Blood RNA Kit, applying both manual or automated extraction procedures (PreAnalytiX # 762 174 for QiaCube, Qiagen, Hilden, Germany) and standard RNA QC.

Experiments of the **training set** were performed using a spotted in-house research microarray addressing approximately 5,000 transcripts with focus on inflammation, and immune response as we have published in detail previously[S5] (Text S5) (Data are available at http://microarray-experiments.analytik-jena.de/ User ID: reviewer; Password: R@tt3n5ch@rf).

Gene expression of the **verification set** was studied using the commercial whole genome microarray BeadChips HumanHT-12 v3 (Illumina, San Diego USA). For improved detection of low abundant transcripts, the β-globin mRNA was reduced using the GLOBINclear™-Human kit (Ambion / Applied Biosystems # AM 1980). 1µg of totaI RNA was processed per sample; amplification of totaI RNA to cRNA with lllumina TotalPrep RNA amplification kit (Ambion/Applied Biosystems #AMIL 1791) with an input of 500 ng β-globin reduced total RNA. Concentration of cRNA was measured spectrophotometrically with the NanoDrop-2000; for hybridization on BeadChips 750 ng cRNA in 5 µI were used for overnight hybridisation at 58 °C. Signals were detected via Cy3-streptavidin staining (GE-Amersham) according to the manufacturer's instructions (lllumina Protocol: Whole-Genome Gene Expression with IntelliHybTM Seal # 1226030 Rev. B 1), and recorded using the BeadArray Reader ® lllumina 500 and the corresponding software lllumina "BeadScan" (Version3.6.17); followed by Image analysis of microarrays applying the lllumina ® software "Genome-studio" (version genomes Studio 2009.2) (Text S1)

For the **confirmation set,** assays were designed to confirm the gene expression pattern for selected genes that were considered suitable for use as biomarkers using a RT-qPCR method, as a potential platform for point-of-care testing. First strand complementary DNA synthesis was performed using 500 ng of isolated RNA from patients of the confirmation sets using SuperScript II enzyme (Invitrogen, Karlsruhe, Germany). Gene specific primers for the selected transcripts and three reference genes were designed using Primer 3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) in order to obtain an annealing temperature of 59°C and an amplicon length between 100-200 bp.

RT-qPCR amplification was performed using an iQ™5 (Bio-Rad, Munich, Germany) in 20 µl reaction volumes using Absolute SYBR Green Mix (Qiagen GmbH, Hilden, Germany) and 0.5 µM of each forward and reverse primer. Each sample was run in triplicate for each of the primer pairs assayed. Using the IQ™5 analysis software relative sample amounts (cycle threshold (Ct) values) were determined, ΔCt-values were obtained for each transcript, where the corresponding Ct-values were normalized to the mean of three reference transcripts.[S6] ΔΔCt-values were obtained, where the individual ΔCt-values were normalized to the mean ΔCt-value of the healthy volunteers[S7]. For RT-qPCR results, the genomic score (GES) was obtained using the ΔCt-values of corresponding markers.

For all tests described performers of the tests and readers of the results were unaware of clinical informations of the patients.

Text S4: ***Statistical analysis***

For the **training set**, 364 cases, representing several heterogeneous phenotypes of ICU patients were screened following selection criteria, which were already advantageous in microarray experiments[S8], where study cases from representative, well defined, and homogenous subgroups were selected. Moreover, samples of the control group were matched to the samples of the target group for the number of organ dysfunctions, thus avoiding the confounding effect of disease severity as patients with sepsis usually have higher or broad scattering degrees of severity than most other patient groups.

96 RNA samples from 96 ICU patients were hybridized against the in-house research microarray. Hybridization signals were extracted using the GenePix Analysis Software. For microarray data pre-processing the Box-Cox transformation was applied,[S9] data were finally normalized by median and median absolute deviation (MAD).

