(12) STANDARD PATENT   (11) Application No. **AU 2005313114 B2**
(19) AUSTRALIAN PATENT OFFICE

(54)   Title
           Early detection of sepsis

(51)   International Patent Classification(s)
           C12Q 1/68 (2006.01)   ***G06F 19/00*** (2006.01)
           G01N 33/68 (2006.01)

(21)   Application No: **2005313114**   (22)   Date of Filing: **2005.12.09**

(87)   WIPO No: **WO06/061644**

(30)   Priority Data

(31)   Number   (32)   Date   (33)   Country
           0426982.5   **2004.12.09**   **GB**

(43)   Publication Date: **2006.06.15**
(44)   Accepted Journal Date: **2012.01.19**

(71)   Applicant(s)
           Secretary of State for Defence

(72)   Inventor(s)
           Yates, Amanda Marie;Brookes, Timothy Jonathon;Lukaszewski, Roman Antoni;Pearce,
           Martin Julian;Jackson, Matthew Christopher

(74)   Agent / Attorney
           Davies Collison Cave, 1 Nicholson Street, Melbourne, VIC, 3000

(56)   Related Art
           US 2003/0207278 A1 (Khan et al) 6 November 2003
           WO 2004/043236 A2 (Becton, Dickinson and Company) 27 May 2004

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: JACKSON, Matthew, Christopher [GB/GB]; c/o DSTL, Por-ton Down, Salisbury, Wiltshire, SP4 0JQ (GB). LUKASZEWSKI, Roman, Antoni [GB/GB]; c/o DSTL, Porton Down, Salisbury, Wiltshire, SP4 0JQ (GB). YATES, Amanda, Marie [GB/GB]; c/o DSTL, Porton Down, Salisbury, Wiltshire, SP4 0JQ (GB). PEARCE,

Martin, Julian [GB/GB]; c/o DSTL, Porton Down, Sal-isbury, Wiltshire, SP4 0JQ (GB). BROOKES, Timothy, Jonathon [GB/GB]; c/o DSTL, Porton Down, Salisbury, Wiltshire, SP4 0JQ (GB).

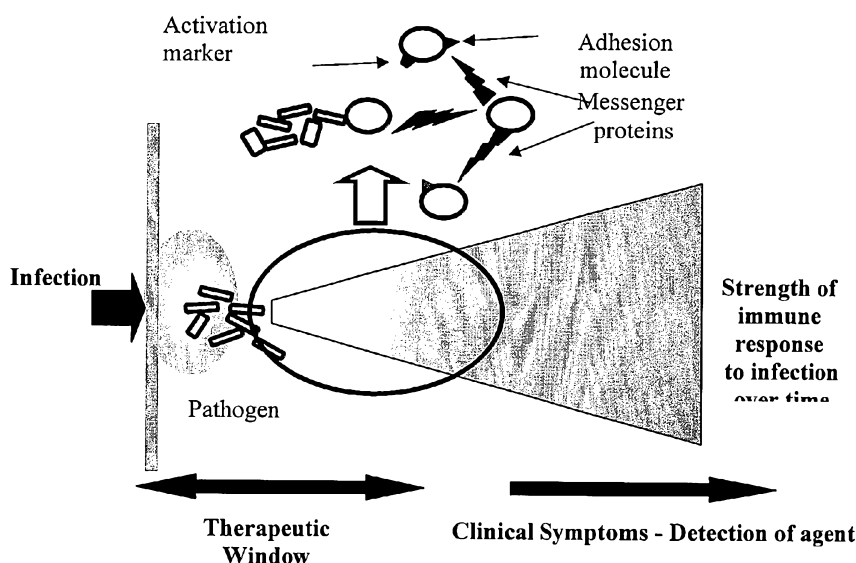(74) Agents: AJELLO, Michael, John et al.; Harrison God-dard Foote, Orlando House, 11c Compstall Road, Marple Bridge, Stockport SK6 5HH (GB).

(54) Title: EARLY DETECTION OF SEPSIS

(57) Abstract: The invention describes a system and method for detecting early signs of infection and, in particular, for identifying individuals most likely to develop sepsis. Measurement of expression levels of particular combinations of cytokines and/or cellular activation markers, optionally combined with the use of predictive algorithms, allows a high degree of accuracy of prediction. The method is applicable in both civilian and military contexts.

Early detection of sepsis

Background

Despite greatly improved diagnosis, treatment and support, serious infection and sepsis remain significant causes of death and often result in chronic ill-health or disability in those who survive acute episodes. Although sudden, overwhelming infection is comparatively rare amongst otherwise healthy adults, it constitutes an increased risk in immunocompromised individuals, seriously ill patients in intensive care, burns patients and young children. In a proportion of cases, an apparently treatable infection leads to the development of sepsis; a dysregulated, inappropriate

10   response to infection characterised by progressive circulatory collapse leading to renal and respiratory failure, abnormalities in coagulation, profound and unresponsive hypotension and, in about 30% of cases death. The incidence of sepsis in the population of North America is about 0.3% of the population annually (about 750,000 cases) with mortality rising to 40% in the elderly and to 50% in cases of the most severe form, septic shock (Angus *et al*, 2001, Crit Care Med <u>29</u>: 1303–1310).

Following infection with infectious micro-organisms, the body reacts with a classical inflammatory response and activation of, first, the innate, non-specific immune response, followed by a specific, acquired immune response. In the case of bacterial infections, bacteraemia leads to the rapid

20   (within 30–90 minutes) onset of pyrexia and release of inflammatory cytokines such as interleukin-1 (IL-1) and tumour necrosis factor-α (TNF-α) triggered by the detection of bacterial toxins, long before the development of a specific, antigen-driven immune response.

In Gram-negative bacteraemia due to infections such as typhoid, plague, tularaemia and brucellosis, or peritonitis from Gram-negative gut organisms such as *Escherichia coli*, *Klebsiella*, *Proteus* or *Pseudomonas* this is largely a response to lipopolysaccharide (LPS) and other components derived from bacterial cell walls. Circulating LPS and, in particular, its constituent lipid A, provokes a wide range of systemic reactions. It is probably contact with Kupffer cells in the liver that first leads to IL-1 release and the onset of pyrexia. Activation of circulating monocytes and

30   macrophages leads to release of cytokines such as IL-6, IL-12, IL-15, IL-18, TNF-α, macrophage migration inhibitory factor (MIF), and cytokine-like molecules such as high mobility group B1 (HMGB1), which, in turn activate neutrophils, lymphocytes and vascular endothelium, up-regulate cell adhesion molecules, and induce prostaglandins, nitric oxide synthase and acute-phase proteins. Release of platelet activating factor (PAF), prostaglandins, leukotrienes and thromboxane activates vascular endothelium, regulates vascular tone and activates the extrinsic

coagulation cascade. Dysregulation of these responses results in the complications of sepsis and septic shock in terms of peripheral vasodilation leading to hypotension, and abnormal clotting and fibrinolysis producing thrombosis and intravascular coagulation (Cohen, 2002, Nature 420: 885–891).

LPS primarily acts on cells by binding to a serum LPS-binding protein (LBP) and CD14 expressed on monocytes and macrophages. On binding a complex of LPS and LBP, CD14 acts with a co-receptor, Toll-like receptor 4 (TLR-4) and a further component, MD-2, to form a signalling complex and initiate activation of macrophages and release of cytokines (Pålsson-McDermott & O'Neill,

10   2004, Immunology 113: 153–162). The Toll-like receptor family is a group of cell surface receptors involved in a range of bacterial and fungal ligands that act as triggers for innate immune system, including Gram-positive cell wall structures, flagellin, and CpG repeats characteristic of bacterial DNA.

In the case of infection with Gram-positive pathogens, septic shock is associated with the production of exotoxins. For instance, toxic shock syndrome, a particularly acute form of septic shock that often affects otherwise healthy individuals is due to infection with particular strain of *Staphylococcus aureus*, which produces an exotoxin known as toxic shock syndrome toxin-1 (TSST-1). A similar syndrome is caused by invasive infection with certain group A *Streptococcus*

20   *pyogenes* strains, and is often associated with streptococcal pyogenic enterotoxin A (SPE-A). Some Gram-positive exotoxins (including TSST-1)are thought to exert their effects predominantly as a result of their superantigen properties. Superantigens are able to non-specifically stimulate T lymphocytes by cross-linking MHC Class II molecules on antigen presenting cells to certain classes of T cell receptors. Usually, T cell receptor (TCR) -Major Histocompatibility Complex (MHC) interactions are highly specific, with only T cells carrying TCRs that specifically recognise short antigen-derived peptides presented by the MHC able to bind and be activated, ensuring an antigen-specific T cell response. Superantigens bypass this mechanism resulting in massive and inappropriate activation of T cells. However, SPE-A is not an efficient superantigen and some further mechanism must be implicated.

30   It should be noted that clinical sepsis may also result from infection with some viruses (for example Venezuelan Equine Encephalitis Virus, VEEV) and fungi, and that other mechanisms are likely to be involved in such cases.

The ability to detect potentially serious infections as early as possible and, especially, to predict the onset of sepsis in susceptible individuals is clearly advantageous. A considerable effort has been expended over many years in attempts to establish clear criteria defining clinical entities such as shock, sepsis, septic shock, toxic shock and systemic inflammatory response syndrome (SIRS).

Similarly, many attempts have been made to design robust predictive models based on measuring a range of clinical, chemical, biochemical, immunological and cytometic parameters and a number of scoring systems, of varying prognostic success and sophistication, proposed.

According to the 1991 Consensus Conference of the American College of Chest Physicians (ACCP) and Society of Critical Care Medicine (SCCM) "SIRS" is considered to be present when patients have more than one of the following: a body temperature of greater than 38°C or less than 36°C, a heart rate of greater than 90/min, hyperventilation involving a respiratory rate higher than 20/min or $PaCO_2$ lower than 32mm Hg, a white blood cell count of greater than 12000 cells /μl or

10   less than 4000 cells /μl (Bone *et al*, 1992, Crit Care Med 20: 864–874).

"Sepsis" has been defined as SIRS caused by infection. It is accepted that SIRS can occur in the absence of infection in, for example, burns, pancreatitis and other disease states. "Infection" was defined as a pathological process caused by invasion of a normally sterile tissue, fluid or body cavity by pathogenic or potentially pathogenic micro-organisms.

"Severe sepsis" was defined as sepsis complicated by organ dysfunction, itself defined by Marshall et al (1995, Crit Care Med 23: 1638–1652) or the Sequential Organ Failure Assessment (SOFA) score (Ferreira et al, 2002, JAMA 286: 1754–1758).

20

"Septic shock" refers (in adults) to sepsis plus a state of acute circulatory failure characterised by a persistent arterial hypotension unexplained by other causes.

In order to evaluate the seriousness of sepsis in intensive care patients and to allow rational treatment planning, a large number of clinical severity models have been developed for sepsis, or adapted from more general models. The first generally accepted system was the Acute Physiology and Chronic Health Evaluation score (APACHE, and its refinements APACHE II and III) (Knaus *et al*, 1985, Crit Care Med 13: 818–829; Knaus *et al*, 1991, Chest 100: 1619–1636), with the Mortality Prediction Model (MPM) (Lemeshow *et al* ,1993, JAMA 270: 2957–2963) and the Simplified Acute

30   Physiology (SAPS) score (Le Gall et al, 1984, Crit Care Med 12: 975–977) also being widely used general predictive models. For more severe conditions, including sepsis, more specialised models such as the Multiple Organ Dysfunction Score (MODS) (Marshall *et al*, 1995, Crit Care Med 23: 1638–1652), the Sequential Organ Failure Assessment (SOFA) score (Ferreira et al, 2002, JAMA 286: 1754–1758) and the Logistical Organ Dysfunction Score (LODS) (Le Gall et al, 1996, JAMA 276: 802–810) were developed. More recently, a specific model, PIRO (Levy et al, 2003, Intensive Care Med 29: 530–538), has been proposed. All of these models use a combination of a wide

4

range of general and specific clinical measures to attempt to derive a useful score reflecting the seriousness of the patient's condition and likely outcome.

In addition to the standard predictive models described above, the correlation of sepsis and a number of specific serum markers has been extensively studied with a view to developing specific diagnostic and prognostic tests, amongst which are the following.

C-reactive protein (CRP) is a liver-derived serum acute phase protein that is well-known as non-specific marker of inflammation. More recently (Toh *et al*, 2003, Intensive Care Med 29: 55–61) a
10   calcium dependent complex of CRP and very low density lipoprotein (VLDL), known as lipoprotein complexed C-reactive protein (LCCRP), has been shown to be involved in affecting the coagulation mechanism during sepsis. In particular, a common test known as the activated partial thromboplastin time develops a particular profile in cases of sepsis, and this has been proposed as the basis for a rapid diagnostic test.

TNF-α and IL-1 are archetypal acute inflammatory cytokines long known to be elevated in sepsis (Damas *et al*, 1989, Critical Care Med 17: 975–978) and have reported to be useful predictors of organ failure in adult respiratory distress syndrome, a serious complication of sepsis (Meduni *et al*, 1995, Chest 107: 1062–1073)
20
Activated complement product C3 (C3a) and IL-6 have been proposed as useful indicators of host response to microbial invasion, and superior to pyrexia and white blood cell counts (Groeneveld *et al*, 2001, Clin Diagn Lab Immunol 8: 1189–1195). Secretary phospholipase A_2 was found to be a less reliable marker in the same study.

Procalcitonin is the propeptide precursor of calcitonin, serum concentrations of which are known to rise in response to LPS and correlate with IL-6 and TNF-α levels. Its use as a predictor of sepsis has been evaluated (Al-Nawas *et al*, 1996, Eur J Med Res 1: 331–333). Using a threshold of 0.1ng /ml, it correctly identified 39% of sepsis patients. However, other reports suggest that it is
30   less reliable than the use of serial CRP measurements (Neely *et al*, 2004, J Burn Care Rehab 25: 76–80), although superior to IL-6 or IL-8 (Harbarth *et al*, Am J Resp Crit Care Med 164: 396–402).

Changes in neutrophil surface expression of leukocyte activation markers (such as CD11b, CD31, CD35, L-selectin, CD16) have been used as a marker of SIRS and have been found to correlate with IL-6 and subsequent development of organ failure (Rosenbloom *et al*, 1995, JAMA 274: 58–65). Similarly, expression of platelet surface antigens such as CD63, CD62P, CD36 and CD31 have been examined, but no reliable predictive model constructed.

Finally, it has been shown that downregulation of monocyte HLA-DR expression is a predictor of a poor outcome in sepsis and may be an indication of monocyte deactivation, impairing TNF-α production. Treatment with IFN-γ has been shown to be beneficial in such cases (Docke *et al*, 1997, Nature Med 3: 678–681).

However, although many of these markers correlate with sepsis and some give an indication of the seriousness of the condition, no single marker or combination markers has yet been shown to be a reliable diagnostic test, much less a predictor of the development of sepsis. The 2001 International
10   Sepsis Definition Conference concluded that "the use of biomarkers for diagnosing sepsis is premature" (Levy *et al*, 2003, Intensive Care Med 29: 530–538).

Extracting reliable diagnostic patterns and robust prognostic indications from changes over time in complex sets of variables including traditional clinical observations, clinical chemistry, biochemical, immunological and cytometric data requires sophisticated methods of analysis. The use of expert systems and artificial intelligence, including neural networks, for medical diagnostic applications has been being developed for some time (Place *et al*, 1995, Clinical Biochemistry 28: 373–389; Lisboa, 2002, Neural Networks 15: 11–39). Specific systems have been developed in attempts to predict survival of sepsis patients (Flanagan *et* al, 1996, Clinical Performance & Quality Health
20   Care 4: 96–103) by use of multiple logistic regression and neural network models using APACHE scores and the 1991 ACCP/SCCM SIRS criteria described above (Bone *et al*, 1992, Crit Care Med 20: 864–874). Such studies suggest that, although both approaches can give good predictive results, neural network systems are less sensitive to preselected threshold values (results of a number of studies reviewed by Rosenberg, 2002, Curr Opin Crit Care 8:321–330). Brause *et al* (2004, Journal für Anästhesie und Intensivbehandlung 11: 40–43) provides an example of a neural network model being used for sepsis prediction. This model (MEDAN) analysed a range of standard clinical measure and compared its results with those obtained by using the APACHE II, SOFA, SAPS II, and MODS models. The study concluded that, of the markers available, the most informative were systolic and diastolic blood pressure, and platelet count.

