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(54) Title: DELTA-LIKE LIGAND 1 FOR DIAGNOSING SEVERE INFECTIONS

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(54) **Title:** DELTA-LIKE LIGAND 1 FOR DIAGNOSING SEVERE INFECTIONS

(57) **Abstract:** The invention refers to a method for in vitro diagnosis of a severe infection comprising determining delta-like ligand 1 protein or a nucleotide sequence coding for delta-like ligand 1 protein in a biological sample wherein an elevated level of expression of delta- like ligand 1 protein or a nucleotide sequence coding for delta-like ligand 1 protein is indicative of a severe infection; and the use of delta-like ligand 1 protein as a biomarker for in vitro diagnosis of a severe infection such as sepsis.

Delta-like ligand 1 for diagnosing severe infections

TECHNICAL FIELD OF THE INVENTION

5 The invention relates to the use of delta-like ligand 1 as a biomarker in the diagnosis of severe infections and to a method for a quick *in vitro* diagnosing of severe infections such as sepsis by controlling the delta-like ligand 1 level in a biological sample.

BACKGROUND OF THE INVENTION

10

Biomarkers are measurable characteristics of an organism that reflect a particular physiological state. In medicine, biomarkers are often compounds isolated from biological tissue that can be used as an indicator of the presence or severity of a particular disease state or monitor the effectiveness of a given intervention.

15

Biomarkers are particularly useful in the diagnosis and possibly prognosis of a disease, as well as in monitoring disease progression or response to treatment. The ideal biomarker should be easily obtained and measured and should be reliable in that it shows both a high sensitivity and specificity for a disease.

20

Severe infections, and in particular sepsis, are particularly evasive medical conditions for which no highly reliable biomarkers are present. Severe infection including sepsis, are identified by and/or may result in life-threatening organ failure evoked by a dysregulated immune response to infection. In sepsis, the host response that is
25 triggered by microbial pathogens peaks in a pathological syndrome that is characterized by exaggerated inflammation and a subsequent immune suppression.

Despite the steady improvements in critical care medicine and anti-microbial therapies, such infection remains a leading cause of death in intensive care units across all age groups worldwide. In order to improve outcomes and simultaneously avoid unnecessary antibiotic treatment, a rapid and reliable test for diagnosing a
5 severe infection is essential.

Sepsis is a severe and thus life-threatening infection. Up to now, blood cultures remain the gold standard in diagnosing sepsis, however blood cultures take time and many patients who have signs and symptoms of sepsis have negative blood cultures.
10 Therefore, a supplementary approach for diagnosing a severe infection, and in particular for diagnosing sepsis is urgently needed.

C. Pierrakos and J.-L. Vincent (2010) *Critical Care*, 14:R15 describe biomarkers known in the art to be useful for identifying or ruling out sepsis. These include various
15 biomarkers directed to cytokines/chemokines, cells, receptors, coagulation, vascular endothelial damage, vasodilation, organ dysfunction, and acute phase proteins. Notch ligands, and in particular delta-like ligand 1, are not mentioned.

Van Engelen et al. (2018) "Biomarkers in Sepsis", *Critical Care Clinics*, 34(1):129-152
20 describes various biomarkers for diagnosing sepsis and in particular points to the omics (i.e. genomics, epigenetics, transcriptomics, proteomics, and metabolomics) field of systems biology as being a promising tool for the discovery of novel biomarkers.

25 WO 2016/145426 A1 describes a method for diagnosing sepsis using expression levels of CEACAM1, ZDHHC19, C9orf95, GNA15, BATF, C3AR1, KIAA1370, TGFBI, MTCH1, RPGRIP1, and HLA-DPB1 as biomarkers.

US 2005/059093 A1 describes a method for detecting modulators of Notch signaling
30 comprising the step of monitoring Notch signaling in a cell of the immune system in

the presence and absence of a candidate modulator and determining whether the candidate modulator modulates Notch signaling.

WO 2017/004159 A1 describes compositions that bind to and inhibit the biological
5 activity of soluble biomolecules to inhibit the target or pathogen from interacting with other molecules or cells. Notch ligands, including Delta-like ligand 1 (DLL1) are mentioned as being particularly useful for treating or preventing atherosclerosis, calcific aortic valve stenosis, heart failure, stroke, and cancer. To treat or prevent sepsis associated with an infection caused by a pathogen, WO 2017/004159A1
10 proposes selectively binding TNFα, interleukin 1, interleukin 6, interleukin 8, interleukin 12, interferon gamma, macrophage migration inhibitory factor, GM-CSF, and/or a blood clotting factor.