For the classification task the linear discriminant analysis (LDA) was applied.[S10] Therefore samples of the training set were assigned into a target group (with infection) and a control group (without infection). The set of relevant transcripts, used as classification features, were selected in concordance with the best estimates of Wilcoxon test applied gene by gene (i.e. transcripts with $p < 0.001$ ordered by the difference of the Hodges-Lehmann estimate).[S11] The classification step was performed for 5 and up to 100 transcripts. The classification power was proven by tenfold cross validation in 20 replications, where the concordance between molecular and clinical classification was evaluated. The set of marker candidates were determined as the subset of transcripts corresponding to the lowest classification error (Figure S1).[S12]

For the **verification set**, study cases of six clinical phenotypes were selected according to strict criteria regarding the status of inflammation and infection. The sample size was estimated with approximately 12 cases per group, where false discovery rate of 0.05, test power of 0.8 and the effect versus standard deviation ratio of 1 were controlled.[S13] The gene-expression data were normalized and log2 transformed.[S14] One-way analysis of variance with 6 groups was applied to each bead type with sufficient signal intensity. In order to control the false discovery rate occurring in multiple comparisons, a q-value was determined for each p-value.[S15] Finally, 4761 Bead Types were included in the final gene list, for which the corresponding q-value was less than 0.0024 and the absolute mean difference was higher than 0.8 between at least two study groups. Gene expression signals of 4761 selected BeadTypes were sorted into 5 clusters by k-means cluster algorithm, where the correlation distance and average linkage method were used.[S16]

In order to develop a genomic score (GES), the gene expression of each sample was sorted depending on its similarity with the patterns of healthy donors and with patterns of BSI and signs of systemic inflammation using an appropriate score, which quantified the relative distance to these two groups.[S17] The score was set to zero for the mean healthy gene expression pattern and to 100 for the mean BSI pattern. If an arbitrary gene expression pattern was more similar to the mean healthy pattern than to the mean BSI pattern the corresponding score was less than 50; otherwise the score was above 50. The genome wide origin of the score was computed incorporating expression signals of all 4761 transcripts selected.

In the next step, we then tried to identify a particularly reduced subset of single representative genes with highest phenotypic separation capacity to determine marker candidates. Therefore, the genomic score was computed using reduced sets of markers. In multitudinous in silico simulations with random selection of 10 to 50 features, subsets of marker combinations were determined reflecting this course with very similar capability. However, the marker groups varied from set to set and no preferred markers candidates were revealed.[S17] Finally, a screening for overlapping of these subgroups with the marker candidates revealed in the training set, identified a

subset of seven genes used to differentiate presence of infection in the training set, which could be applied to represent the whole genomic version of GES with a satisfying precision (Pearson correlation coefficient R=0·96, median difference (IQR) 3 (10) score points).

For the sample size assessment of the **confirmation set**, the sensitivity of the prediction of negative outcome was considered, where H_0: sensitivity < 70% vs. H_A: sensitivity > 85% and a mortality rate of 30% were assumed. Using the one-sample-design formula,[S18] a total of 217 cases were necessary to confirm the alternative hypothesis with a power of 90% and 266 cases with a power of 95%, where the type I error was 5%.

Results of the two cohorts enrolled in the confirmation set were expressed as individual data, means ± SD for variables with normal distribution of values and as medians and 95% confidence intervals (CI) or interquartile ranges (IQR) for variables with non-normal distribution of values. Comparisons between groups were done by t-test or one-way-anova test for normal distribution variables or by the Kruskall Wallis test for non-normal distribution variables. Bonferroni adjustments were done for multiple *post-hoc* comparisons using t-test or Wilcoxon-test respectively. We used receiver operator characteristics (ROC) curves to evaluate if the changes over time of the generated scores can distinguish mortality; areas under curve (AUC) and 95%CI were calculated. Comparisons between groups for dichotomous variables were done by the Chi-square test. Odds ratios (OR) and 95%CI were calculated by Mantel-Haenszel statistics. Step-wise forward regression analysis for outcome was conducted. Outcome was the dependent variable and disease severity and changes of generated scores were the independent variables. OR and 95%CI were calculated. Correlation between two non-normal distribution variables was done according to Spearman. Any value of p below 0.05 was considered significant.

Text S5: Microarray **E**xperiment **D**escription **F**ile (according to the MIAME Checklist)

In-house research microarray experiments

Experiment Design:

The goal of the experiment: To assess transcriptomic patterns of systemic inflammation in sepsis
Keywords: gene expression, systemic inflammation, sepsis
Experimental factors: Response to severe sepsis /septic shock and sterile SIRS using whole blood samples from ICU patients.
Experimental design - relationships between samples, treatments, extracts, labeling, and arrays (e.g., a diagram or table):
Samples were grouped regarding clinical assessment of inflammation and infection status (Table I in Attachment).
Quality control steps taken:
Presence of repetitive elements (retroposons), unspecific binding, traces of spotting buffer, RNA isolation yield, possible degradation of one and/or both RNA specimens, reverse transcription processivity and efficiency and efficiency, dye coupling efficiency, hybridization uniformity, scanning output, spot finding and flagging.
Links to the publication, any supplemental websites or database accession numbers: Not available

Samples used, extract preparation and labelling:

The origin of each biological sample:

Organism (NCBI taxonomy): Human (*Homo sapiens*).