30

Neural networks are non-linear functions that are capable of identifying patterns in complex data systems. This is achieved by using a number of mathematical functions that make it possible for the network to identify structure within a noisy data set. This is because data from a system may produce patterns based upon the relationships between the variables within the data. If a neural network sees sufficient examples of such data points during a period known as "training", it is capable of "learning" this structure and then identifying these patterns in future data points or test data. In this way, neural networks are able to predict or classify future examples by modelling the

patterns present within the data it has seen. The performance of the network is then assessed by its ability to correctly predict or classify test data, with high accuracy scores, indicating the network has successfully identified true patterns within the data. The parallel processing ability of neural networks is dependent on the architecture of its processing elements, which are arranged to interact according to the model of biological neurones. One or more inputs are regulated by the connection weights to change the stimulation level within the processing element. The output of the processing element is related to its activation level and this output may be non-linear or discontinuous. Training of a neural network therefore comprises an adjustment of interconnected weights depending on the transfer function of the elements, the details of the interconnected

10   structure and the rules of learning that the system follows (Place *et al*, 1995, Clinical Biochemistry <u>28</u>: 373–389). Such systems have been applied to a number of clinical situations, including health outcomes models of trauma patients (Marble & Healy (1999) Art Intell Med <u>15</u>: 299–307).

Warner *et al* (1996, Ann Clin Lab Sci <u>26</u>: 471–479) describe a multiparametric model for predicting the outcome of sepsis, using measures of 'septic shock factor' (which appears to be simply whether the patients have signs of septic shock on admission), IL-6, soluble Il-6 receptor (as measured by enzyme-linked immunosorbent assay) and the APACHE II score as components of a four-input algorithm in a multi-layer, feed-forward neural network model. However, this system is not predictive for individuals who do not yet have clinical signs and, arguably, by the time serum

20   levels of cytokines such as IL-6 are raised, the diagnosis, if not the outcome, is clinically obvious.

Dybowski *et al* (1996, Lancet <u>347</u>: 1146–1150) use Classification and Regression Trees (CART) to select inputs from 157 possible sepsis prediction criteria and then use a neural network running a genetic algorithm to select the best combination of predictive markers. These include many routine clinical values and proxy indicators rather than serum or cell surface biomarkers. However, the problem being addressed is the prognosis of patients who already have a clear diagnosis of sepsis and are already critically ill.

A further refinement of the genetic algorithm approach involves the use of Artificial Immune

30   Systems, of which one version is the Artificial Immune Recognition System (AIRS) (Timmis *et al*, An overview of Artificial Immune Systems. In: Paton, Bolouri, Holcombe, Parish and Tateson (eds.) *"Computation in Cells and Tissues: Perspectives and Tools for Thought"*, Natural Computation Series, pp51–86, Springer, 2004; Timmis (L.N. De Castro and J, Timmis. *Artifical Immune Systems: A New Computational Intelligence Approach*. Springer-Verlag, 2002).which are adaptive systems inspired by the clonal selection and affinity maturation processes of biological immune systems as applied to artificial intelligence.

Immunologically speaking, AIRS is inspired by the clonal selection theory of the immune system (F. Burnett. *The Clonal SelectionTheory of Acquired Immunity.* Cambridge University Press, 1959). The clonal selection theory attempts to explain that how, through a process of matching, cloning, mutation and selection, anti-bodies are created that are capable of identifying infectious agents. AIRS capitalises on this process, and through a process of matching, cloning and mutation, evolves a set of memory detectors that are capable of being used as classifiers for unseen data items. Unlike other immune inspired approaches, such as negative selection, AIRS is specifically designed for use in classification, more specifically one-shot supervised learning.

10   US patent application 2002/0052557 describes a method of predicting the onset of a number of catastrophic illnesses based on the variability of the heart-rate of the patient. Again, a neural network is among the possible methods of modelling and analysing the data.

International patent application WO 00/52472 describes a rapid assay method for use in small children based on the serum or neutrophil surface levels of CD11b or 'CD11b complex' (Mac-1, CR3). The method uses only a single marker, and one which is, arguably, a well-known marker of neutrophil activation in response to inflammation.

The alternative approach to analysing such complex data sets where the data are often qualitative and discrete, rather than quantitative and continuous, is to use sophisticated statistical analysis

20   techniques such as logistic regression. Where logistic regression using qualitative binary dependent variables is insufficiently discriminating in terms of selecting significant variables, multivariate techniques may be used. The outputs from both multiple logistic regression models and neural networks are continuously variable quantities but the likelihoods calculated by neural network models usually fall at one extreme or the other, with few values in the middle range. In a clinical situation this is often helpful and can give clearer decisions (Flanagan *et* al, 1996, Clinical Performance & Quality Health Care 4: 96–103).

The ability to detect the earliest signs of infection and / or sepsis has clear benefits in terms of allowing treatment as soon as possible. Indications of the severity of the condition and likely

30   outcome if untreated inform decisions about treatment options. This is relevant both in vulnerable hospital populations, such as those in intensive care, or who are burned or immunocompromised, and in other groups in which there is an increased risk of serious infection and subsequent sepsis. The use or suspected use of biological weapons in both battlefield and civilian settings is an example where a rapid and reliable means of testing for the earliest signs of infection in individuals exposed would be advantageous.

Statement of Invention

Following infection, cells of the immune system recognise and respond to a pathogen by becoming activated. This results in the production of different messenger proteins (e.g. cytokines and chemokines) and expression of activation markers and adhesion molecules on the cell surface (Figure 1). The production of these facilitates communication between cells and results in a co-ordinated immune response against a particular agent. Since this inflammatory immune response is relatively constant in response to infection, and occurs in the very earliest stages of the disease process, monitoring changes in the expression of such markers predict the early stages of sepsis development. It is an object of the invention to provide a means of detecting serious infections at

10 an early stage, preferably, during the therapeutic window of intervention, prior to the onset of clinical symptoms and disease (Figure 1).

In a first aspect, the invention describes a system and methods of detecting early signs of infection, SIRS or sepsis several days before clinical signs become apparent. It also provides methods capable of predicting the timing of the clinical course of the condition. The system comprises analysing the results of one or more sets of tests based on biological samples, preferably blood samples. Optionally, other routine clinical measurements may be included for analysis.

The first set of test comprises determining the level of expression of a panel of chemokines and

20 cytokines in blood leukocytes by amplifying cytokine mRNA by reverse transcription polymerase chain reaction (RT-PCR). This group comprises CD178 (FAS-L), MCP-1 (monocyte chemotactic protein-1), TNF-α, IL-1β, IL-6, IL-8, IL-10, INF-α and INF-γ. CD178 is encoded and expressed as a type-II membrane protein, but may be considered as a cytokine since it is cleaved by a metalloprotease to release a soluble homotrimer, soluble FasL or sFasL.

The second set of tests comprises determining cell surface expression of a group of surface markers comprising HLA-DR, CD54, CD11b, CD31, CD69, CD80, CD62L (L-selectin), CD25 and CD97. This may done by any standard method, preferably by labelling the cells with specific, labelled antibodies or fragments thereof and analysing by flow cytometry.

30

A third set of tests that may be included in the analysis comprise routine clinical data including temperature, heart rate, total and differential white blood cell count (monocytes, lymphocytes, granulocytes, neutrophils), platelet count, serum creatinine, urea, lactate, base excess, pO_2, HCO_3^- , and C-reactive protein .

The results of one or more sets of tests are analysed, preferably by means of a neural network program enabling a yes /no prediction of the patient from whom the sample was taken developing sepsis to be calculated.

In the case of a positive prediction, preferably a further analysis is then performed allowing an estimate to be made as to the time to onset of overt clinical signs and symptoms. Alternatively, both analyses may be performed by multivariate logistic regression.

Analysis of the test groups can be performed individually or simultaneously. Preferably clinical

10   data are entered into the neural net as supplementary data to the PCR data. At the same time flow cytometry data can be processed by the neural network. Only one set of data is required for processing through the neural net although there are advantages in inputting one, two or all three data sets as these additional examples help "train" the neural net and improve confidence in the output from the program.

In a further aspect the neural network is used to process pre-recorded clinical data or a database of such data may be used to train the neural network and improve its predictive power.

The method may be used as part of routine monitoring for intensive care patients, where regular

20   blood samples are taken for other purposes. Other hospital patients who may be predisposed to infections and/or sepsis may also be monitored. Such predisposing conditions include inherited or acquired immunodeficiencies (including HIV/AIDS) or immunosuppression (such as general surgery patients, transplant recipients or patients receiving steroid treatment), diabetes, lymphoma, leukaemia or other malignancy, penetrating or contaminated trauma, burns or peritonitis. In another aspect, the method of the invention may be used to screen individuals during an outbreak of infectious disease or alternatively individuals who have been, or who are suspected of having been, exposed to infectious pathogens, whether accidentally or deliberately as the result of bioterrorism or of use of a biological weapon during an armed conflict.

30   The invention therefore provides a method for screening a biological sample to detect early stages of infection, SIRS or sepsis comprising the steps of: detecting expression of a set of informative biomarkers by RT-PCR and/or detecting expression of a set of informative cell surface biomarkers by means of flow cytometry and/or monitoring a set of standard clinical measurements; analysing the results of detection by means of a neural network or multivariate statistical analysis; classifying said sample according to the likelihood and timing of the development of overt infection. Preferably, said biological sample is a blood sample. Further preferably, the results of the RT-PCR and/or the flow cytometry and/or standard clinical measurements are subjected to a first analysis

yielding a prediction as to the probability of SIRS or sepsis developing. In one alternative embodiment, this is expressed as a probability. In an alternative embodiment it is expressed as a binary yes/no result.

Optionally, where the first analysis suggests that SIRS or sepsis are probable (as defined as exceeding a predetermined arbitrary threshold probability, or a 'yes' prediction, said results are subjected to a second analysis to determine the likely time to development of overt clinical signs, or to give an indication of probable severity of the clinical disease.

10   Preferably, the set of informative biomarkers, expression of which is detected by means of RT-PCR consists of at least 4, preferably at least 6, more preferably at least 7, most preferably all selected from the list consisting of CD178 (FasL), MCP-1 (alternatively known as CCL-2), TNFα, IL-1β, IL-6, IL-8, IL-10, INF-α and INF-γ .

Alternatively, the set of informative cell surface biomarkers the expression of which is detected by means of flow cytometry consists of at least one, preferably at least two and most preferably at least three selected from the list consisting of CD31, HLA-DR, CD54, CD11b, CD62L, CD 25, CD69, CD80 and CD97.

20   Where standard clinical measurement are analysed these are consist of at least one, preferably at least three, more preferably at least five selected from the list consisting of temperature, heart rate, total and differential white blood cell count (monocytes, lymphocytes, granulocytes, neutrophils), platelet count, serum creatinine, urea, lactate, base excess, pO_2, HCO_3^-, and C-reactive protein

In one highly favoured embodiment, said analysis is by means of a neural network. Most preferably it is a multilayered perceptron neural network

Preferably such a neural network is capable of correctly predicting SIRS or sepsis in greater than 70% of cases (determined in trials where such development is not prevented by prophylactic
30   treatment in a control group), more preferably in at least 80% of cases, even more preferably in at least 85% of cases and most preferably in at least 95% of cases. It is preferred that SIRS or sepsis is can be predicted at least one day before the onset of overt clinical signs, more preferably, at least two days, still more preferably at least three days and most preferably more than three days before SIRS or sepsis is diagnosed.

In another favoured embodiment analysis is by means of multivariate statistical analysis, preferably comprising principle component analysis and/or discriminant function analysis. It is more preferred that the multivariate statistical analysis comprises discriminate function analysis.

In a further aspect, the invention provides a system for screening a biological sample to detect early stages of infection, SIRS or sepsis comprising: a means of extracting and purifying RNA from cells obtained from said sample, a thermal cycler or other means to amplify selected RNA sequences by means of reverse transcription polymerase chain reaction (RT-PCR), a means of detecting and quantifying the results of said RT-PCR, a computer-based neural network trained so

10   as to be able to analyse such results and a display means whereby the conclusion of the neural network analysis may be communicated to an operator.
Note: in this aspect the results of the RT-PCR may be analysed using discriminate function analysis, but the neural network is the preferred embodiment.

Alternatively, system comprises: a means of labelling specific cell surface markers on cells obtained from a biological sample and quantifying expression of said markers, a means of detecting and quantifying the results of said labelling and quantification, a computer-based neural network trained so as to be able to analyse such results and a display means whereby the conclusion of the neural network analysis may be communicated to an operator. Preferably, the

20   labelling is by means of labelled antibodies or antibody fragments and the quantification is by means of fluorescence-activated cell sorting (FACS) or other form of flow cytometry.

Preferably, said system comprises means for both RT-PCR and FACS analysis and more preferably can also analyse other routine clinical test results, integrating all such results into its analysis.

In a further aspect the invention provides analysis according to any of embodiment of the method described above for the preparation of a diagnostic means for the diagnosis of SIRS, sepsis or infection, or the use of the system described above for the preparation of a diagnostic means for

30   the diagnosis of infection.

Also provided is a method of early diagnosis of SIRS, infection or sepsis according to the method as described above.

Detailed description of the invention

The invention will be described in further detail with reference to the following Figures and
Examples

Figure 1: Following infection, cells of the immune system recognise and respond to a pathogen by
becoming activated. This results in the production of different messenger proteins (e.g. cytokines
and chemokines) and expression of activation markers and adhesion molecules on the cell
surface. The production of these facilitates communication between cells and results in a co-
10   ordinated immune response against a particular agent. Since this inflammatory immune response
is relatively constant in response to infection, and occurs in the very earliest stages of the disease
process, monitoring changes in the expression of such markers can be used to predict the early
stages of sepsis development. Ideally this is done during the therapeutic window of intervention,
prior to the onset of clinical symptoms and disease.

Figure 2: A plot of the CD31 expression measured on granulocytes by flow cytometry. From
blood samples taken from patients three days before diagnosis of sepsis (n=6), and in ICU patients
who did not go on to develop sepsis (n=24). Each symbol represents a measurement from one
patient.

20

Figure 3: **Design of neural network analysing clinical data according to Table 4, model 2.**
WCC= white cell count, CRP= C-reactive protein.

Figure 4: **Change in cytokine profile obtained following** *in vitro* **blood infection with** *S.*
aureus. Data from blood taken from three volunteers as detailed in Example 8.

Figure 5: **Results of neural network analysis of** *S. aureus in vitro* **sepsis model.**

Example 1: **Prediction of sepsis by neural network analysis of cytokine expression,**
30   **cell surface markers and clinical measures.**

Study design and patients
The study into the onset of sepsis from the ICU department of Queen Alexandra hospital resulted
in a cohort of ninety-one patients (Dstl/CR08631). Blood samples were collected daily from these
patients throughout their stay in the ICU and in total, twenty-four patients were diagnosed as
developing sepsis. Samples taken on the day clinical sepsis was diagnosed (Day 0), back through
to six days prior to sepsis diagnosis (Day -6) were analysed by RT-PCR and flow cytometry for the

expression of activation markers and cytokine mRNA respectively. In addition, standard hospital data and clinical observations were recorded. Samples from control patients were also processed in the same manner to provide data for traditional statistical analysis.

RT-PCR was performed according to commonly-used laboratory techniques. Briefly, in the case of a blood sample, whole blood was taken and cells then lysed in the presence of an RNA stabilising reagent. RNA was separated by affinity binding of beads, which were isolated by centrifugation (or magnetically, as appropriate), contaminating DNA removed by DNase digestion and the RNA subjected to RT-PCR.

10

Fluorescence activated cell sorting (FACS) flow cytometry is very well-known in the art and any standard technique may be used.

Data analysis

The complexity of biological systems and intricate relationships between the markers used in this study caused standard linear techniques of data analysis to give inconclusive results. Consequently it was unclear whether any patterns existed in the data and a more powerful technique, capable of non-linear modelling, was sought to cope with the complexity of the data sets.

20

For analysis, data was collated from patients 1 to 4 days prior to the onset of sepsis and compared with an age/sex matched control group consisting of ICU patients who did not develop sepsis. Individual samples provided data measuring up to 56 different parameters and selective combinations of variables were fed into a multi-layered perceptron neural network (Proforma, Hanon Solutions, Glasgow, Scotland).

Each network was trained with a random 70% selection of balanced sepsis and control data using back propagation algorithms and then tested with the remaining 30% of the data. Five attempts were made at modelling the data within this network, each model differing in its ability to generalise to the data. The most successful model was the one most capable of correctly classifying previously unseen patients as being from either the sepsis or non-sepsis control group.

30

Results

Table 2 shows an example of a successful model that classified or "scored" 29/35 (or 82.9%) test patients correctly.

Table 2. Classification readout using cytokine mRNA variables (Days 1 to 4)

	Score	% of sample correctly predicted
Total patients	29/35	82.9%
Control patients	16/20	80.0 %
Sepsis patients	13/15	86.7%

To increase confidence in this model, this was carried out five times, each time using a different random selection of data for which to train and test the network. Once completed, the scores for the individual models were averaged to give an overall indication of the networks ability to classify patients into the correct sepsis or non-sepsis control group.