US 2006/140943 A1 describes a use of a modulator of Notch signaling for the
15 preparation of a medicament for treatment of Graft Versus Host Disease (GVHD) and diseases and conditions caused by or associated with transplants such as organ, tissue and/or cell transplants (e.g. bone marrow transplants), wherein the modulator is used to reduce the reactivity of cells of the immune system.

20 WO 2012/092539 A2 describes an antibody against DLL4 and a method for treating a DLL4-associated disease, such as a cell proliferative disorder or a pathological condition associated with angiogenesis. WO 2012/092539 A2 describes that dysregulation of angiogenesis can lead to neoplastic and non-neoplastic disorders, and names sepsis as one of many of such non-neoplastic disorders.

25

US 9,731,024 B2 and US 2017/0240590 A1 describe materials and methods of conjugating a water soluble polymer to an oxidized carbohydrate moiety of a therapeutic protein. One of many proteins listed as therapeutic proteins is delta-like protein 1.

30

Several biomarkers are currently used to diagnose a severe infection such as sepsis. The acute phase proteins procalcitonin (PCT) and C-reactive protein (CRP), together with leukocyte count, have been most widely used.

5 Nonetheless, the effectiveness of PCT and CRP are restricted by their lack of specificity and sensitivity for sepsis. In particular, it remains difficult to differentiate sepsis from other non-infectious causes of inflammation. Therefore, new sepsis biomarkers with higher reliability are required.

10

SUMMARY OF THE INVENTION

The problem underlying the invention is providing a biomarker that can be used to diagnose a severe infection with a high level of reliability.

15

This problem is solved by the use of delta-like ligand 1 protein (DLL1) or a nucleotide sequence coding for delta-like ligand 1 protein as a biomarker for the *in vitro* (ex vivo) diagnosis of a severe infection. Accordingly, an elevated level of delta-like ligand 1 protein or a nucleotide sequence coding for delta-like ligand 1 protein in a patient's
20 biological sample is indicative for the presence of a severe infection.

Further, the invention concerns a method for in vitro diagnosis of a severe infection comprising determining delta-like ligand 1 protein or a nucleotide sequence coding for delta-like ligand 1 protein in a biological sample wherein an elevated level of
25 expression of delta-like ligand 1 protein or of a nucleotide sequence coding for delta-like ligand 1 protein is indicative of an infection.

Surprisingly, it was found that delta-like ligand 1 acts as a biomarker for severe infections, in particular sepsis with a high level of reliability. Advantages associated
30 with this diagnostic biomarker of the invention are earlier diagnosis of the infection, timely treatment, and improved disease outcome. It will also reduce unnecessary

costs associated with testing other biomarkers that show lower sensitivity and selectivity than delta-like ligand 1.

DETAILED DESCRIPTION OF THE INVENTION

5

Delta-like proteins are single-pass transmembrane proteins known for their role in Notch signaling as homologs of the Notch Delta ligand first described in *Drosophila*. Synonyms of DLL-1 are delta-like-ligand 1, delta-like protein, H-Delta, 1, drosophila Delta homolog 1, delta like canonical Notch ligand 1, DL1, Notch ligand deltal-like1. In
10 mammals, there are three delta-like genes encoding delta-like ligand 1 (*DLL1* encoding DLL1), delta-like ligand 3 (*DLL3* encoding DLL3), and delta-like ligand 4 (*DLL4* encoding DLL4), all ligands comprising a conserved cysteine-rich region known as the DSL (Delta, Serrate, Lag2) domain, several epidermal growth factor (EGF)-like repeats, and a transmembrane domain. The amino acid sequence of delta-like ligand 1
15 protein and the nucleotide sequence coding for delta-like ligand 1 protein are known. For example, an amino acid sequence of DLL1 is described in American Journal of Pathology, Vol. 154, No.3, March 1999, 785 -794 or in the database of the National Center for Biotechnology Information (*https://www.ncbi.nlm.nih.gov/protein/ NP_005609.3).* The chromosomal location of the human orthologue is 6q27.

20

The delta-like ligand 1 protein as used in the invention can be a protein that is encoded by the nucleotide sequence SEQ ID NO: 1 or a nucleotide sequence which is at least 80%, preferably 85% or 90 % identical to SEQ ID NO: 1.

25 Further, the delta-like ligand 1 protein can be a protein having at least 90% identity, particularly 95% identity with amino acid sequence SEQ ID NO: 2 or SEQ ID NO: 3.