25 ml of whole blood were collected from ICU patients on preselected days. Blood samples were collected into heparinized phlebotomy tubes for each patient.

Manipulation of biological samples and protocols used (e.g., growth conditions, treatments, separation techniques): Not relevant
Growth conditions: not relevant.
in vivo treatments (organism or individual treatments): None

in vitro treatments (cell culture conditions): PaxGene tubes (Qiagen, Hilden Germany) were used collecting whole blood and therefore for leukocyte sampling under controlled venous stasis.

Compound treatment: none

separation technique (e.g., none, trimming, microdissection, FACS): none.
Experimental factor value for each experimental factor, for each sample (e.g., '*time* = 30 min' for a sample in a time course experiment).
Technical protocols for preparing the hybridization extract (e.g., the RNA or DNA extraction and purification protocol), and labeling.
Extraction method: RNA isolation was performed according to PAXgene Blood RNA Tubes manufacturer's recommendations (Qiagen GmbH, Germany). Total RNA was extracted from the blood samples.

No RNA amplification method was used.

Label used: monofunctional NHS-(succin-imidyl)-esters derivatives for both AlexaFluor 555 and AlexaFluor 647.

Label incorporation method: in first step, a reverse transcription was used to produce cDNA from above amount of total RNAs. In the dNTP mix used for the reaction, a fraction of dTTP was substituted by aminoallyl-dUTP (AA-dUTP) in proportion 1:4. Upon mRNA sequence composition, each successfully transcribed cDNA molecule acquires a defined substitution pattern of dTTP by AA-dUTP. Afterwards, conversion of formed RNA/cDNA duplexes into single-stranded cDNA was performed via RNA alkaline hydrolysis. Then, resulting cDNAs were labeled with Alexa-647 and Alexa-555 monofunctional NHS-esters according to the

cohybridization experimental schemes given above. The labelling proceeded through chemical coupling of dye monofunctional esters to the incorporated AA-dUTP. After labeling cDNA samples were purified using a PCR clean-up kit.

External controls (spikes), if used:
Ten mRNAs, each complementary to the polynucleotide probe for ten cognate *A. thaliana* genes, were used
Hybridization procedures and parameters:
Samples were hybridized according to company internal protocol.
Wash procedure:

soaking of hybridization chamber with wash buffer I (2xSSC, 0.03%SDS) for 30 seconds;

double washing in buffer I for 1.5 min at room temperature each, with soaking of hybridization chamber with wash buffer Ifor 30 seconds in between;

soaking of hybridization chamber with wash buffer II (1xSSC) for 30 seconds;

double washing in buffer II for 1.5 min at room temperature each, , with soaking of hybridization chamber with wash buffer II for 30 seconds in between;

soaking of hybridization chamber with wash buffer III (0.2xSSC) for 30 seconds;

washing in buffer III for 1.5 min at room temperature;

array surface drying by application of a nitrogen under pressure 2.5 bar for 2.5 min at 30°C.

Quantity of labeled target used: whole amount of obtained labeled cDNA starting from 10µg tRNA was used in each hybridization.

Time, concentration, volume, temperature:

Hybridization time= 14 hours;

volume = 80 µl;

Hybridization temperature = 42°C.

description of the hybridization instruments:

The HS 400 Hybridization Station is a compact system that fully automates the process of hybridization of arrays spotted on microscopic slides and is part of Tecan's Array Suite (Tecan). It consists of a built-in Liquid Distribution Unit, a waste system and one module with a heating block and a chamber frame for securing the slide adapter with 4 slides, thus, it has the ability to perform hybridization with a maximum of 4 slides which can be manipulated simultaneously. The slides are temperature-controlled and can be heated and cooled between 4°C and 85°C. Vacuum-driven agitation of the hybridization mixture produces a homogeneous DNA concentration over array area and augments a slow diffusion by liquid movement along the long slide axis. This results in higher spot signals and homogeneous low background level over the whole slide. The station has ports for 6 reagent bottles, 4 of which are temperature-controlled. They are used for hosting and applying pre-hybridization, washing buffers etc. The On-Board slide drying allows the slides to be automatically dried by using pressurized nitrogen. The HS 400 Hybridization Station is controlled by an external PC with HS Control Manager Software.