A series of 5 datasets gives a mean accuracy of prediction of approximately 80%, as shown by Table 3 below

Table 3: neural network predicting sepsis using RT-PCR data only (Classification Performance Analysis of 5 projects)

	Hits/Occurred	%	Hits/Predicted	%	Chance %	Improvement %	Ratio
MODEL 1							
Condition	23/27	85.2	N/A	N/A	50.0	35.2	1.7:1
Sepsis	9/13	69.2	9/9	100.0	48.1	51.9	2.1:1
Control	14/14	100.0	14/18	77.8	51.9	25.9	1.5:1
MODEL 2							
Condition	29/35	82.9	N/A	N/A	50.0	32.9	1.7:1
Sepsis	13/15	86.7	13/17	76.5	42.9	33.6	1.8:1
Control	16/20	80.0	16/18	88.9	57.1	31.7	1.6:1
MODEL 3							
Condition	32/40	80.0	N/A	N/A	50.0	30.0	1.6:1
Sepsis	20/22	90.9	20/26	76.9	. 55.0	21.9	1.4:1
Control	12/18	66.7	12/14	85.7	45.0	40.7	1.9:1
MODEL 4							
Condition	21/26	80.8	N/A	N/A	50.0	30.8	1.6:1
Sepsis	9/12	75.0	9/11	81.8	46.2	35.7	1.8:1
Control	12/14	85.7	12/15	80.0	53.8	26.2	1.5:1

MODEL 5							
Condition	23/29	79.3	N/A	N/A	50.0	29.3	1.6:1
Control	12/15	80.0	12/15	80.0	51.7	28.3	1.5:1
Sepsis	11/14	78.6	11/14	78.6	48.3	30.3	1.6:1

128 / 157 = **81.5%**

79.3 + 85.2 + 80 + 82.9 + 80.8 = 408.2 / 5 = **81.64%**

Table 4 lists the averaged prediction accuracy values for a range of networks constructed using differing combinations of variables.

The most successful model was constructed using cytokine mRNA expression combined with CD31 % expression from the flow cytometry data (average 81.0% accuracy, Table 3, model 1) with clinical data also scoring highly (80.4%, Table 3, Model 2).

Table 4. The results from the neural network analysis.

Model	Markers	Prediction Accuracy (%)
1	FasL, MCP-1, TNF-α, IL-1β, IL-6, IL-8, IL-10	81.6
2	Creatinine, Monocytes, CRP, Lymphocytes, Temperature, Neutrophils, White Cell Count	80.4
3	FasL, MCP-1, IL-8, White cell count, Temperature, Creatinine	79.0
4	FasL, MCP-1, TNF-α, IL-1β, IL-6, IL-8 & IL-10, %CD31 & Creatinine	78.7
5	FasL, TNF-α, IL-1β, IL-6, IL-8 & IL-10	78.1
6	FasL, MCP-1, TNF-α, IL-1β, IL-6, IL-8, IL-10, Creatinine, Monocytes, CRP, Lymphocytes, Temperature, Neutrophils, White Cell Count	76.0
7	MCP-1, TNF-α, IL-1β, IL-6, IL-8, IL-10	76.0
8	Platelets, HCO3, PO2, Urea, Creatinine, heart rate	70.7

To further test our predictive model, we trained the network on up to 100% of the cytokine data obtained from 1 to 4 days prior to the onset of clinical symptoms. We then selected test data comprising "Day 0" sepsis patients and those from Day -5 and Day -6. day 0, 5 and 6 and also selected 14 control patients from a separate volunteer study, 7 of which developed symptoms of an Upper Respiratory Tract Infection (URTI) within 9 days of sampling (Dstl/CR08631). The results are shown below in table 5.

Table 5. Performance of cytokine mRNA model (Days -4 to -1) in prediction of other groups

Test set	Score	% of sample correctly predicted
Day 0 sepsis	8/9	89%
Day -5 sepsis	7/9	78%
Day -6 sepsis	5/6	83%

This table shows that our model, built from patterns expressed by sepsis patients up to 4 days before the onset of clinical sepsis, correctly identified, or "scored", 89% of day 0 sepsis patients, 78% of Day -5 sepsis patients and 83% of Day -6 sepsis patients. Overall, analysis using neural networks has led to the creation of a number of predictive models for sepsis. Models built using only cytokine data have proved consistently capable of successfully distinguishing between individuals who will develop sepsis from those that will not.

Example 2: Lack of false positive results from non-sepsis volunteers using neural network model

Table 6 shows the results of testing a group of volunteers by cytokine RT-PCR, none of whom developed signs of SIRS or sepsis.

Table 6

Name	Hits/Occurred	%	Hits/Predicted	%	Chance	Improvement	Ratio
Total	13/13	100.0	N/A	N/A	50.0%	50.0%	2.0:1
Control	13/13	100.0	13/13	100.0	100.0%	0.0%	1.0:1
Sepsis	0/0	N/A	0/0	0.0	0.0%	0.0%	N/A

Example 3: Neural network sepsis prediction of more than 90% accuracy using Clinical Data.

Neural network model tested using clinical data set defined in Table 4 model 2, using the parameters as described in Table 7 below and further illustrated in Figure 3:

Table 7: Neural network parameters to analyse clinical data

Weights from input to hidden					
Input	Unit 1	Unit 2	Unit 3	Unit 4	Unit 5
1	-3.04389	0.783085	-8.14579	-5.31918	-11.699
2	-2.40492	-3.28341	6.81119	-2.22889	10.3357
3	2 0.0835039	-2.25136	15.2575	-3.38677	11.4842
4	-16.2918	3.45666	-5.86258	8.93773	-1.57967
5	12.7828	4 5.2618	6.36791	7.45053	17.1156
6	34.3201	7.31471	-18.392	16.806	10.2057
7	-3.31337	-1.41443	-10.2639	-3.68324	1.4483
Weights from bias to hidden					
1	-0.407035	-9.38879	4.06257	-12.6877	-10.7582
Weights from hidden to output					
1	-8.79483	8.82026			
2	8.79794	-8.38064			
3	3.60783	-3.62177			
4	3.00073	-3.83778			
5	4.85609	-4.85559			
Weights from bias to output					
1	-2.64015	2.65373			

Table 8

Name	Hits/Occurred	%	Hits/Predicted	%	Chance	Improvement	Ratio
Condition	14/15	93.3	N/A	N/A	50.0%	43.3%	1.9:1
Sepsis	8/8	100.0	8/9	88.9	53.3%	35.6%	1.7:1
Control	6/7	85.7	6/6	100.0	46.7%	53.3%	2.1:1

Example 4: Use of Artificial Immune Recognition System

10   <u>Representation</u>

The initial AIRS system (A. Watkins. *An Artificial Immune Recognition System*. Mississippi Sate University: MSc Thesis., 2001) employed simple real-value shape space. Recently, other people have extended the representation to Hamming shape space (J. Hanamaker and L. Boggess. The effect of distance metrics on AIRS. In *Proc. Of Congress on Evolutionary Computation (CEC)*. IEEE, 2004) and natural language (D. Goodman, L. Boggess and A. Watkins. "An investigation into the source of power for AIRS, an artificial immune classification system". In *Proc. Int Joint Conference on Neural Networks*, pp1678–1683. IEEE, 2003). AIRS maintains a set of Artificial Recognition Balls (ARBs) that contain a vector of the data being learnt, a stimulation level and a number of resources. During training, the stimulation level is calculated by assessing the affinity of

20   the data vector in the ARB against a training item, the stronger the match, the greater the stimulation. This stimulation level is used to dictate how many clones the ARB will produce, and affects survival of the ARB.

Affinity Measure

This is dependent on the representation employed. A number of affinity measures for use in AIRS have been proposed, including Hamming distance, Euclidean distance and so on. In this study, both Euclidean and Hamming distance metrics were used, with Euclidean giving the best results.

Immune Algorithm

Essentially, AIRS evolves with two populations, a memory pool and an ARB pool **C**. It has a separate training and test phase, with the test phase being akin to a k-nearest neighbour classifier.

10   During the training phase, a training data item is presented to **M**. This set can be seeded randomly, and experimental evidence would suggest that AIRS is insensitive to the initial starting point. The training item is matched against all memory cells in the set **M**, and a single cell is identified as the higher match *MCmatch*. This *MCmatch* is then cloned and mutated. Cloning is performed in proportion to stimulation (the higher the stimulation, the higher the clonal rate), and mutation is inversely proportional (the higher the stimulation, the lower the mutation rate). These clones are inserted into the ARB pool, **C**. The training item is then presented to the members of the ARB pool, where an iterative procedure is adopted which allows for the cloning and mutation of new candidate memory cells. Through a process of population control, where survival is dictated by the number of resources an ARB can claim, a new candidate memory cell is created. This mechanism

20   is based on the resource allocation algorithm proposed in J. Timmis and M. Neal. A Resource Limited Artificial Immune System. *Knowledge Based Systems*m 14(3/4): 121-130, 2001. This new candidate is compared against the *MCmatch*, with the training item. If the affinity between the candidate cell and *MVCmatch* is higher, then the memory cell is replaced with the candidate cell.

This process is performed for each training item, whereupon the memory set will contain a number of cells capable of being used for classification. Classification of an unseen data item is performed in a k-nearest neighbour fashion.

Experimental Setup

30   An attempt was made to use an experimental procedure that was comparable to the application of neural networks to this data set. For all studies, the marks: asL, MCP-1, TNF-α, IL-β, IL-6, IL-8 and IL-10 were used. However, it was not possible to completely reproduce exactly the data set, due to incomplete information regarding the pre-processing of the data during the neural network study.

Experiment One

In the first set of experiments, data collected from patients on days 1 through 4 prior to the onset of sepsis, along with data from a control set of patents as training data were used. Specifically, the combined data from days 1, 2, 3 and 4 for patients who showed signs of sepsis were used, and a random collection of control patients in order to train AIRS. In total, 59 training data items were used. To test AIRS, a random collection of patients from the control group and combined data from all days (excluding data that had been used in the training process) was used. In total, 34 test data points were used. The settings for AIRS are shown in table 9:

10

Table 9: Parameter Settings for AIRS

Parameter	Setting
Epochs Clonal Rate	10
Mutation Rate	0.8
Initial size of population	5
Affinity Threshold	0.2
Stimulation Threshold	0.8
Number of resources	200

Experiment Two

For our second set of experiments, patients were classified who showed signs of sepsis using data for days 0, 5 and 6 and control patients. The AIRS system was trained using the same data as for Experiment One, whilst making use of the same parameters.

Results

The results are not directly comparable with the results obtained from the neural network analysis
20   due to the fact thatit was difficult to ascertain from the original report exactly how the data had been first combined over a period of days, and then divided into training and test sets. Therefore, the results obtained should be considered with this in mind.

Experiment One

Ten independent runs of the AIRS algorithm were run, then the average and standard deviation calculated. It was found that AIRS was capable of achieving on average 73(2.96)% classification accuracy. This is approximately 10% lower than the neural network analysis, (using the same markers). However, care has to be taken with a direct comparison.

Experiment Two

Again, ten independent runs of the AIRS algorithm were undertaken, and the average and standing deviation taken. This time, preceding days (0, 5 and 6) before the onset of sepsis were analysed, and the control group. Again, AIRS was trained on data taken from days 1 through 4 and the control group. These results are presented in Table 10.

Table 10: Prediction in Other Groups (standard deviation in braces)

Test Set	Accuracy
Day 0 sepsis	83(7.6)%
Day 5 sepsis	93(6.1)%
Day 6 sepsis	91(8.2)%
Control	70(12.8)%

As can be seen from Table 10, AIRS identifies a high percentage of sepsis cases (being able to outperform the neural network on day 5 and day 6, but again with the comparative caveat). The control group did not fair as well, being a lower than expected result, and significantly lower than the neural network approach. This may be due to the fact that AIRS has biased towards the sepsis patients due to the larger amount of data available for training with those, than for non-sepsis.

Conclusions

AIRS appears capable of identifying potential cases of sepsis in advance, and comparable at a certain level to neural network approaches.

Example 5: Use of CD31 expression to predict sepsis

Study design and patients

See Example 1.

Flow Cytometry

Blood was collected into sodium heparin containers (HM&S, Chessington, Surrey) and transported to the laboratory at room temperature. 100 µl aliquots of blood were mixed with immunofluorescent stains using the volumes recommended by the manufacturer (Beckman Coulter limited, High Wycombe, Buckinghamshire, and Becton Dickinson UK Limited, Cowley, Oxford). T helper cells were identified by co-staining for CD3 and CD4 and T cytotoxic cells were identified by staining for both CD3 and CD8. These cell populations were stained for HLA-DR, CD25, CD54 and CD69. B cells were identified by staining for CD19 and were interrogated with CD80, CD86, CD25 CD54.

Natural killer cell were distinguished by staining with CD56 and interrogated with CD11b, CD25, CD54 and CD69. The monocyte population was selected by staining for CD14, these cells were probed with CD11b, CD54, CD80, CD86 and HLA-DR stains. Gating was used in order to identify the granulocyte population, which was stained for CD11b, CD69, CD31, CD54 and CD62L. The stains were incubated at room temperature for 20 minutes. 500 µl of Optilyse C (Beckman Coulter limited) was added to each tube and vortex mixed immediately. The samples were incubated at room temperature for 10 minutes to lyse the red blood cells and 500 µl of Isoton (Beckman Coulter limited) were then added in order to fix the stains. The tubes were vortex mixed immediately and incubated at room temperature for 10 minutes. The cells were then counted on a Beckman Coulter
10   Epics XL System 2 Flow Cytometer.

StatisticsData was analysed using a Binary Logistic Regression model on the SPSS software package version 11.0. This analysis compared the control group means for immune modulator expression with the means obtained from the sepsis patients at seven time points: 6 days before diagnosis, 5, 4, 3, 2, and 1 day before diagnosis and 0, on the day of diagnosis of sepsis. Where data points were missing, averaged values for the group were substituted in order to maintain acceptable n values. Results from the model were only reported if the substituted data points did not involve markers that were highlighted by the model as possible predictors.

20   Results

Analysis of the data found that there was weak evidence of a predictor effect = 0.114. Decreased expression of CD31 was indicated to be a possible predictor of sepsis three days before diagnosis p=0.037 (n=6). The results obtained for 6 days before diagnosis were inconclusive because of the small sample size for this date (n=4). There were no statistically significant predictors found for 5, 4, 2 or 1 day before diagnosis, or for the day of diagnosis.

Conclusions
The flow cytometry data obtained from patients prior to the development of sepsis, and from
30   patients who did not develop this disease were collated. Groups were constructed using results from patients in the days before diagnosis of sepsis, with a control group consisting of measurements taken from age matched patients who did not develop sepsis. Examination of bar graphs displaying the medians and 90th and 10th percentiles were difficult to interpret because of the spread of the data and hence statistical analysis was performed.

When the raw data for this is plotted (see Figure 2) it could be seen that 4 out of 6 (66.6 %) of the sepsis patients had CD 31 expression that was lower than that of the control group. It can be seen

that the control group (non sepsis) data points are distributed between 11.8 % and 100 %, while four of the six data points in the three days before diagnosis measurements were less than 9 %. Therefore it is possible that CD31 may therefore be used to predict the onset of sepsis three days prior to the appearance of clinical signs and symptoms. This suggests that CD31 could be a useful predictive marker, particularly in combination with other informative sepsis biomarkers.

Example 6: Multivariate statistical analysis to predict sepsis

Introduction

Multivariate data analysis procedures were applied to data collected from patients 1-6 days prior to

10   development of symptoms of Sepsis. Measurements included flow cytometry, PCR and classical clinical observations. Principle component analysis (PCA) was applied to the data matrix considering each of the three classes of observations individually and combined as a complete data set. Discriminant Function Analysis (DFA) was used to determine whether groups differ with regard to the mean of a variable, and then to use that variable to predict group membership (e.g., of new cases). This was performed on the results from PCA and on the three classes of observations individually and combined as a complete data set.

Data description, manipulation and multivariate techniques

Prior to PCA, the data was summarised by producing probability density functions As normality of distribution is required prior to PCA and DFA, non-normal data were transformed using the

20   Johnson transformation algorithm.

PCA is a dimensionality reducing technique which endeavours to decompose a multivariate data matrix into a few latent variables, composed of linear combinations of the variables, which explain the bulk of the variance of the original matrix. In this way correlations (positive or negative) of parameters within the data set can be established.

Essentially, DFA is similar in approach to Analysis of Variance (ANOVA). The DFA problem can be rephrased as a one-way analysis ANOVA problem. Specifically, one can ask whether two groups are significantly different from each other with respect to the mean of a particular variable.

30   However, it should be clear that, if the means for a variable are significantly different in different groups, then it may be concluded that this variable discriminates between the groups.