Data:

Measurement data and specifications

- *Scanning hardware* – GenePix 4000B confocal epifluorescent scanner (Axon Instruments);

- *scanning software* – GenPix Pro 4.0;

- *Scan parameters*

Laser power: Cy3 channel – 100%

Cy5 channel – 100%

PMT voltage: Cy3 channel – 700 V;

Cy5 channel – 800 V;

Spatial resolution (pixel space) – 10 µm.

Data extraction and processing protocols:
Within the scope of the experiment, 1784 patients samples were used. Each RNA pair (sample vs. reference) was hybridised against a microarray. Generally, the RNA of a patient sample was labelled by red dye and reference samples were green-labelled. Digital images resulting from posthybridization array scanning were quantified using of GenePix Pro 4.0 software (Axon Instruments). For the spot detection, quantification and quality flagging, the GenePix™ Analysis Software was used. The raw expression signals for each spot were quantified as the median spot intensity in the red and green channel, corrected by the median local background intensity. The spots were flagged corresponding to the settings of the GenePix™ Software (100 = "good", 0 = "weak /saturation", -50 = "not found", -100 = "bad"). The raw signals of a microarray were summarised in a *.gpr file (cf. Table 1)).

Normalization, transformation and data selection procedures and parameters:
Normalization and transformation: For the microarray data pre-processing the Box-Cox transformation was applied (cf. *Box GEP, Cox DR. (1964) An analysis of transformations. J Roy Stat Soc B 26: 211-252*); the data were finally normalized by median and MAD. This approach made a self-sufficient normalization of each sample possible. Data quality was proven before and after the normalization step following recommendations of Buneβ at al. (*Buneβ A, Huber W, Steiner K, Sültmann H, Poustka A. (2005) arrayMagic: two-colour cDNA microarray quality control and preprocessing. Bioinformatics 21: 554 - 556*).

Array Design:

General array design, including the platform type

Array design name: middle-density polynucleotide array developed for internal R&D purposes .

Platform type: spotted microarrays.
Surface and coating specification: Epoxysilane coated glass slides for covalent immobilization of amino-modified DNA or polynucleotides. The surface-modifiedslides Typ E were developed at Schott-Nexterion. (Schott Group Germany)
Physical dimensions of array support: 26x76x1 mm microscope glass slide.
Number of features on the array: The microarray consists of 5308 probes (addressing to 4868 transcripts of approximately 3704 human genes) and is cuts into 28 subarrays. 6 out of the 28 subarrays contain a set of 17 standard control features
availability: The aforementioned microarray is not available for purchase.
Spot dimensions: diameter 120±10 µm. Spotting pattern 15x15 spots using 28 SMP 4 Spilt pins (Telechem Inc. USA)
Attachment chemistry: covalent.
Array feature and reporter annotation

Type of reporter: synthetic polynucleotides, 56-70 deoxynucleotide residues long.
single-stranded.
Reporter sequence
Sequence or PCR primer information: not available.
Sequence information: see supplemental material Tomic et al.[5]
Composite sequence
Polynucleotide probes for different regions of 3'-part of human β-actin gene and glyceraldehyde-3-phosphate dehydrogenase gene located on the distance of approximately 200 bases along their cognate mRNA sequences.

Control elements on the array

Positive control elements: spots of the 5'-end Cy3-labeled "alien" oligonucleotide without a significant homology to any human coding sequences; spots of probes for different regions of 3'-part of human β-actin gene, glyceraldehyde-3-phosphate dehydrogenase gene and genes for two isoforms of human tubulin alpha; Negative control elements: blank spots; spotting buffer spots; spots containing human Cot-1 DNA; spots containing polynucleotide probes originating from the sequences of ten *E. coli* plasmid and phage cloning vectors and their marker genes which have no homology with any human coding sequences.
Control elements with specialized functions: spots containing polynucleotide probes against five "alien" artificial sequences and an *Arabidopsis thaliana* gene, all without a significant homology to any human coding sequences, which may serve both functions as being negative or spiking control elements depending on a presence of cognate mRNAs in samples under investigation; spots containing polynucleotide probes for 24 annotated human genes known to maintain their RNA expression rate at constant level in most investigated biological systems and their responses to perturbed environment ("in vitro" genes); spots containing polynucleotide probes for 12 human ESTs selected on the basis of constant expression in sepsis/sirs patients vs. healthy controls studies (sequences encoding for "house-keeping" genomic functional units); spots containing polynucleotide probes for 16 human genes encoding for different alpha and beta chain paralogues among major histocompatibility complex II receptors as well as CD markers specific for defined sets and subsets of leukocytes (surface markers).

Feature location on the array: all information concerning the location of the reporters on the microarray have already been described in detail.[5]

All information concerning the characteristics of the reporter molecule on the microarray have already been described in detail.[5]

All information concerning the biological annotation of each of the reporter molecules on the microarray have already been described in detail.[5]

Principle array organism(s)

Human sequence probe

Illumina BeadChips experiments

Experimental Design:

The goal of the experiment

Capability of the gene expression patterns to reflect the host response evoked by acute inflammation with /without infection

A brief description of the experiment

In this study, patients represented six clinical phenotypes, which reflected stages of low-grade and high-grade inflammation with and without infection

Keywords: Microarray, gene expression profiling, Illumina

Experimental factors: Sterile vs. infectious causes in local and systemic inflammation

Experimental design: Table II in attachment

Quality control steps taken:

Qualitycontrol of isolated / delivered RNA is performed by

Bioanalyzer® 2100 RNA Nano 6000 LabChips (Agilent Technologies)

measuring the A260/A280 ratio with a Nanodrop 2000 Spectro-Photometer

Quality control of hybridisation / data generating, -processing is performed by seven control categories incl. negative control built into the chip to check every aspect of the array experiment, e.g. the biological specimen (housekeeping), conditions of hybridization and signal generation.

Similarity analyses, data normalization, boxplots of raw and normalized data, mean-sd-plots during data processing

Links to the publication, any supplementary websites or database accession numbers

www.illumina.com

Samples used, extract preparation and labelling
The origin of each biological sample

Organism (NCBI taxonomy): Homo Sapiens

Sample origin: whole blood samples

Manipulation of biological samples and protocols used: none

Experimental factor value for each experimental factor, for each sample: none

Technical protocols for preparing the hybridization extract and labelling

Extraction method:

Total RNA was extracted from PAXGene whole Blood samples using the PAXGene Blood RNA Kit (Qiagen) according to manufactors' instructions. Total RNA extracts were quantified with a NanoDrop 2000 spectrophotometer (NanoDrop-Technologies) and quality checked with the Agilent 2100 Bioanalyzer Total - RNA LabChip Nano 6000.

Hybridisation sample preparation:

To specify the GeneExpression profile, overwhelming ßGlobin mRNA content of total RNA was reduced applying GLOBINclear ^{TM}-Human, (Ambion /Applied Biosystems), following manufactors' instructions. Subsequently each of 600 ng of Glb-minus RNA were reversely transcribed, amplified and biotinylated using the Illumina TotalPrep RNA Amplification Kit (Ambion). Yields of cRNA were quantified with a NanoDrop 2000 spectrophotometer (Nanodrop-Technologies). Sample preparation for hybridization on Illumina "Gene Expression" BeadChips was carried out according manufactors' "Gene Expression on Sentrix® Arrays Direct Hybridization System Manual" (Illumina).

External controls (spikes), if used: none

Hybridization procedures and parameters
Hybridization is performed according to the Illumina "Gene Expression on Sentrix® Arrays Direct Hybridization System Manual" (Illumina).

Quantity of target used: 750 ng cRNA of each sample were hybridized on a Sentrix® BeadChip Array human HT12v3 (Illumina Inc) targeting more than 25.000 annotated gene targets with more than 48.000 oligonucleotide probes per array

Hybridization time: 16 h

Volume: 15 µl

Temperature: 58° C

Label incorporation method:

Hybridized arrays were stained with 1µg/µl Streptavidin-Cy3 (FluoroLink® Cy3, GE Biosciences), washed, dried and scanned immediately on a Illumina BeadArray Reader.

Label used: Streptavidin-Cy3 (FluoroLink® Cy3, GE Biosciences)

Measurement data and specifications

Data extraction and processing protocols

Data extraction
Scanning hardware : Illumina BeadArray Reader
Scanning software : BeadScan 3.6.17
Spatial resolution (pixel space) 0,8 µm
Data analysis software: BeadStudio Gene Expression Module v3.2.