In the case of a single variable, the final significance test of whether or not a variable discriminates between groups is the F-test. F is essentially computed as the ratio of the between-groups

variance in the data over the pooled (average) within-group variance. If the between-group variance is significantly larger then there must be significant differences between means.

When considering multiple variables, it is possible to establish which of several variables contribute to the discrimination between groups. This results in a matrix of total variances and covariances; and likewise, a matrix of pooled within-group variances and covariances. These matrices are then compared via multivariate F-tests in order to determine whether or not there are any significant differences (with regard to all variables) between groups. This procedure is identical to multivariate analysis of variance or <u>MANOVA</u>. As in MANOVA, the multivariate test is performed, and, if

10   statistically significant, which of the variables have significantly different means across the groups is examined. Thus, even though the computations with multiple variables are more complex, the principal reasoning still applies, namely, that variables that discriminate between groups are sought, as evident in observed mean differences.

DFA was performed on clinical, flow cytometry and RT-PCR data using the complete data matrix (including substituted mean values) and by exclusion of data points for which one or more parameters contained substituted mean values. Analogous models were developed to allow analysis of PCA scores from models developed in Model 1. The purpose of the latter was to establish if transformed data matrices (PCA) could be used to classify observations.

20   *Model 1. Principle Component Analysis (PCA) of observation data*

        i)   PCA model based on clinical data

The number of PCs derived and used in a given model is usually defined as those having an Eigenvalue of >1. 6 PCs meet this criterion for clinical data and explain a total of 74.3% of the variance of the data set. Since each of the PCs is orthogonal (uncorrelated) with respect to the other PCs, the association of a clinical parameter with a particular PC defines the PC and illustrates how the parameter influences the variance of the data set.

Table 12 summarises the loadings of each parameter with the six derived PCs from the clinical
30   data. Loading values of >0.5 indicate a strong contribution of a particular parameter to a given PC. The PCs derived from the data set may be interpreted as follows:

PC1   this is dominated by the strong correlation of WCC, monocytes, neutrophils and platelets. A strong correlation exists between creatinine and lactate. Both these groups have a negative relationship (opposite ends of PC1 scale) and are therefore negatively correlated. BXS and HCO_3^- are highly correlated and contribute to PC1 and PC2 equally. The latter parameters

are contrasted by creatinine and lactate in PC1. These correlations are summarised in Table 2 and Figure 2

PC2   shows a negative correlation between the group composed of WCC, monocytes, neutrophils and platelets and the group composed of BXS and HCO_3^-

PC3   this PC is characterised by the strong relationship between temp, HR and CRP as shown in Figure 3

10   PC4   although many parameters approach significance for this PC, only CRP is definitively associated with this PC as demonstrated by Figure 4.

PC5 pO_2 is contrasted with both urea and MAP in this component.

PC6   this PC exclusively explains the variance introduced into the data set by lymphocytes.

In interpreting the PC loadings, correlated clinical parameters suggest levels of these species/physical parameters will be elevated or decreased in patients belonging characterised as belonging to PC1, 2 etc. This will be performed in the discriminant analysis section.

20

ii)   *PCA model based on flow cytometry data*

The parameters were abbreviated for clarity and abbreviations listed in Table 13. Nine PCs account for 80.5% of the variance of the data set as shown in the eigenvalue matrix in Table 14 and associated loadings are summarised in Table 15. The correlations between the measured parameters are shown in Table 16 with strong correlations within the derived PCs between the following:

fc 1 and fc 3

fc 5 and fc 6

30   fc 7 and fc 8

fc 9 with fc 10 and fc 11,

fc 12 and fc 13

fc 11 and fc 14

fc 17 with fc 20 and fc 23

fc 21 with fc 17, 20 and 22

fc 23 with fc 20 and fc 22

fc 28 and fc 29

The PC structure may be interpreted as follows:

PC1   correlates CD3 CD4 CD25 in CD3 CD4, CD3 CD4 HLA-DR in CD3 CD4, CD3 CD8 CD25
      in CD3 CD8, CD19 CD80 in CD19, CD19 CD86 in CD19, CD14 CD80 in CD14, CD14
      CD86 in CD14, CD19 CD54 in CD19, CD19 CD25 in CD19 and CD56 CD54 in CD56.

PC2   contrasts CD19 CD86 in CD19 with CD14 HLA-DR in CD14, CD14 HLA-DR CD11B in
      CD14, CD14 HLA-DR CD11B CD54 in CD14.

PC3   CD3 CD4 CD54 in CD3 CD4, CD3 CD4 CD69 in CD3 CD4, CD3 CD8 CD54 in CD3 CD8,
      CD3 CD8 CD69 in CD3 CD8

PC4   CD56 CD69 in CD56, CD69 (%), CD11B CD69 (%)

PC5   CD3 CD8 CD54 in CD3 CD8, CD62L (%)

PC6   CD31 (%)

PC7   no significant components with EV>0.5

PC8   CD54 (%)

PC9   CD14 CD11B in CD14

By considering only one parameter of a pair or group, it would be possible to remove 9 parameters
thus increasing the y component of the data matrix. However, it was decided that CD31 (%), CD54
(%), CD62L (%), CD11B (%), CD69 (%) and CD11B CD69 (%) only be subjected to statistical
analysis (fc 24-fc 29).

The eigenvalue matrix of the selected flow cytometry variables is shown in Table 17 and loadings
of the PCA model constructed summarised in Table 18. The use of 3 PCs explains 76.6% of the
variance of the data set. The PC model shows the following:

PC1   correlates CD69 (%) and CD11B CD69 (%)

PC2   correlates CD31 (%), CD62L (%) and CD11B (%)

PC3   is composed of the variance associated with CD54 (%)

iii)   *PCA model based on RT-PCR data*

Table 19 indicates that 72.9% of the variance of the RT-PCR data is explained by only 3 PCs. The
loading for this model are shown in Table120. The correlation of variables with each PC are shown
in Figures 23 and 24 and reveal the following:

PC1   correlates Fas-L, MCP-1, TNF-alpha, IL-6 and Il-8
PC2   correlates IL-1 and IL 10
PC3   contrasts IL-1 and IL-10

The correlation of IL-1 and IL-10 in PC2 and subsequent contrast of these variables in PC3 is interesting. It appears that in some patients these vairables may be highly correlated or contrasted possibly providing a powerful means of discriminating patients.

iv)   *PCA model based on combined clinical, flow cytometry and RT-PCR data*

Table 21 summarises the parameters included in this final model and the associated Eigenvalues for the correlation matrix. Table 21 indicates that 9 PCs have an Eigenvalue greater than 1 which explain 68.7% of the data variance. The loadings of the model are shown in Table 22. Analysis allows the following interpretation of the PCA model:

PC1   shows positive correlation between WCC, Neutrophils, Monocytes, APTR, HCO3-, BXS, Platelets, CD69 (%) and CD11B CD69 (%). This PC also contrasts the above with Lactate and Creatinine which are correlated.

PC2   correlates CD69 (%), CD11B CD69 (%), WCC, Neutrophils, Monocytes and INR. These parameters are contrasted with TNF-alpha.

PC3   strongly correlates the PCR parameters Fas-L, MCP-1, TNF-alpha, IL-6 and Il-8

PC4   contrasts CRP and IL-10.

PC5   correlates the flow cytometry parameters CD31 (%),CD54 (%),CD62L (%) and CD69 (%).

PC6   correlates CD62L (%) and HR.

PC7   is associated with Temp.

PC8   is associated with IL-1

PC9   is associated with PO2

Model 2: Discriminant Function Analysis (DFA) based on observations and PCA score data

The terminology common to all model definition in the DFA models developed is explained below and numerical values shown in Table 23.

Model

The object of the analysis is to build a "model" of how to best predict to which group a case belongs. In the following discussion the term "in the model" will be used in order to refer to

variables that are included in the prediction of group membership, and "not in the model" if they are not included.

- **Forward stepwise analysis**

In stepwise discriminant function analysis, a model of discrimination is constructed step-by-step. Specifically, at each step all variables are reviewed and evaluated to establish which one will contribute most to the discrimination between groups. That variable will then be included in the model.

10   - **Backward stepwise analysis**

It is possible to step backwards; in that case the programme first includes all variables in the model and then, at each step, eliminates the variable that contributes least to the prediction of group membership. Thus, as the result of a successful discriminant function analysis, one would only keep the "important" variables in the model, that is, those variables that contribute the most to the discrimination between groups.

- **F to enter, F to remove**

The stepwise procedure is "guided" by the respective F to enter and F to remove values. The F value for a variable indicates its statistical significance in the discrimination between groups, that

20   is, it is a measure of the extent to which a variable makes a unique contribution to the prediction of group membership. In general, the programme continues to choose variables to be included in the model, as long as the respective F values for those variables are larger than the user-specified F to enter; and excludes (removes) variables from the model if their significance is less than the user-specified F to remove.

- **Tolerance**

The tolerance value of a variable is computed as $1-R^2$ of the respective variable with all other variables in the model. Thus, the tolerance is a measure of the respective variable's redundancy. For example, a tolerance value of .10 means that the variable is 90% redundant with the other

30   variables in the model.

- **Wilks' λ**

This parameter gives a measure of the discriminatory power of the model and can assume values in the range of 0 (perfect discrimination) to 1 (no discrimination).

- Partial λ

This is the Wilks' λ associated with the unique contribution (measured orthogonally) of the respective variable to the discriminatory power of the model.

As a point of note, a common misinterpretation of the results of stepwise discriminant analysis is to take statistical significance levels at face value. When the programme decides which variable to include or exclude in the next step of the analysis, it actually computes the significance of the contribution of each variable under consideration. Therefore, by nature, the stepwise procedures will capitalize on chance because they "pick and choose" the variables to be included in the model
10   so as to yield maximum discrimination. Thus, when using the stepwise approach awareness that the significance levels do not reflect the true alpha error rate, that is, the probability of erroneously rejecting H0 (the null hypothesis that there is no discrimination between groups) must be maintained

- Canonical Correlation Analysis (CCA)

This is an additional procedure for assessing the relationship between variables. Specifically, this manipulation allows the elucidation of the relationship between two sets of variables. Parameters which characterise this analysis are detailed below.

20   - Significance of roots (χ^2 test)

The term root is used to describe the individual discriminant functions (DFs). The statistical significance of the derived DFs, is tested by the χ^2 test of successive DFs. A report of the step-down test of all canonical roots is obtained containing the significance of all DFs followed by the second line which reports the significance of the remaining roots, after removing the first root, and so on. Thus the number of DFs to interpret is obtained.

- Discriminant function coefficients

Two outputs are produced, one for the Raw Coefficients and one for the Standardized Coefficients. Raw here means that the coefficients can be used in conjunction with the observed data to
30   compute (raw) discriminant function scores. The standardized coefficients are the ones that are customarily used for interpretation, because they pertain to the standardized variables and therefore refer to comparable scales.

• Eigenvalues

An Eigenvalue for each DF and the cumulative proportion of explained variance accounted for by each function is obtained. This value is defined in an identical way in PCA and DFA. The larger the value, the greater the amount of variance explained by that DF.

• Factor structure coefficients

These coefficients represent the correlations between the variables and the DFs and are commonly used in order to interpret the "meaning" of discriminant functions. In an analogous way to PCA, the interpretation of factors should be based on the factor structure coefficients.

• Means of canonical variables

When knowledge of how the variables participate in the discrimination between different groups is obtained, the next logical step is to determine the nature of the discrimination for each DF. The first step to answer this question is to look at the canonical means. The larger the canonical mean for a given DF and group of observations, the greater the discriminatory power of that DF.

i)   DFA model based on PCA scores of clinical data

a)   Containing substituted mean values

Table 19 summarises the results of this analysis. The Wilks' λ value of 0.4 indicates a relatively inefficient classification model. The three derived DFs account for a total of 89.9% of the variance of the data set and the DFs are composed mainly of PCs 1, 3 and 4. The factor structure coefficients indicate that:

• DF1 is composed of the variance explained by PC1 and to a lesser extent with PC4

• DF2 is exclusively composed of the variance explained by PC3

• DF3 is composed of the variance explained by PC4

These correlations are confirmed by the standardised coefficients. The means of cannonical variables indicate that:

• DF1 negatively correlates days 1, 2 and 3 with days 5 and 6

• DF2 defines control group observations

• DF3 defines observations for day 2

A summary of the classification of this model and its discriminative nature in relation to the PCs is shown in Table 25. The classification matrix for the model is shown in Table 26. Table 26 suggests a good classification can be obtained for control and 6 day data with 80 and 83 % respectively of

observations being classified correctly. However the overall classification power of the model is poor with only 48 % of all observations being correctly classified.

b)   Excluding substituted mean values

Table 24 summarises the results of this analysis. The Wilks' λ value of 0.45 indicates a relatively inefficient classification model. The three derived DFs account for a total of 95 % of the variance of the data set and the DFs are composed mainly of PCs 1, 3 and 5. The factor structure coefficients indicate that:

10

- DF1 is composed of the variance explained by the negative correlation between PC1 and PC5

- DF2 is composed of the variance explained by PC3

- DF3 is composed of the variance explained by PC3 but to a lesser degree than DF2

These correlations are confirmed by the standardised coefficients. The means of cannonical variables indicate that:

20   - DF1 negatively correlates days 1, 2 and 3 with days 5 and 6

- DF2 negatively correlates days 1 and 6 with the control group

- DF3 defines observations for day 6

A summary of the classification of this model and its discriminative nature in relation to the PCs is shown in Table 25. The classification matrix for the model is shown in Table 26. Table 26 suggests a good classification can be obtained for control and 6 day data with 83 and 67 % respectively of observations being classified correctly. However the overall classification power of the model is 30   poor with only 44 % of all observations being correctly classified, less than that using mean substituted variables.

The similar prediction efficiency with and without mean substituted values validated the subsequent approach to perform DFA with the inclusion of these values.

ii)   DFA model based on transformed values of clinical data

In an effort to improve the classification of observations, the transformed variable values from the original data matrix were subjected to DFA. The thesis was that since PCA is a dimensionality

reducing technique, perhaps some data quality is lost and performing DFA on PCA scores leads to a model with less predictive power.

Table 27 summarises the results of this analysis. The Wilks' λ value of 0.22 is an improvement on the PCA scores classification models. The five derived DFs account for a total of 99 % of the variance of the data set and the DFs are composed of BXS, CRP, lactate, urea, temperature, creatinine, neutrophils, pO_2 and HCO_3^- with the other clinical variable having no influence on the classification of observations. The factor structure coefficients indicate that:

10 • DF1 classifies the correlation between BXS and HCO_3^- which are negatively correlated with lactate

    • DF2 classifies observations showing a high degree of correlation between BXS, CRP and HCO_3^-

    • DF3 classifies samples with a negative correlation between temperature and creatinine

    • DF4 classifies samples with a negative correlation between temperature and PO2

20 • DF5 classifies samples with a negative correlation between urea and neuts

A summary of the classification of this model and its discriminative nature in relation to the clinical variables is shown in Table 31. The classification matrix for the model is shown in Table 32. Table 32 suggests a good classification can be obtained for control and 6 day data with 80 and 83 % respectively of observations being classified correctly. Days 1, 2 and 5 are greatly improved compared to the PCA scores models but the overall classification power of the model is poor with 55 % of all observations being correctly classified.

30 *iii)   DFA model based on PCA scores of flow cytometry*

Table 33 summarises the results of this analysis. The Wilks' λ value of 0.39 indicates a relatively inefficient classification model. The two derived DFs account for a total of 71% of the variance of the data set and the DFs are composed mainly of PCs 1, 5 and 5. The factor structure coefficients indicate that:

    • DF1 is composed of the variance explained by PC1 and PC8

- DF2 is exclusively composed of the variance explained by PC5

These correlations are confirmed by the standardised coefficients. The means of cannonical variables indicate that:

- DF1 negatively correlates day 1 with days 5 and 6

- DF2 defines day 3 observations

10

A summary of the classification of this model and its discriminative nature in relation to the PCs is shown in Table 34. The classification matrix for the model is shown in Table 35. Table 35 suggests a reasonable classification can be obtained for control and 6 day data with 66% of observations being classified correctly in both groups. However the overall classification power of the model is poor with only 44 % of all observations being correctly classified.

iv)   DFA model based on flow cytometry data

A summary of the classification of this model and its discriminative nature in relation to the variables is shown in Table 36. The Wilks' λ value of 0.034 indicates an excellent classification

20   model. The three derived DFs account for a total of 74% of the variance of the data set and the DFs are composed mainly of fc7-8, fc11, fc16, fc25, fc28, fc29. The factor structure coefficients indicate that:

- DF1 correlates fc7, 16, 28 and 29 and contrasts these to fc8 and 25

- DF2 correlates fc7, 8, 16 and 25 and contrasts these with fc12

- DF3 is correlated with fc11

30   Table 37 summarises this information.

These correlations are confirmed by the standardised coefficients. The means of cannonical variables indicate that:

- DF1 contrasts 1 day with days 5 and 6

- DF2 contrasts the control group with days 3, 4 and 5 and correlates the control with day 6

- DF3 contrasts days 2 and 3 with day 4

Table 38 suggests a good classification can be obtained all groups. The overall classification power of the model is impressive with 76.6% of all observations being correctly classified.