Data processing protocol: The BeadChips were scanned according to the protocol described in the "Illumina Whole Genome Gene Expression for BeadStation Manual v3.2, Revision A".

Data:
The first level of raw data generated by the Illumina BeadArray Reader are Scan-images (*.Tif)
image data files (*.idat) and technical information files data obtained by the Illumina's "Genome Studio" data analysis software (2009 v2) are further proceeded to .txt files, which feed the biostatistical analysis.

Normalization, transformation and data selection procedures and parameters
Normalization and transformation: gene-expression data were variance stabilized normalized (21) and log2 transformed (c*f. Huber W, Heydebreck A; Sueltmann H; Poustka A; Vingron M. (2002) Variance stabilization applied to microarray data calibration and to the quantification of differential expression. Bioinformatics 18: 96-104*).
Filtering: The bead types were filtered using Illumina detection p value. Only bead types with detection value equal or smaller than 0.01 on at least one array were further analyzed.

Array Design
General array design, including the platform type
Array design name: Illumina Expression BeadChip Array human HT12v3

Platform type:

DNA oligonucleotide random Bead-Array by, Illumina (The platform, its reproducibility and sensitivity have been described in the FDA-guided Microarray Quality Control study (MAQC)[1])

Surface and coating specification: glass slide with wells to hold the beads, transcript specific oligonucleotides are covalently linked to silica beads of 3µm diameter

Physical dimensions of array support: 26 x 76 x 1 mm glass slide

Number of features on the array: 48.803 bead types (each feature is represented in average 15 times, beads are assembled randomly on array surface).

Sequence orientation on the bead surface: 5´ proximal to surface, linked to a 23 bp identifier-sequence which is decoded by the manufacturer in order to determine the location of the feature on the array surface

Availability: The aforementioned microarray is commercially available (Illumina, Inc., San Diego)

Attachment chemistry: covalent, adhaesive

[1] MAQC Consortium. (2006). The MicroArray Quality Control (MAQC) project shows interplatform reproducibility of gene expression measurements. Nature Biotechnology **24**(9), 1151-1161.

Array feature and reporter annotation
Type of reporter: synthetic polynucleotides, 50 deoxynucleotide residues long; single-stranded.
Reporter sequence / Sequence information:
The features represent the currently known RefSeq (Build36.2, Rel 22) and UniGene ((Build 199) databases (latest release, http://www.ncbi.nlm.nih.gov/RefSeq/
Control elements on the array:
There are sample-dependent and –independent control features present, like housekeepers, low-, medium- and high stringency-control features as well as hybridization control probes to control successful hybridization and signal generation that are content of the hybridization buffer, as delivered by the manufacturer.
Further information concerning the arrays is available at http:// www.illumina.com
Principle array organism(s): human

Attachment Text S5:

Table I: Mapping of patients samples and microarray experiment ID (Raw-Data-Files)

Clinical phenotype	Patient ID	Experi- ment ID
septic Shock	714	id131
septic Shock	782	id134
severe SIRS	790	id135
severe SIRS	814	id176
severe SIRS	844	id178
none/ SIRS	856	id181
severe SIRS	865	id183
severe SIRS	869	id183
septic Shock	877	id185
severe SIRS	883	id403
severe SIRS	901	id187
severe SIRS	919	id188
septic Shock	933	id191
severe SIRS	936	id192
septic Shock	1015	id11
septic Shock	1021	id19
septic Shock	1035	id31
severe SIRS	2038	id51
severe Sepsis	6002	id102
septic Shock	6008	id204
septic Shock	6009	id216
septic Shock	6011	id255
severe Sepsis	6014	id307
septic Shock	6022	id395
septic Shock	6023	id422
septic Shock	6025	id447
septic Shock	6032	id528
septic Shock	6035	id567
septic Shock	6038	id600
septic Shock	6040	id614
septic Shock	6046	id643
septic Shock	6048	id657