The DFA models for RT-PCR were so poor for both PCA scores and transformed data, with The Wilks' λ values >0.8, they were discarded and will not be considered further.

10   v)   *DFA model based on combined clinical, flow cytometry and RT-PCR data*

A summary of the classification of this model and its discriminative nature in relation to the variables is shown in Table 39. The Wilks' λ value of 0.0087 indicates an excellent classification model. The four derived DFs account for a total of 89.2% of the variance of the data set and the DF factor structure indicates that:BXS, fc 25, fc 22, fc 11, Temp, CRP, fc 18, fc 6, IL-6, INR, APTR, fc 16, Urea, Lactate, Fas-L, fc 13, fc 24, fc 1, fc 3, MCP-1, fc 28, Il-10, fc 27, fc 26, Neutrophils, fc 14, WCC, fc 29, Platelets, pO_2 are included in the model. All other parameters fail to meet the stepwise criteria and hence were eliminated from the model.

The means of canonical variables indicate that:

20

- DF1 contrasts 1 day with days 5 and 6

- DF2 correlates the control group with day 6 and contrasts these with days 3, 4 and 5

- DF3 correlates the control group with day 5 and contrasts these to days 2, 3 and 6

- DF4 contrasts days 4 and 5

Table 40 shows an excellent classification can be obtained for all groups with a minimum correct 30   assignment rate of 76%. The overall classification power of the model is impressive with 86.9% of all observations being correctly classified.

When each DF is applied to the data using this model,the groups of patients can clearly be seen to cluster and are spatially separated from the other groups.

Conclusions

PCA has highlights correlations between measured variables for all classes of patients. Many of the correlations are expected from a molecular biology standpoint. Some of the PCA models greatly reduced the dimensionality of the data set but the resulting scores did not spatially separate the groups of patients.

40

DFA on scores obtained from PCA showed disappointing results. The discriminatory power of the models ranged from 44 – 56 % when PCA scores were used. The low discriminatory power of these models may be a result of the reduction in dimensionality of the data set during PCA with significant detail being lost. Using transformed variables in DFA gave much improved models. The discriminatory power of the clinical and flow cytometry models was 55 and 76 % respectively. When DFA was performed on the complete data set (clinical, flow cytometry and RT-PCR variables) a prediction efficiency of 86.9% was observed. Therefore it is recommended that the variables included in this latter model (Table 36) be measured and used to classify new patients suspected of being susceptible to sepsis.

10

The most impressive feature of the model is its ability to correctly assign patience correctly 6 days before the onset of symptoms. Therefore key discriminatory variables could be monitored and threshold levels established at which medical treatment must be administered. Using the parameters shown in Table 34 it is possible to acquire data from patients and using transformation algorithms input the data into the DFA model. This is then capable of classifying patients into the appropriate groups with an efficiency of approaching 90%. This could of great value when used in clinical laboratories.

Table 11. Eigenvalues of correlation matrix, and related statistics for clinical observations

PC	Eigenvalue	% Total variance	Cumulative Eigenvalue	Cumulative variance %
1	3.73	24.87	3.73	24.87
2	2.50	16.70	6.23	41.58
3	1.53	10.23	7.77	51.81
4	1.22	8.14	8.99	59.95
5	1.10	7.39	10.10	67.35
6	1.04	6.96	11.14	74.31
7	0.87	5.80	12.01	80.11
8	0.77	5.19	12.79	85.31
9	0.66	4.42	13.46	89.73
10	0.54	3.62	14.00	93.36
11	0.44	2.99	14.45	96.35
12	0.29	1.95	14.74	98.30
13	0.210	1.40	14.95	99.70
14	0.02	0.16	14.98	99.87
15	0.01	0.12	15.00	100.00

20

Table 12. Loadings of clinical measurements for each PC. (associations with Eigenvalues >0.5 shown for 95% CL).

Clinical measurement	PC1	PC 2	PC 3	PC 4	PC 5	PC 6
Temp	0.14	0.08	**0.60**	-0.36	0.14	0.40
HR	0.15	-0.06	**0.58**	-0.48	0.11	-0.37
MAP	0.22	0.21	-0.29	-0.48	**-0.54**	-0.14
WCC	**0.63**	**-0.74**	0.00	0.05	0.02	-0.01
Neuts	**0.58**	**-0.75**	0.01	0.17	0.00	0.05
Lymphocytes	0.35	0.00	-0.14	-0.16	-0.19	**-0.62**
Monocytes	**0.62**	**-0.66**	0.08	-0.05	0.01	-0.01
Platelets	**0.53**	-0.09	-0.37	0.05	0.22	0.04
CRP	-0.15	0.11	**0.62**	**0.50**	0.08	-0.36
PO2	-0.26	0.07	-0.36	0.12	**0.53**	-0.39
HCO3-	**0.76**	0.47	0.16	0.23	-0.13	-0.07
BXS	**0.78**	0.49	0.15	0.20	-0.11	-0.05
Lactate	**-0.55**	-0.46	0.16	-0.12	-0.08	-0.22
Urea	-0.30	-0.17	0.09	0.44	**-0.58**	0.04
Creatinine	**-0.69**	-0.44	0.05	0.02	-0.19	-0.06

Table 13. Abbreviations used in analysis of flow cytometry data

Abbreviation	flow cytometry parameter
fc 1	CD3 CD4 CD25 in CD3 CD4
fc 2	CD3 CD4 HLA-DR in CD3 CD4
fc 3	CD3 CD8 CD25 in CD3 CD8
fc 4	CD3 CD8 HLA-DR in CD3 CD8
fc 5	CD3 CD4 CD54 in CD3 CD4
fc 6	CD3 CD4 CD69 in CD3 CD4
fc 7	CD3 CD8 CD54 in CD3 CD8
fc 8	CD3 CD8 CD69 in CD3 CD8
fc 9	CD19 CD80 in CD19
fc 10	CD19 CD86 in CD19
fc 11	CD14 CD80 in CD14
fc 12	CD14 CD86 in CD14
fc 13	CD19 CD54 in CD19
fc 14	CD19 CD25 in CD19
fc 15	CD56 CD54 in CD56
fc 16	CD56 CD69 in CD56
fc 17	CD14 CD54 in CD14
fc 18	CD14 HLA-DR in CD14
fc 19	CD14 CD11B in CD14
fc 20	CD14 CD54 HLA-DR in CD14
fc 21	CD14 HLA-DR CD11B in CD14
fc 22	CD14 HLA-DR CD11B CD54 in CD14
fc 23	CD14 CD11B CD54 in CD14
fc 24	CD31 (%)
fc 25	CD54 (%)
fc 26	CD62L (%)
fc 27	CD11B (%)
fc 28	CD69 (%)
fc 29	CD11B CD69 (%)

Table 14. Eigenvalues of correlation matrix, and related statistics for flow cytometry data

PC	Eigenvalue	% Total variance	Cumulative Eigenvalue	Cumulative variance %
1	7.16	24.68	7.16	24.68
2	3.81	13.12	10.96	37.80
3	2.63	9.07	13.59	46.88
4	2.43	8.39	16.03	55.27
5	1.90	6.54	17.93	61.81
6	1.62	5.59	19.55	67.41
7	1.46	5.05	21.01	72.45
8	1.21	4.19	22.23	76.64
9	1.10	3.78	23.32	80.42
10	0.87	3.00	24.19	83.42
11	0.85	2.93	25.04	86.36
12	0.71	2.44	25.75	88.80
13	0.63	2.17	26.38	90.97
14	0.53	1.82	26.91	92.78
15	0.39	1.36	27.30	94.14
16	0.35	1.21	27.65	95.35
17	0.34	1.16	27.99	96.51
18	0.31	1.07	28.30	97.58
19	0.20	0.68	28.50	98.26
20	0.12	0.42	28.62	98.68
21	0.10	0.34	28.72	99.02
22	0.08	0.27	28.79	99.29
23	0.07	0.23	28.86	99.52
24	0.05	0.18	28.91	99.70
25	0.04	0.13	28.95	99.83
26	0.02	0.08	28.98	99.91
27	0.01	0.05	28.99	99.96
28	0.01	0.03	29.00	99.99
29	0.00	0.01	29.00	100.00

Table 15. Loadings for all flow cytometry data for each PC. (associations with Eigenvalues >0.5 shown for 95% CL)

	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8	PC9
fc 1	**0.59**	-0.36	0.19	0.06	-0.13	-0.28	0.28	-0.11	-0.33
fc 2	**0.61**	-0.07	-0.20	-0.21	0.11	0.41	0.34	-0.01	-0.17
fc 3	**0.62**	-0.31	-0.07	0.14	0.14	-0.11	0.21	0.05	-0.31
fc 4	0.24	-0.03	-0.26	-0.19	0.11	0.44	0.46	-0.24	-0.03
fc 5	-0.06	-0.01	**0.89**	-0.24	-0.10	0.07	0.20	0.07	-0.10
fc 6	-0.11	-0.11	**0.62**	-0.02	-0.45	0.06	0.42	0.15	-0.07
fc 7	0.08	0.18	**0.64**	-0.39	**0.50**	0.05	-0.25	-0.09	-0.06
fc 8	0.14	0.29	**0.63**	-0.41	0.43	0.03	-0.24	-0.12	-0.07
fc 9	**0.70**	-0.52	0.17	0.05	-0.15	-0.01	-0.05	-0.03	0.18
fc 10	**0.58**	**-0.57**	0.05	0.14	-0.01	-0.06	-0.25	-0.35	0.11
fc 11	**0.79**	-0.35	-0.06	-0.09	0.02	0.30	0.04	0.03	0.14
fc 12	**0.53**	-0.36	0.06	0.15	-0.22	-0.09	-0.33	-0.44	0.02

Table 15 continued

fc 13	**0.57**	-0.27	0.23	-0.09	0.17	-0.06	0.01	-0.15	0.24
fc 14	**0.74**	-0.44	-0.01	0.03	0.09	0.13	0.00	0.22	0.16
fc 15	**0.54**	-0.19	0.11	0.18	0.08	0.13	-0.30	0.46	0.14
fc 16	0.31	-0.09	-0.21	**-0.63**	-0.07	0.45	-0.07	-0.03	-0.10
fc 17	**0.73**	0.26	0.02	0.26	0.03	-0.15	-0.13	0.19	-0.28
fc 18	0.49	**0.64**	-0.02	-0.10	-0.39	0.23	-0.15	-0.06	-0.07
fc 19	0.28	0.28	0.00	-0.27	-0.01	-0.32	0.40	0.01	**0.60**
fc 20	**0.64**	**0.64**	-0.05	0.09	-0.18	0.07	-0.16	0.01	-0.19
fc 21	**0.52**	**0.65**	0.01	-0.15	-0.38	0.08	-0.02	-0.07	0.16
fc 22	**0.66**	**0.66**	-0.02	0.01	-0.19	-0.07	-0.03	-0.01	0.06
fc 23	**0.79**	0.31	0.05	0.16	0.01	-0.29	0.02	0.15	0.05
fc 24	0.45	0.19	-0.05	-0.21	0.23	**-0.53**	0.33	-0.02	-0.04
fc 25	0.29	-0.15	-0.12	0.00	0.29	0.03	-0.01	**0.54**	-0.12
fc 26	0.10	0.35	-0.24	-0.03	**0.55**	0.12	0.02	0.04	0.24
fc 27	0.25	0.16	-0.37	-0.17	0.35	-0.29	0.03	-0.29	-0.27
fc 28	-0.08	-0.30	-0.25	**-0.77**	-0.27	-0.26	-0.19	0.16	-0.05
fc 29	-0.03	-0.29	-0.26	**-0.77**	-0.26	-0.29	-0.17	0.17	-0.04

Table 16. Correlation matrix of PCA model using flow cytometry variables

	fc 1	fc 2	fc 3	fc 4	fc 5	fc 6	fc 7	fc 8	fc 9	fc 10	fc 11	fc 12	fc 13	fc 14	fc 15
fc 1	1.00	0.34	0.69	0.09	0.13	0.16	-0.02	0.00	0.59	0.47	0.43	0.38	0.46	0.42	0.19
fc 2	0.34	1.00	0.43	0.53	-0.06	-0.08	0.02	0.03	0.33	0.26	0.57	0.17	0.22	0.54	0.24
fc 3	0.69	0.43	1.00	0.14	-0.11	-0.12	-0.03	-0.05	0.46	0.41	0.54	0.29	0.31	0.58	0.36
fc 4	0.09	0.53	0.14	1.00	-0.12	-0.07	-0.06	-0.02	0.10	0.06	0.32	0.06	0.10	0.13	0.00
fc 5	0.13	-0.06	-0.11	-0.12	1.00	0.80	0.53	0.51	0.09	-0.07	-0.05	-0.04	0.10	-0.04	-0.01
fc 6	0.16	-0.08	-0.12	-0.07	0.80	1.00	-0.02	-0.02	0.10	-0.07	-0.06	-0.03	0.03	-0.05	-0.09
fc 7	-0.02	0.02	-0.03	-0.06	0.53	-0.02	1.00	0.92	0.00	-0.04	0.00	-0.03	0.16	0.01	0.10
fc 8	0.00	0.03	-0.05	-0.02	0.51	-0.02	0.92	1.00	-0.02	-0.04	-0.01	-0.02	0.23	-0.04	0.07
fc 9	0.59	0.33	0.46	0.10	0.09	0.10	0.00	-0.02	1.00	0.73	0.76	0.61	0.52	0.76	0.46
fc 10	0.47	0.26	0.41	0.06	-0.07	-0.07	-0.04	-0.04	0.73	1.00	0.57	0.82	0.55	0.60	0.35
fc 11	0.43	0.57	0.54	0.32	-0.05	-0.06	0.00	-0.01	0.76	0.57	1.00	0.45	0.51	0.82	0.49
fc 12	0.38	0.17	0.29	0.06	-0.04	-0.03	-0.03	-0.02	0.61	0.82	0.45	1.00	0.30	0.41	0.30
fc 13	0.46	0.22	0.31	0.10	0.10	0.03	0.16	0.23	0.52	0.55	0.51	0.30	1.00	0.50	0.28
fc 14	0.42	0.54	0.58	0.13	-0.04	-0.05	0.01	-0.04	0.76	0.60	0.82	0.41	0.50	1.00	0.58
fc 15	0.19	0.24	0.36	0.00	-0.01	-0.09	0.10	0.07	0.46	0.35	0.49	0.30	0.28	0.58	1.00
fc 16	0.06	0.47	0.12	0.27	-0.01	-0.07	0.11	0.10	0.15	0.11	0.52	0.07	0.20	0.23	0.07
fc 17	0.41	0.31	0.38	0.00	-0.06	-0.09	0.03	0.09	0.34	0.26	0.37	0.28	0.32	0.38	0.44
fc 18	0.04	0.27	0.04	0.06	-0.02	-0.02	0.03	0.16	0.06	-0.06	0.21	0.13	0.08	0.07	0.12
fc 19	0.06	0.13	0.04	0.08	0.04	0.01	0.07	0.09	0.10	-0.06	0.15	-0.07	0.19	0.11	-0.01
fc 20	0.16	0.32	0.18	0.07	-0.10	-0.12	0.03	0.17	0.12	0.04	0.25	0.17	0.14	0.16	0.24
fc 21	0.06	0.25	0.05	0.12	0.00	-0.02	0.05	0.20	0.11	-0.05	0.22	0.14	0.10	0.10	0.09
fc 22	0.16	0.29	0.19	0.13	-0.07	-0.11	0.07	0.22	0.17	0.04	0.27	0.19	0.12	0.20	0.20
fc 23	0.40	0.29	0.40	0.05	-0.03	-0.08	0.06	0.14	0.41	0.28	0.44	0.31	0.33	0.44	0.44
fc 24	0.36	0.26	0.30	0.12	-0.01	-0.08	0.12	0.17	0.15	0.09	0.17	0.09	0.24	0.18	0.07
fc 25	0.07	0.23	0.22	0.08	-0.09	-0.09	0.00	0.03	0.20	0.12	0.28	0.04	0.14	0.37	0.26
fc 26	-0.24	0.15	-0.05	0.09	-0.20	-0.29	0.10	0.12	-0.17	-0.10	0.03	-0.23	0.09	0.01	0.01
fc 27	0.02	0.20	0.18	0.10	-0.25	-0.32	0.05	0.03	-0.04	0.09	0.11	0.11	0.07	0.07	-0.16
fc 28	0.03	0.00	-0.07	-0.01	-0.05	-0.04	-0.02	-0.02	0.07	0.01	0.01	0.01	-0.02	0.02	-0.07
fc 29	0.06	0.03	-0.04	-0.01	-0.05	-0.04	-0.04	-0.03	0.09	0.04	0.04	0.03	0.02	0.04	-0.04

Table 16 continued

	fc 16	fc 17	fc 18	fc 19	fc 20	fc 21	fc 22	fc 23	fc 24	fc 25	fc 26	fc 27	fc 28	fc 29
fc 1	0.06	0.41	0.04	0.06	0.16	0.06	0.16	0.40	0.36	0.07	-0.24	0.02	0.03	0.06
fc 2	0.47	0.31	0.27	0.13	0.32	0.25	0.29	0.29	0.26	0.23	0.15	0.20	0.00	0.03
fc 3	0.12	0.38	0.04	0.04	0.18	0.05	0.19	0.40	0.30	0.22	-0.05	0.18	-0.07	-0.04
fc 4	0.27	0.00	0.06	0.08	0.07	0.12	0.13	0.05	0.12	0.08	0.09	0.10	-0.01	-0.01
fc 5	-0.01	-0.06	-0.02	0.04	-0.10	0.00	-0.07	-0.03	-0.01	-0.09	-0.20	-0.25	-0.05	-0.05
fc 6	-0.07	-0.09	-0.02	0.01	-0.12	-0.02	-0.11	-0.08	-0.08	-0.09	-0.29	-0.32	-0.04	-0.04
fc 7	0.11	0.03	0.03	0.07	0.03	0.05	0.07	0.06	0.12	0.00	0.10	0.05	-0.02	-0.04
fc 8	0.10	0.09	0.16	0.09	0.17	0.20	0.22	0.14	0.17	0.03	0.12	0.03	-0.02	-0.03
fc 9	0.15	0.34	0.06	0.10	0.12	0.11	0.17	0.41	0.15	0.20	-0.17	-0.04	0.07	0.09
fc 10	0.11	0.26	-0.06	-0.06	0.04	-0.05	0.04	0.28	0.09	0.12	-0.10	0.09	0.01	0.04
fc 11	0.52	0.37	0.21	0.15	0.25	0.22	0.27	0.44	0.17	0.28	0.03	0.11	0.01	0.04
fc 12	0.07	0.28	0.13	-0.07	0.17	0.14	0.19	0.31	0.09	0.04	-0.23	0.11	0.01	0.03
fc 13	0.20	0.32	0.08	0.19	0.14	0.10	0.12	0.33	0.24	0.14	0.09	0.07	-0.02	0.02
fc 14	0.23	0.38	0.07	0.11	0.16	0.10	0.20	0.44	0.18	0.37	0.01	0.07	0.02	0.04
fc 15	0.07	0.44	0.12	-0.01	0.24	0.09	0.20	0.44	0.07	0.26	0.01	-0.16	-0.07	-0.04
fc 16	1.00	0.06	0.30	0.05	0.18	0.19	0.08	-0.01	-0.02	0.06	0.11	0.16	0.39	0.41
fc 17	0.06	1.00	0.43	0.04	0.77	0.33	0.60	0.84	0.34	0.24	0.15	0.24	-0.22	-0.18
fc 18	0.30	0.43	1.00	0.16	0.82	0.90	0.74	0.40	0.13	-0.01	0.07	0.11	-0.10	-0.09
fc 19	0.05	0.04	0.16	1.00	0.11	0.42	0.39	0.42	0.41	-0.02	0.12	0.15	0.05	0.08
fc 20	0.18	0.77	0.82	0.11	1.00	0.72	0.87	0.67	0.28	0.03	0.22	0.20	-0.21	-0.18
fc 21	0.19	0.33	0.90	0.42	0.72	1.00	0.86	0.50	0.25	0.01	0.06	0.09	-0.06	-0.05
fc 22	0.08	0.60	0.74	0.39	0.87	0.86	1.00	0.77	0.38	0.05	0.19	0.18	-0.16	-0.13
fc 23	-0.01	0.84	0.40	0.42	0.67	0.50	0.77	1.00	0.46	0.20	0.14	0.24	-0.18	-0.14
fc 24	-0.02	0.34	0.13	0.41	0.28	0.25	0.38	0.46	1.00	0.15	0.22	0.37	0.08	0.13
fc 25	0.06	0.24	-0.01	-0.02	0.03	0.01	0.05	0.20	0.15	1.00	0.05	0.16	0.03	0.03
fc 26	0.11	0.15	0.07	0.12	0.22	0.06	0.19	0.14	0.22	0.05	1.00	0.20	-0.17	-0.14
fc 27	0.16	0.24	0.11	0.15	0.20	0.09	0.18	0.24	0.37	0.16	0.20	1.00	0.05	0.09
fc 28	0.39	-0.22	-0.10	0.05	-0.21	-0.06	-0.16	-0.18	0.08	0.03	-0.17	0.05	1.00	0.98
fc 29	0.41	-0.18	-0.09	0.08	-0.18	-0.05	-0.13	-0.14	0.13	0.03	-0.14	0.09	0.98	1.00

Table 17. Eigenvalues of correlation matrix, and related statistics for selected flow cytometry variables (fc 24-fc 29)

PC	Eigenvalue	% Total variance	Cumulative Eigenvalue	Cumulative variance %
1	2.06	34.26	2.06	34.26
2	1.60	26.64	3.65	60.90
3	0.94	15.71	4.60	76.60
4	0.76	12.70	5.36	89.31
5	0.63	10.45	5.99	99.76
6	0.01	0.24	6.00	100.00

Table 18. Loadings for selected flow cytometry data for each PC. (associations with Eigenvalues >0.5 shown for 95% CL)

	PC1	PC2	PC3
fc 24	-0.23	**-0.72**	-0.13
fc 25	-0.10	-0.40	**0.87**
fc 26	0.20	**-0.60**	-0.39
fc 27	-0.19	**-0.72**	-0.06
fc 28	**-0.98**	0.14	-0.05
fc 29	**-0.98**	0.09	-0.08

Table 19. Eigenvalues of correlation matrix, and related statistics for RT-PCR data

PC	Eigenvalue	% Total variance	Cumulative Eigenvalue	Cumulative variance %
1	2.9	41.0	2.9	41.0
2	1.2	17.6	4.1	58.6
3	1.0	14.4	5.1	73.0
4	0.6	8.3	5.7	81.3
5	0.6	7.9	6.2	89.2
6	0.4	6.2	6.7	95.4
7	0.3	4.6	7.0	100.0

Table 20. Loadings for RT-PCR data for each PC. (associations with Eigenvalues >0.5 shown for 95% CL)

	PC1	PC2	PC3
Fas-L	**0.68**	-0.01	0.340
MCP-1	**0.71**	-0.38	-0.27
TNF-alpha	**0.77**	0.21	-0.25
IL-1	0.37	**0.64**	**0.56**
IL-6	**0.72**	-0.22	-0.20
II-8	**0.70**	-0.28	0.389
II-10	0.34	**0.698**	**-0.48***

- borderline significance at 95% CI

10

Table 21. Eigenvalues of correlation matrix, and related statistics for combined clinical, RT-PCR and flow cytometry variables

	Eigenvalue	% Total variance	Cumulative Eigenvalue	Cumulative %
1	4.550	15.167	4.550	15.167
2	3.861	12.870	8.411	28.037
3	3.295	10.982	11.706	39.019
4	2.028	6.760	13.734	45.779
5	1.816	6.052	15.549	51.831
6	1.520	5.065	17.069	56.896
7	1.234	4.113	18.303	61.010
8	1.153	3.844	19.456	64.854
9	1.139	3.797	20.595	68.650
10	0.958	3.192	21.553	71.842
11	0.931	3.104	22.484	74.946
12	0.906	3.021	23.390	77.968
13	0.825	2.748	24.215	80.716
14	0.740	2.466	24.955	83.182
15	0.685	2.284	25.640	85.466
16	0.611	2.036	26.251	87.502
17	0.559	1.863	26.810	89.365
18	0.542	1.808	27.352	91.173
19	0.480	1.601	27.832	92.774

Table 21 continued

20	0.441	1.469	28.273	94.244
21	0.371	1.235	28.644	95.479
22	0.333	1.108	28.976	96.588
23	0.299	0.998	29.276	97.586
24	0.250	0.833	29.526	98.419
25	0.175	0.585	29.701	99.003
26	0.139	0.462	29.840	99.465
27	0.114	0.381	29.954	99.846
28	0.020	0.068	29.974	99.914
29	0.018	0.059	29.992	99.973
30	0.008	0.027	30.000	100.000

Table 22. Loadings for combined clinical, RT-PCR and flow cytometry data for each PC. (associations with Eigenvalues >0.5 shown for 95% CL)

parameter	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8	PC9
CD31 (%)	-0.02	-0.03	0.42	0.18	0.52	-0.07	-0.40	-0.17	0.03
CD54 (%)	-0.16	0.04	0.20	-0.09	0.48	0.48	0.06	0.18	-0.19
CD62L (%)	-0.41	-0.09	0.20	0.28	0.16	-0.21	0.01	-0.01	0.39
CD11B (%)	-0.13	-0.16	0.22	0.19	0.54	-0.13	-0.09	-0.19	0.05
CD69 (%)	0.49	-0.55	0.23	-0.27	0.13	-0.16	0.00	0.40	-0.25
CD11B CD69 (%)	0.48	-0.53	0.29	-0.27	0.14	-0.16	-0.03	0.40	-0.22
Temp	0.20	0.06	0.30	-0.35	-0.02	0.37	0.47	0.17	0.30
HR	0.15	-0.12	0.13	-0.35	0.20	0.65	-0.03	-0.32	0.00
MAP	0.19	0.19	-0.15	0.10	-0.44	0.31	-0.30	0.04	-0.35
WCC	0.57	-0.64	0.14	0.38	0.01	0.06	0.02	-0.08	0.08
Neuts	0.53	-0.64	0.21	0.38	-0.01	-0.05	0.00	-0.06	0.12
Lymphocytes	0.31	-0.10	-0.32	0.01	-0.02	0.09	-0.06	-0.30	-0.28
Monocytes	0.56	-0.61	0.04	0.25	-0.04	0.19	0.06	-0.04	0.09
Platelets	0.49	-0.02	-0.31	0.29	0.01	-0.15	0.26	0.04	-0.01
INR	-0.21	-0.71	0.09	0.10	-0.25	0.21	-0.24	-0.12	-0.04
APTR	-0.50	-0.49	-0.04	0.07	-0.33	0.11	-0.22	-0.08	-0.08
CRP	-0.07	-0.09	0.26	-0.63	0.14	-0.32	-0.23	-0.18	0.02
PO2	-0.23	0.13	-0.10	0.18	0.45	-0.10	0.14	-0.12	-0.49
HCO3-	0.79	0.32	-0.07	-0.25	-0.03	-0.12	-0.21	-0.06	0.01
BXS	0.80	0.33	-0.07	-0.24	-0.04	-0.11	-0.22	-0.08	0.03
Lactate	-0.50	-0.41	-0.02	-0.15	0.03	0.06	0.22	0.08	-0.08
Urea	-0.25	-0.15	0.21	-0.12	-0.24	-0.32	0.16	-0.08	-0.19
Creatinine	-0.68	-0.45	0.16	-0.06	-0.15	-0.04	-0.03	0.10	-0.18
Fas-L	-0.05	0.12	0.77	-0.08	0.01	0.11	-0.16	-0.05	-0.05
MCP-1	0.12	0.19	0.61	0.07	-0.36	-0.09	0.20	-0.32	-0.13
TNF-alpha	0.14	0.49	0.57	0.32	0.01	0.24	0.13	0.05	-0.12
IL-1	-0.22	0.28	0.20	0.20	-0.12	0.11	-0.45	0.49	0.16
IL-6	0.07	0.19	0.66	0.15	-0.15	-0.20	0.20	-0.05	-0.25
IL-8	0.02	0.16	0.73	-0.05	-0.27	-0.04	-0.03	-0.01	0.16
IL-10	0.14	0.44	0.02	0.51	0.09	0.03	-0.05	0.21	-0.19

Table 23. Model definition used in all DFA models

parameter	value
variable introduction	forward stepwise
tolerance	0.010
terms included in model	as derived
F to enter	1.00
F to remove	0.00
number of steps	as significant

Table 24. Summary of DFA model based on PCA scores of clinical data containing substituted mean values

parameter		value/term		
Wilks' λ		< 0.40		
variables included in model		PC1, PC3, PC4		
χ^2		3 sig. DFs		
cumulative proportion		DF1	DF2	DF3
		56.7%	79.3%	89.9%
Eigenvalue		0.60	0.24	0.11
factor structure	PC1	0.63	0.02	0.15
	PC3	0.09	-0.87	0.39
	PC4	0.44	0.03	-0.51
standardised coefficients	PC1	**0.78**	0.02	0.13
	PC3	0.12	**-0.89**	0.35
	PC4	**0.61**	0.04	**-0.48**
means of cannonical variables	control	-0.15	0.63	0.04
	day 1	0.51	-0.39	0.02
	day 2	0.57	-0.17	-0.64
	day 3	0.72	0.02	0.35
	day 4	0.21	0.02	0.43
	day 5	-1.80	-0.01	-0.28
	day 6	-1.33	-1.14	0.37

Table 25. summary of variable association in the discriminative DFA model based on PCA scores of clinical data containing substituted mean values

DF	PC	components of each PC
1	1	WCC, monocytes, neuts and platelets, BXS and HCO_3^-–ve corr. with creatinine and lactate
	4	CRP
2	3	HR and CRP
3	4	CRP

Table 26. Classification matrix of DFA model based on PCA scores of clinical data containing substituted mean values

group	percent correct	control p=.28	1 day p=.23	2 days p=.14	3 days p=.08	4 days p=.11	5 days p=.09	6 days p=.05
control	80	24	4	0	1	0	0	1
1 day	36	12	9	3	0	0	0	1
2 days	40	5	2	6	0	1	0	1
3 days	11	1	5	1	1	1	0	0
4 days	8	7	3	0	0	1	1	0
5 days	50	2	2	0	0	0	5	1
6 days	83	1	0	0	0	0	0	5
Total	48	52	25	10	2	3	6	9

Table 27. Summary of DFA model based on PCA scores of clinical data without substituted mean values

parameter		value/term		
Wilks' λ		< 0.45		
variables included in model		PC1, PC3, PC5		
χ^2		3 sig. DFs		
cumulative proportion		DF1	DF2	DF3
		52%	89%	95%
Eigenvalue		0.49	0.35	0.05
factor structure	PC1	**0.65**	0.09	0.08
	PC3	-0.13	**0.80**	**0.45**
	PC5	**-0.61**	-0.32	-0.07
standardised coefficients	PC1	0.79	0.10	0.06
	PC3	-0.15	0.87	0.38
	PC5	-0.73	-0.34	-0.06
means of cannonical variables	control	-0.18	**0.56**	-0.10
	day 1	**0.54**	**-0.59**	0.07
	day 2	**0.45**	-0.14	0.36
	day 3	**0.81**	0.40	-0.19
	day 4	0.22	-0.05	-0.27
	day 5	**-1.61**	-0.00	0.33
	day 6	**-1.40**	**-1.90**	**-0.59**

10   **Table 28.** summary of variable association in the discriminative DFA model based on PCA scores of clinical data without substituted mean values

DF	PC	components of each PC
1	1	WCC, monocytes, neuts and platelets, BXS and HCO_3^-–ve corr. with creatinine and lactate
	5	PO2 is contrasted with both urea and MAP
2	3	HR and CRP
3	3	HR and CRP

Table 29. Classification matrix of DFA model based on PCA scores of clinical data without substituted mean values

group	percent correct	control p=.34	1 day p=.21	2 days p=.13	3 days p=.06	4 days p=.10	5 days p=.09	6 days p=.03
control	83	25	2	1	1	0	1	0
1 day	47	9	9	1	0	0	0	0
2 days	0	6	6	0	0	0	0	0
3 days	0	3	3	0	0	0	0	0
4 days	0	5	3	0	0	0	1	0
5 days	25	4	2	0	0	0	2	0
6 days	67	0	0	0	0	0	1	2
Total	44	52	25	2	1	0	5	2