Clinical phenotype	Patient ID	Experi- ment ID
septic Shock	6057	id687
septic Shock	6062	id727
septic Shock	6063	id734
septic Shock	6065	id746
severe Sepsis	6070	id764
septic Shock	6073	id787
septic Shock	6075	id400
septic Shock	6084	id832
septic Shock	6085	id841
septic Shock	6088	id867
septic Shock	6096	id930
severe Sepsis	6104	id405
septic Shock	6108	id100
septic Shock	6109	id100
septic Shock	6118	id401
septic Shock	6127	id109
septic Shock	6130	id111
septic Shock	6132	id112
septic Shock	6138	id116
septic Shock	6141	id118
septic Shock	6142	id119
severe SIRS	8001	id136
severe SIRS	8002	id138
severe SIRS	8009	id140
severe SIRS	8010	id142
severe SIRS	8012	id142
severe SIRS	8025	id145
severe SIRS	8026	id146
severe SIRS	8030	id147
severe SIRS	8032	id148
severe SIRS	8049	id152
severe SIRS	8051	id153

Clinical phenotype	Patient ID	Experi- ment ID
severe SIRS	8056	id153
severe SIRS	8058	id154
severe SIRS	8068	id155
severe SIRS	8076	id157
severe SIRS	8084	id160
severe SIRS	8086	id160
severe Sepsis	8089	id162
severe SIRS	8094	id164
severe SIRS	8096	id165
severe SIRS	8102	id167
severe SIRS	8111	id170
severe SIRS	8112	id171
severe SIRS	8116	id172
severe SIRS	8122	id173
severe SIRS	KG15132	
severe SIRS	P10134	
severe SIRS	KG147912	
severe SIRS	KG4138	
severe SIRS	KG101318	
severe SIRS	KG161314	
severe SIRS	KG111320	
severe SIRS	KG171326	
severe SIRS	KG61323	
severe SIRS	P71324	
severe SIRS	KG12101232	
severe SIRS	KG51333	
severe SIRS	P211328	
severe SIRS	P41336	
severe SIRS	P17945	
severe SIRS	P61346	
severe SIRS	P8138	
severe SIRS	P5139	

Attachment Text S5:

Table II: Mapping of patients samples and microarray ID

microarray_chipID	Subjects Group	Sample-No	microarray_chipID	Subjects Group	Sample-No
5361198024_J	Healthy	1	5361198011_I	SIRS w/o infection	37
5361198011_L	Healthy	2	5361198011_J	SIRS w/o infection	38
5361198014_K	Healthy	3	5361198015_G	SIRS w/o infection	39
5361198024_K	Healthy	4	5361198013_J	SIRS w/o infection	40
5361198024_L	Healthy	5	5361198011_K	SIRS w/o infection	41
5361198013_K	Healthy	6	5361198014_I	SIRS w/o infection	42
5361198013_L	Healthy	7	5361198014_J	SIRS w/o infection	43
5361198015_J	Healthy	8	5361198013_I	SIRS w/o infection	44
5361198015_L	Healthy	9	5361198015_H	SIRS w/o infection	45
5361198014_L	Healthy	10	5361198024_I	SIRS w/o infection	46
5361198024_C	local sterile inflammation	11	5361198015_I	SIRS w/o infection	47
5361198011_D	local sterile inflammation	12	5361198024_F	local infection with SIRS	48
5361198014_B	local sterile inflammation	13	5361198013_G	local infection with SIRS	49
5357965011_K	local sterile inflammation	14	5361198013_H	local infection with SIRS	50
5361198024_D	local sterile inflammation	15	5361198012_E	local infection with SIRS	51
5357965012_J	local sterile inflammation	16	5361198012_F	local infection with SIRS	52
5361198011_C	local sterile inflammation	17	5357965012_L	local infection with SIRS	53
5361198014_A	local sterile inflammation	18	5361198011_G	local infection with SIRS	54
5361198013_C	local sterile inflammation	19	5361198011_H	local infection with SIRS	55
5361198015_C	local sterile inflammation	20	5361198015_F	local infection with SIRS	56
5357965011_J	local sterile inflammation	21	5361198014_E	local infection with SIRS	57
5361198015_D	local sterile inflammation	22	5361198014_F	local infection with SIRS	58
5361198013_E	local infection w/o SIRS	23	5361198014_G	local infection with SIRS	59
5361198013_F	local infection w/o SIRS	24	5361198014_H	local infection with SIRS	60
5361198012_C	local infection w/o SIRS	25	5361198024_B	blood stream infection with SIRS	61
5361198012_D	local infection w/o SIRS	26	5361198013_A	blood stream infection with SIRS	62
5357965012_K	local infection w/o SIRS	27	5361198013_B	blood stream infection with SIRS	63
5361198015_E	local infection w/o SIRS	28	5361198011_B	blood stream infection with SIRS	64
5361198014_C	local infection w/o SIRS	29	5361198015_A	blood stream infection with SIRS	65
5357965011_L	local infection w/o SIRS	30	5361198015_B	blood stream infection with SIRS	66
5361198024_E	local infection w/o SIRS	31	5357965011_G	blood stream infection with SIRS	67
5361198011_E	local infection w/o SIRS	32	5357965011_H	blood stream infection with SIRS	68
5361198011_F	local infection w/o SIRS	33	5361198024_A	blood stream infection with SIRS	69
5361198014_D	local infection w/o SIRS	34	5357965012_H	blood stream infection with SIRS	70
5361198024_G	SIRS w/o infection	35	5357965012_I	blood stream infection with SIRS	71
5361198024_H	SIRS w/o infection	36	5361198011_A	blood stream infection with SIRS	72