Table 30. Summary of DFA model based on transformed clinical data with substituted mean values

parameter		value/term				
Wilks' λ		0.25				
variables included in model		BXS, CRP, lactate, urea, temperature, creatinine, neuts, PO2, HCO_3^-				
χ^2		5 sig. DFs				
cumulative proportion		DF1	DF2	DF3	DF4	DF5
		46%	74%	85%	94%	99%
Eigenvalue		0.78	0.47	0.18	0.15	0.09
factor structure	BXS	**-0.62**	**0.58**	-0.09	0.21	0.22
	CRP	0.12	**0.63**	0.16	-0.03	-0.31
	temp	0.17	0.03	**-0.56**	**0.62**	0.11
	lactate	**0.65**	0.03	0.05	0.05	0.01
	urea	-0.19	-0.18	-0.19	0.17	**-0.57**
	creatinine	0.42	-0.26	**0.51**	0.20	-0.19
	HCO_3^-	**-0.54**	**0.67**	-0.13	0.19	0.16
	PO2	0.16	-0.05	-0.04	**-0.67**	-0.31
	neuts	-0.14	0.05	-0.24	-0.18	**0.70**
standardised coefficients	BXS	**-1.45**	**-0.78**	**0.84**	0.09	**1.00**
	CRP	0.13	**0.65**	0.07	-0.20	-0.20
	temp	0.41	-0.02	**-0.64**	**0.53**	0.02
	lactate	**0.56**	0.34	-0.22	-0.01	0.21
	urea	-0.52	-0.22	**-0.51**	0.09	**-0.60**
	creatinine	0.13	-0.11	**0.93**	**0.57**	0.10
	HCO_3^-	**1.02**	**1.45**	**-0.53**	0.32	**-0.88**
	PO2	0.19	0.07	-0.28	**-0.61**	-0.21
	neuts	-0.10	0.01	-0.30	-0.32	**0.67**
means of canonical variables	control	**-0.54**	**-0.90**	-0.10	-0.14	-0.07
	day 1	-0.22	**0.53**	-0.35	-0.03	0.40
	day 2	-0.19	**0.84**	0.20	**-0.58**	-0.36
	day 3	-0.42	**0.64**	0.41	**0.62**	-0.28
	day 4	-0.34	-0.09	0.43	**0.61**	0.06
	day 5	**2.05**	-0.37	**0.61**	-0.23	0.27
	day 6	**1.95**	0.03	**-1.05**	0.47	**-0.54**

Table 31. Summary of variable association in the DFA model based on clinical data with substituted mean values

DF	clinical variables defining DFs
1	BXS and HCO_3^- which are negatively correlated with lactate
2	correlation between BXS, CRP and HCO_3^-
3	negative correlation between temperature and creatinine
4	negative correlation between temperature and PO2
5	negative correlation between urea and neutrophilss

Table 32. Classification matrix of DFA model based on clinical data with substituted mean values

group	percent correct	control p=.28	1 day p=.23	2 days p=.14	3 days p=.08	4 days p=.11	5 days p=.09	6 days p=.05
control	80	24	5	1	0	0	0	0
1 day	56	4	14	5	0	1	0	1
2 days	60	2	2	9	1	0	0	1
3 days	11	1	4	2	1	1	0	0
4 days	8	7	4	0	0	1	0	0
5 days	50	1	2	1	0	0	5	1
6 days	83	0	0	0	0	0	1	5
Total	55	39	31	18	2	3	6	8

Table 33. Summary of DFA model based on PCA scores of flow cytometry data containing substituted mean values

parameter		value/term	
Wilks' λ		< 0.39	
variables included in model		PC1, PC5, PC8	
χ^2		2 sig. DFs	
cumulative proportion		DF1	DF2
		46 %	71 %
Eigenvalue		0.49	0.26
factor structure	PC1	0.30	-0.04
	PC5	-0.19-	**-0.59**
	PC8	**0.419**	-0.14
standardised coefficients	PC1	**0.40**	-0.04
	PC5	-0.25	**-0.67**
	PC8	**0.56**	-0.16
means of canonical variables	control	-0.21	-0.35
	day 1	**-0.71**	-0.12
	day 2	-0.24	0.16
	day 3	0.14	**1.44**
	day 4	0.48	0.32
	day 5	**0.88**	-0.28
	day 6	**1.99**	-0.43

Table 34. Summary of variable association in the DFA model based on PCA scores of flow cytometry data containing substituted mean values

DF	PC	components of each DF
1	1	fc 1-3, 9-15, 17, 20-23
	8	fc 19
2	5	fc 7 + 26

Table 35 Classification matrix of DFA model based on PCA scores of flow cytometry data containing substituted mean values

group	percent correct	control p=.28	1 day p=.23	2 days p=.14	3 days p=.08	4 days p=.11	5 days p=.09	6 days p=.05
control	66.6	20	7	0	1	0	1	1
1 day	36.0	12	9	2	0	0	2	0
2 days	13.3	4	8	2	1	0	0	0
3 days	44.4	2	2	0	4	0	1	0
4 days	33.3	3	2	1	0	4	1	1
5 days	40.0	4	1	0	0	0	4	1
6 days	66.6	1	1	0	0	0	0	4
Total	43.9	46	30	5	6	4	9	7

Table 36 Summary of DFA model based on flow cytometry data containing substituted mean values

parameter		value/term		
Wilks' λ		< 0.034		
variables included in model		fc7-8, fc11, fc16, fc25, fc28, fc29		
χ^2		3 sig. DFs		
cumulative proportion		DF1	DF2	DF3
		46%	62%	74%
Eigenvalue		2.17	0.94	0.63
factor structure	fc7	**0.06**	**-0.16**	-0.10
	fc8	**-0.06**	**-0.19**	-0.07
	fc11	0.02	**0.13**	**-0.24**
	fc16	**0.05**	**-0.14**	0.14
	fc25	**-0.34**	**-0.23**	-0.08
	fc28	**0.04**	-0.02	0.02
	fc29	**0.06**	-0.03	0.01
standardised coefficients	fc7	1.84	0.20	-1.03
	fc8	-1.66	-1.00	0.32
	fc11	0.87	1.89	-1.16
	fc16	-0.73	-1.33	0.80
	fc25	-0.86	-0.29	0.02
	fc28	-2.34	0.65	0.29
	fc29	2.64	0.12	-0.66
means of canonical variables	control	0.50	**-0.92**	-0.40
	day 1	**1.07**	-0.32	0.12
	day 2	0.22	0.07	**1.10**
	day 3	0.20	**1.75**	**1.26**
	day 4	-0.39	**1.49**	**-1.34**
	day 5	**-1.05**	**0.73**	-0.55
	day 6	**-5.27**	**-1.06**	0.48

10

Table 37 Summary of variable association in the DFA model based on flow cytometry data containing substituted mean values

DF	clinical variables defining DFs
1	correlates fc7, 16, 28 and 29 and contrasts these to fc8 and 25
2	correlates fc7, 8, 16 and 25 and contrasts these with fc12
3	correlated with fc11

Table 38 Classification matrix of DFA model based on clinical data with substituted mean values

group	percent correct	control p=.28	1 day p=.23	2 days p=.14	3 days p=.08	4 days p=.11	5 days p=.09	6 days p=.05
control	80.0	24.0	2.0	0.0	1.0	2.0	1.0	0.0
1 day	68.0	6.0	17.0	1.0	0.0	1.0	0.0	0.0
2 days	73.3	3.0	1.0	11.0	0.0	0.0	0.0	0.0
3 days	66.7	2.0	0.0	0.0	6.0	1.0	0.0	0.0
4 days	91.7	1.0	0.0	0.0	0.0	11.0	0.0	0.0
5 days	80.0	0.0	1.0	1.0	0.0	0.0	8.0	0.0
6 days	83.3	0.0	0.0	0.0	0.0	1.0	0.0	5.0
Total	76.6	36.0	21.0	13.0	7.0	16.0	9.0	5.0

Table 39 Summary of DFA model based on combined clinical, RT-PCR and flow cytometry variables

parameter		value/term			
Wilks' λ		< 0.0087			
variables included in model		fc7-8, fc11, fc16, fc25, fc28, fc29			
χ^2		3 sig. DFs			
cumulative proportion		DF1	DF2	DF3	DF4
		44%	63%	79%	89%
Eigenvalue		3.79	1.60	1.34	0.86
factor structure	BXS	**-0.30**	0.08	**-0.26**	**0.17**
	fc 25	**0.22**	**-0.23**	-0.08	**0.13**
	fc 22	**0.22**	-0.03	0.08	**0.28**
	fc 11	-0.02	**0.12**	0.05	**0.23**
	Temp	0.09	**-0.11**	**-0.13**	**0.20**
	CRP	0.01	**0.16**	**-0.33**	**-0.16**
	fc 18	**0.15**	0.01	-0.12	0.06
	fc 6	-0.03	0.01	-0.02	**0.12**
	IL-6	**-0.11**	-0.06	**0.11**	-0.07
	INR	0.00	0.06	0.03	**0.12**
	APTR	**0.15**	**0.12**	**0.30**	-0.03
	fc 16	-0.07	-0.09	**-0.11**	-0.07
	Urea	-0.02	**-0.10**	0.01	**0.17**
	Lactate	**0.25**	0.04	-0.05	**-0.22**
	Fas-L	0.00	**0.15**	-0.03	0.04
	fc 13	-0.04	-0.01	-0.07	**0.11**
	fc 24	0.00	**-0.15**	**0.11**	**0.15**
	fc 1	-0.01	**0.12**	**-0.12**	**0.22**
	fc 3	-0.02	0.04	-0.02	**0.15**
	MCP-1	-0.06	0.04	0.02	-0.02
	fc 28	-0.05	-0.03	-0.08	-0.01
	Il-10	0.01	**-0.10**	0.09	-0.07

Table 39 contd. Summary of DFA model based on combined clinical, RT-PCR and flow cytometry variables

	fc 27	0.01	**-0.12**	0.08	-0.02
	fc 26	0.04	**-0.10**	**0.19**	-0.06
	Neuts	**-0.11**	-0.07	0.03	-0.04
	fc 14	0.00	0.09	0.08	**0.19**
	WCC	**-0.10**	-0.08	0.03	-0.01
	fc 29	-0.07	-0.04	-0.08	0.03
	Platelets	**-0.11**	0.04	**0.12**	-0.03
	PO2	0.05	-0.07	0.03	**-0.27**
parameter		value/term			
cumulative proportion		DF1	DF2	DF3	DF4
		44%	63%	79%	89%
Eigenvalue		3.79	1.60	1.34	0.86
standardised coefficients	BXS	-0.80	0.33	-0.21	-0.09
	fc 25	0.81	-0.52	-0.29	0.22
	fc 22	0.46	-0.58	0.05	0.68
	fc 11	0.04	2.00	0.53	0.96
	Temp	0.51	-0.41	-0.07	0.23
	CRP	0.07	0.14	-0.58	-0.13
	fc 18	0.64	0.72	-0.53	-0.11
	fc 6	0.02	-0.29	0.17	0.08
	IL-6	-0.69	-0.36	0.28	-0.12
	INR	-0.90	0.03	-0.99	0.94
	APTR	0.82	0.62	1.28	-0.44
	fc 16	-0.63	-1.23	-0.54	-0.69
	Urea	-0.13	-0.38	0.06	0.58
	Lactate	0.14	0.12	-0.06	-0.68
	Fas-L	0.20	0.92	-0.04	0.05
	fc 13	-0.27	-0.82	-0.33	-0.07
	fc 24	-0.46	-0.72	0.11	0.15
	fc 1	1.04	0.84	0.09	0.12
	fc 3	-0.67	-0.46	-0.41	0.06
	MCP-1	0.33	0.01	-0.21	-0.05
	fc 28	3.20	0.89	-2.48	-0.21
	Il-10	0.22	-0.03	0.26	-0.16
	fc 27	0.12	0.14	0.23	-0.13
	fc 26	0.15	0.26	0.27	-0.01
	Neuts	0.83	0.74	0.27	-1.34
	fc 14	-0.43	-0.73	0.32	-0.88
	WCC	-0.70	-1.06	0.05	0.77
	fc 29	-2.73	-0.34	2.71	0.56
	Platelets	-0.47	-0.06	0.33	0.27
	PO2	-0.17	-0.12	0.20	-0.45
means of canonical variables	control	-0.39	**-1.04**	**1.24**	0.23
	day 1	**-1.80**	-0.51	-0.50	-0.19
	day 2	-0.65	0.70	**-1.16**	-1.17
	day 3	-0.40	**1.12**	**-1.19**	0.45
	day 4	0.38	**2.02**	0.20	**1.77**
	day 5	**3.33**	**1.36**	**1.41**	**-1.45**
	day 6	**5.37**	**-2.44**	**-2.21**	0.76

Table 40. Classification matrix of DFA model based on combined clinical, RT-PCR and flow cytometry variables

group	percent correct	control p=.28	1 day p=.23	2 days p=.14	3 days p=.08	4 days p=.11	5 days p=.09	6 days p=.05
control	90.0	27.0	0.0	1.0	1.0	1.0	0.0	0.0
1 day	76.0	3.0	19.0	3.0	0.0	0.0	0.0	0.0
2 days	93.3	0.0	1.0	14.0	0.0	0.0	0.0	0.0
3 days	77.8	1.0	0.0	1.0	7.0	0.0	0.0	0.0
4 days	91.7	0.0	0.0	1.0	0.0	11.0	0.0	0.0
5 days	90.0	0.0	0.0	1.0	0.0	0.0	9.0	0.0
6 days	100.0	0.0	0.0	0.0	0.0	0.0	0.0	6.0
Total	86.9	31.0	20.0	21.0	8.0	12.0	9.0	6.0

Example 7: Binary logistic regression analysis to predict sepsis

A binary logistic regression model was used to analyse the RT-PCR, flow cytometry results and clinical data separately, from the ICU patients who went on to develop sepsis and presented positive microbiology results. This model used results gained from an age matched group of ICU patients who were not diagnosed with sepsis as the control group. Although the model identified numerous possible predictors some appeared to be of limited use since the values obtained for the pre-symptomatic sepsis patients were within those obtained for the non sepsis patients. The potential prediction markers that did yield some pre-sepsis data points that differed from the non sepsis data are listed in Table 36. However when combined, these prediction markers could only have identified 8 out of the 24 pre-sepsis patients.

Table 41. Summary of potential prediction markers identified by binary logistic regression analysis.

Time Point	Overall Predictor effect	Predictor	Probability score
5 days pre-diagnosis	p=0.10	base excess	p=0.047
4 days pre-diagnosis	p=0.042	IL-10	p=0.058
3 days pre-diagnosis	p=0.095	blood bicarbonate	p=0.021
	p=0.114	CD 31	p = 0.037
	p=0.055	IL-10	p=0.052
2 days pre-diagnosis	p=0.10	blood bicarbonate	p=0.017
		C reactive protein	p=0.038
	P=0.085	TNF-α	p=0.022

The discovery of a combination of markers that could possibly predict sepsis in 8 out of 24 patients who later went on to develop SIRS with confirmed infection dos not constitute a diagnostic test. Although the prediction capability for CD31 on granulocytes appeared promising (66%), this marker was only effective three days before the appearance of clinical symptoms. A test based on CD31 alone may not constitute a diagnostic test since to be effective there would need to be a

larger diagnostic window. This could be achieved by the discovery of even more markers. This study may however have found some markers that could form part of a diagnostic test in the future, but caution must be exercised. In the mid 1980s HLA-DR was believed to be prognostic for the development of infections and sepsis (Spittler, A. & Roth, E. 2003, *Intensive Care Med* , vol. 29, pp. 1211-1213More recent studies however have shown that post-operative levels of this marker did not predict the onset of SIRS, sepsis or infectious complications (Oczenski, W. *et al* 2003, *Intensive Care Med*, vol. 29, pp. 1253-1257 and Perry, S.*et al*. 2003, *Intensive Care Med*, vol. 29, pp. 1245-1252.The conflicting reports in the case of HLA-DR illustrates why caution must be applied to the results of this study. These findings could be due to regional factors such as

10   antibiotic policy, diagnostic criteria, clinical practice, surgical procedures, treatment regimes, environmental factors and the patients predisposing factors (Angus, D. & Wax, R. 2001, *Critical Care Medicine*, vol. 29, no. 7 (suppl), pp. 109-116). A larger study that involves more patients from several different hospitals, preferably spanning different health authorities, needs to be conducted to further assess the usefulness of the markers identified for the prediction of sepsis.

Example 8: Sepsis as a model for response to biological weapons

Background and method

Since one of the applications for the claimed invention is the early detection of deliberate infection resulting from exposure to biological weapons, the applicability of sepsis as a model for such

20   infection was examined. Presumptive biological weapons pathogens such as *Burkholderia pseudomallei* and *Francisella tularensis* are predicted to produce severe sepsis (see Table 42), which is difficult to model for obvious reasons.

However, *in vitro* infection of whole blood may be used as a model and the activation marker expression and cytokine response measured. To compare this *in vitro* infection model with the *in vivo* situation, *Staphylococcus aureus* infection was selected as a model infectious agent directly comparable with the *in vivo* hospital-acquired infection data.