Text S6:

References Appendix:

S1. Levy M, Fink MP, Marshall JC et al. 2001 SCCM/ESICM/ACCP/ATS/SIS international sepsis definitions conference. *Crit Care Med* 2003; **31**: 1250-6.

S2. Bloos F, Sachse S, Kortgen A et al. Evaluation of a polymerase chain reaction assay for pathogen detection in septic patients under routine condition: an observational study. *PLoS One* 2012; **7**: e46003.

S3. Fitting C, Parlato M, Adib-Conquy M et al. DNAemia detection by multiplex PCR and biomarkers for infection in systemic inflammatory response syndrome patients. *PLoS One* 2012; **7**: e38916.

S4. Calandra T, Cohen J. The International Sepsis Forum Consensus definitions of infections in the intensive care unit. *Crit Care Med* 2005; **33**: 1639-48.

S5. Tomic V, Russwurm S, Möller E et al. Transcriptomic and proteomic patterns of systemic inflammation in on-pump and off-pump coronary artery bypass grafting. *Circulation* 2005; 112: 2912-20.

S6. WO 2008107114 A3: Control genes for the normalization of gene expression analysis data, patent application id PCT/EP2008/001582, published in 2008.

S7. Kenneth JL, Thomas DS. Analysis of relative gene expression data using real-time quantitative PCR and the ΔΔCT method. *Methods* 2001; **25**: 4028.

S8. Liu H, Li J. and L. Wong. Use of extreme patient samples for outcome prediction from gene expression data. *Bioinformatics* 2005; **21:**3377-84..

S9. Box GEP, Cox DR. An analysis of transformations. *J Roy Stat Soc B* 1964; **26**: 211-52.

S10. Hastie T, Tibshirani R, Friedman J. The elements of statistical learning: data mining, inference, and prediction. Springer Series in Statistics 2001; pp: 11-14, 266-271, 371-389.

S11. Hodges JL, Lehmann EL. Estimation of location based on ranks. *Ann Math Stat* 1963; **34**: 598-611.

S12. WO 2009115478 A3: Method for in vitro detection and differentiation of pathophysiological states, patent application id PCT/EP2009/053042, published in 2009.

S13. Liu P, Hwang JG. Quick calculation for sample size while controlling false discovery rate with application to microarray analysis. *Bioinformatics* 2007; **23:**739-46.

S14. Huber W, von Heydebreck A, Sueltmann H, Poustka A, Vingron M. Parameter estimation for the calibration and variance stabilization of microarray data. *Stat Appl Genet Mol Biol* 2003; **2**: Article 3.

S15. Storey JD, R Tibshirani R. Statistical significance for genomewide studies. *Proc Natl Acad Sci USA* 2003; **100**: 9440-5.

S16. Theodoridis S, Koutroumbas K. Pattern Recognition. Third Edition 2006. Academic Press, San-Diego; pp. 602-22.

S17. WO 2012120026 A1: Method for identifying a subset of polynucleotides from an initial set of polynucleotides corresponding to the human genome for the in vitro determination of the severity of the host response of a patient, patent application id PCT/EP2012/053870, published in 2012.

S18. Machin D, Campbell MJ, Tan SB, Tan SH. Sample Size Tables for Clinical Studies. 3rd edition (ISBN: 978-1-4051-4650-0), Wiley-Blackwell 2009, p. 162