Table 42

Infection	Forms of "Negative" Outcome
Anthrax	Sepsis, septic shock
Brucellosis	Sepsis, septic shock or chronic form
Glanders and Melioidosis	Sepsis, septic shock or persistent form
Plague	Sepsis, septic shock
Tularemia	Sepsis, septic shock
Epidemic Typhus	Sepsis
Q fever	Sepsis

Table 42 continued

Ebola and Marburg hemorrhagic fevers	Sepsis, septic (toxico-infectious, hypovolemic) shock
Japanese encephalitis	Sepsis, septic shock
Smallpox	Sepsis, septic shock
Yellow fever	Sepsis

Blood from 25 healthy volunteers was infected *in vitro* with *Staphylococcus aureus* and the following activation markers and cytokine levels measured at 24 and 48 hours post-infection, as previously described.

FACS

10   Dendritic cells: CD54, CD97, CCR6, CCR7

NK cells: CD25, CD44, CD62L, CD69, CD97

Monocytes: CD44, CD54, CD62L, CD69, CD97, CD107a

Neutrophils: CD44, CD62L, CD69, CD107a

Real time RT PCR

IL-1β, IL-6, IL-8, IL-10, MCP-1, TNF-α, sFasL

Each of these sets of input parameters (ie Dendritic cell markers, NK cell markers, monocyte markers, neutrophil markers, RT PCR data at 24h, RT PCR data at 48h) were used to train its own
20   neural network model. Random selections of infected or non-infected blood samples were used for training (70%) or subsequent testing (30%). The testing phase of the neural network analysis gave a predictive accuracy based on the % of times it would correctly predict that the test set of input parameters was from an infected or non-infected sample. This testing of each set of input parameters was repeated 5 times. Each time the test was conducted a new neural network was constructed using a newly randomised 70% of the infected and non-infected samples. An average predictive accuracy was derived for each set of input parameters by working out the mean from the 5 predictive accuracies calculated from the 5 neural networks constructed on the 5 randomised sets of input data. The methodology was similar to that used in the sepsis patient study.

30   Results

The most consistent results were obtained from the RT PCR results. Figure 4 shows the data obtained from three subjects, which demonstrates the somewhat heterogeneous patterns of change in the profiles. However, when subjected to the neural network analysis described above,

the algorithm achieved a good level of identification of infected sample over uninfected controls (Figure 5).

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", and variations such as "comprises" and "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

The reference in this specification to any prior publication (or information derived from it), or to any matter which is known, is not, and should not be taken as an acknowledgment or admission or any form of suggestion that that prior publication (or information derived from it) or known matter forms part of the common general knowledge in the field of endeavour to which this specification relates.

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1.   Method for screening a biological sample to detect early stages of infection, SIRS or sepsis comprising the steps of:

    a) detecting expression of a set of biomarkers by RT-PCR

    b) analysing the results of detection by means of neural network or multivariate statistical analysis

    c) classifying said sample according to the likelihood of the development of overt infection

characterised in that the set of biomarkers the expression of which is detected by means of RT-PCR consists of at least 4 selected from the list consisting of FasL, MCP-1, TNF-α, IL-1β, IL-6, IL-8, IL-10, INF-α and INF-γ.

2.   Method of claim 1, wherein the expression of at least 7 biomarkers is detected.

3.   Method of claims 1 or claim 2, wherein analysis is by means of multivariate statistical analysis comprising principle component analysis and/or discriminant function analysis.

4.   Method of claim 3, wherein the multivariate statistical analysis comprises discriminant function analysis.

5.   Method of claim 1 or claim 2, wherein analysis is by means of neural network.

6.   Method of claim 5, wherein the neural network is a multilayered perceptron neural network.

7.   Method of any one of claims 3 to 6, wherein the analysis is capable of correctly predicting clinical SIRS or sepsis in greater than 80% of cases.

8.   System for screening a biological sample to detect early stages of infection, SIRS or sepsis comprising: a means of extracting and purifying RNA from cells in said sample, a thermal cycler to amplify selected RNA sequences by means of reverse transcription polymerase chain reaction (RT-PCR), a means of detecting and quantifying the results of

said RT-PCR, a computer-based neural network trained so as to be able to analyse such results and a display means whereby the conclusion of the neural network analysis may be communicated to an operator characterised in that the neural network is trained to correctly predict SIRS or sepsis in greater than 70% of cases from the detection of at least

5   4 biomarkers selected from the list consisting of FasL, MCP-1, TNF-α, IL-1β, IL-6, IL-8, IL-10, INF-α and INF-γ.
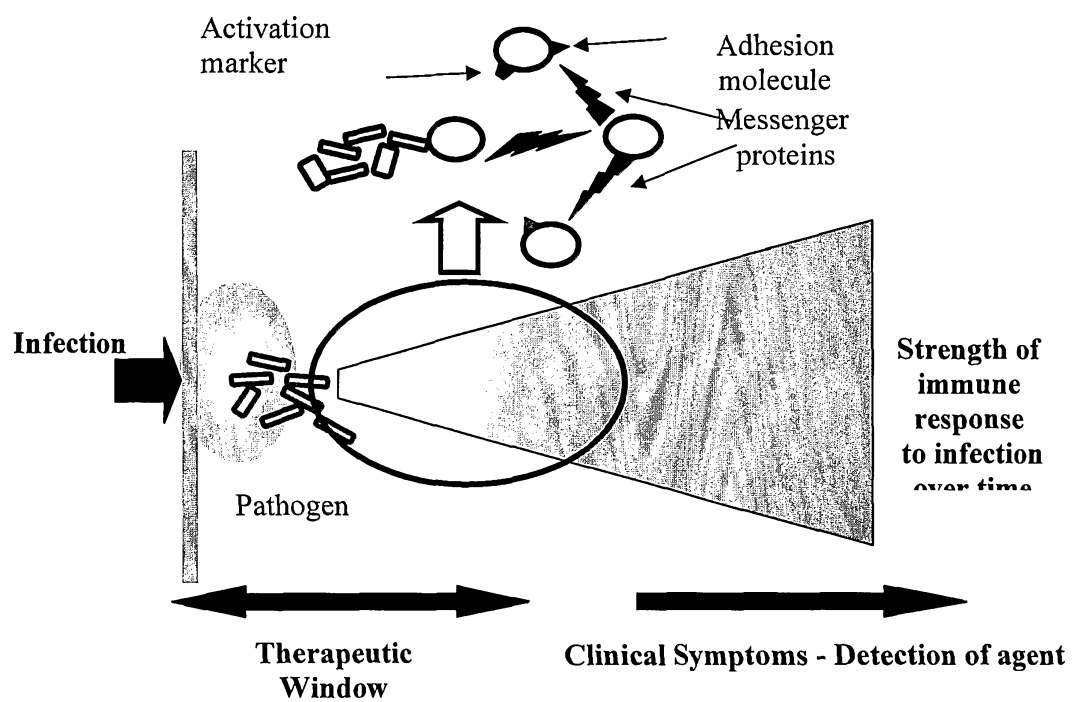
9.   Analysis according to the method of any one of claims 1 to 7 or using the system of claim 8 for the preparation of a diagnostic means for the diagnosis of infection, SIRS or

10   sepsis.

10.   Use of a method according to any one of claims 1 to 7 or the system according to claim 8 for the preparation of a diagnostic means for the diagnosis of infection, SIRS or sepsis.

15
11.   Method of claim 1 or the system of claim 8, substantially as hereinbefore described and with reference to any of the Examples and/or Figures.
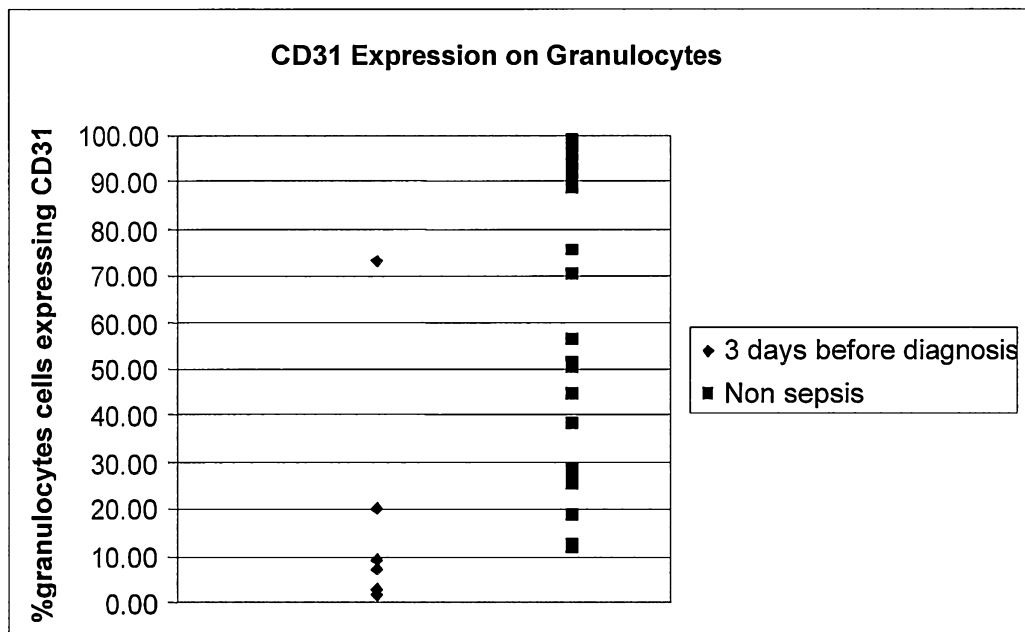
Figure 1

Figure 2

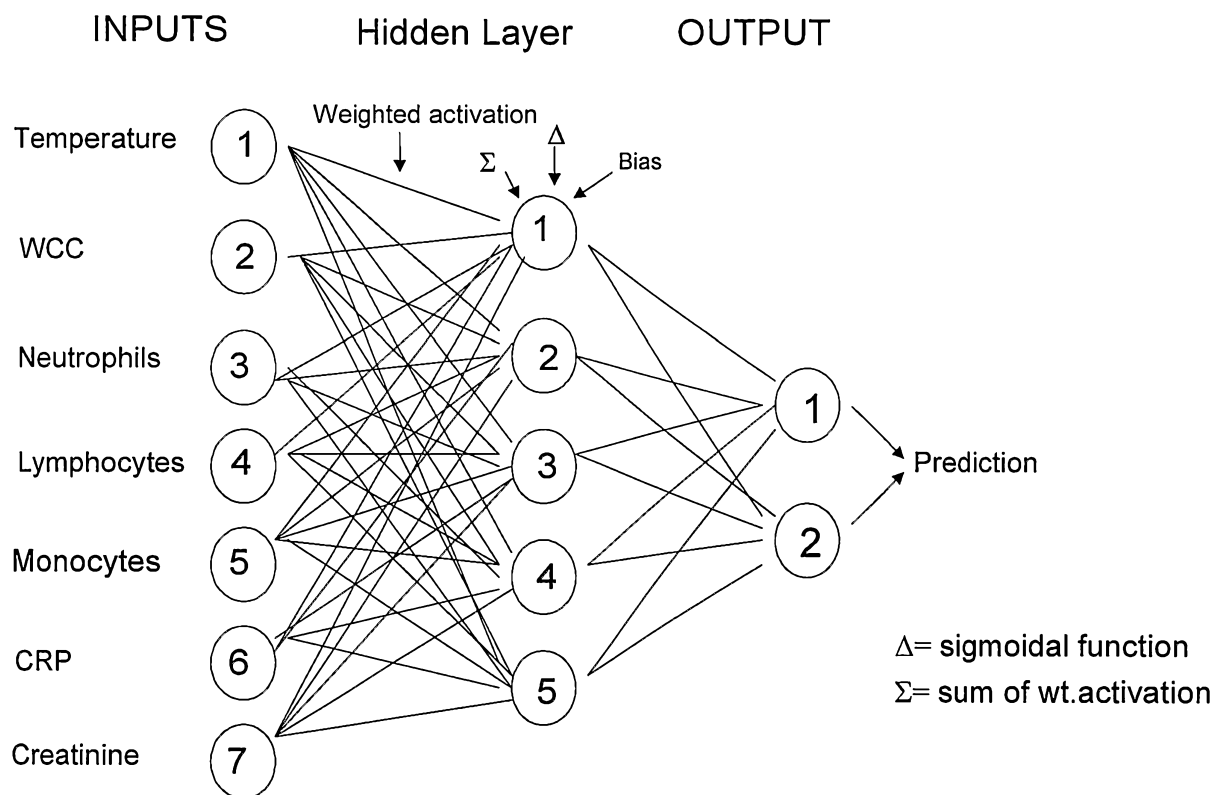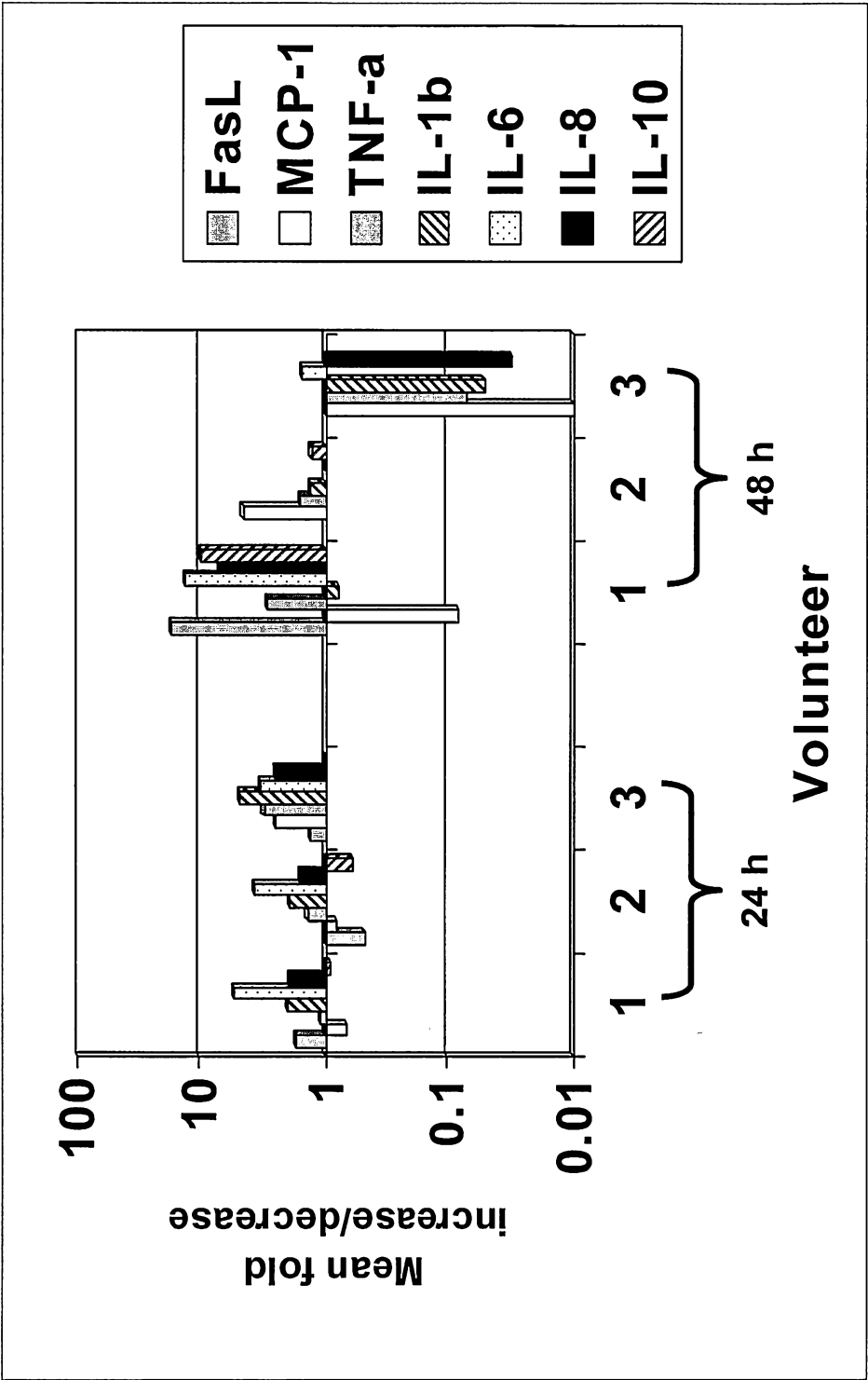CD31 Expression on Granulocytes

♦ 3 days before diagnosis

■ Non sepsis

3/5

Figure 3

INPUTS   Hidden Layer   OUTPUT

Temperature

WCC

Neutrophils

Lymphocytes

Monocytes

CRP

Creatinine

Weighted activation

Σ   Δ   Bias

Prediction

Δ= sigmoidal function
Σ= sum of wt.activation

4/5

Figure 4

Figure 5