The term delta-ligand 1 protein as used herein for the purposes of the invention also includes naturally occurring cleavage products of DLL1. Naturally occurring cleavage
30 products according to the invention comprise extracellular, transmembrane, and intracellular cleavage products. In a preferred embodiment, the naturally occurring

cleavage product is an extracellular cleavage product. Thus, the term delta-ligand 1 protein also includes polypeptides consisting essentially of N- or C-terminal fragments of the protein according to SEQ ID NO: 2 and which are elevated at a severe infection, in particular sepsis. Thus, the term DLL1 protein comprises post-translational
5 modifications, natural proteolytic and processed DLL1 protein. It also comprises the soluble, insoluble DLL1 protein and naturally occurring isoforms of DLL1 protein of SEQ ID NOs: 2 or 3 (UniProtKB – 000548 (DLL1_HUMAN). Such appropriate natural cleavage products of DLL1 are, for example, represented by amino acid SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6 and/or SEQ ID NO: 7. SEQ ID NO: 4 represents the soluble
10 DLL1 protein. SEQ ID NO: 5 represents the transmembrane domain of the DLL1 protein linked to the intracellular domain of the DLL1 protein. SEQ ID NO: 6 represents the intracellular domain of the DLL1 protein. SEQ ID NO: 7 represents the transmembrane domain of the DLL1 protein.

15 Further comprised within the term DLL1 proteins are proteins which are modified, for example, by phosphorylation, methylation, acetylation and glycosylation.

In the invention the level of DLL1 protein and/or the level of a DLL1 nucleotide sequence is/are determined. As used herein, the term nucleotide sequence may refer
20 to DNA, cDNA, RNA, or mRNA. The nucleotide sequence coding for the DLL1 protein or for a protein isoform of DLL1 can also be a splice variant on RNA level. Such splice variants, for example are, nucleotide sequences without the N-terminal or without the C-terminal of the sequence.

25 According to one embodiment of the invention, when using the *DLL1* nucleotide sequence as a biomarker for diagnosing an infection or in a method for *in vitro* diagnosing an infection, an elevated expression level of *DLL1* is indicative of a severe infection. Exemplary nucleotide sequences of DLL1 in the determination of the expression level are SEQ ID NO: 1.

30

References to the nucleotide sequence *DLL1* labeled SEQ ID NO: 1 refer to the DNA sequence in *Homo sapiens*. It will, however, be evident that the invention is not limited to *Homo sapiens*, but rather extends to all mammals.

5 The term "elevated" as used herein means an increased level compared to a control. The control can be any non-infected biological sample or system. Typically, the control is of the same species as the infected biological sample or system. A patient before surgery and/or before the onset of sepsis or another severe infection may, for example, serve as a control. The skilled person in the field of medicine and medical
10 biology will easily be able to identify an appropriate control to which expression levels can be compared. Typically, an elevated amount of DLL1 may be a concentration of DLL1, which is significantly beyond the standard deviation value of the control. According to a preferred embodiment an elevated amount of DLL1 is a concentration, which is two times or in particular three times the standard deviation
15 value of the mean in the control sample. Three times the standard deviation value of the mean in the control sample gives particularly good results.

Alternatively for the determination of an elevated amount of DLL1 or for diagnosis of a severe infection it is also beneficial to define a cut-off value and to take into account
20 such cut-off value for the diagnosis of sepsis when a patient's sample is tested in line with the invention. A cutoff value will allow differentiation between the groups of patients having a severe infection or not having a severe infection. A reasonable cut-off value for the case the severe infection is sepsis is about 36331 pg DLL1 protein per ml. A particularly beneficial diagnostic cut-off value for the case where the severe
25 infection is sepsis can be about 29,538 pg DLL1 protein per ml.

The expression level of *DLL1* may be measured in any biological sample. The term "biological sample" as used herein comprises the whole of an organism or any part of an organism. Typically, a biological sample is removed from the organism for *ex vivo*
30 analysis. A biological sample may include single cells and/or cell cultures and/or tissue cultures. Biological samples also include, but are not limited to, tissues, such as

epithelial, muscular, connective, and nervous tissue. The biological sample may comprise, for example, whole blood, buffy coat, plasma, serum, peripheral blood mononucleated cells (PBMCS), neutrophils, monocytes, T cells, urine, spinal fluid, lymph fluid, external secretions of the skin, tears, and/or saliva.

5

In one embodiment of the invention, the expression level of *DLL1* is measured from a single cell or a cell culture. The cell or cell culture may comprise cells of the immune system, in particular. Typically, the cell or cell culture comprises an immune cell. Preferably, the cell is a leukocyte. More preferably, the cell is a monocyte. Preferably,
10 the cell culture comprises leukocytes. More preferably, the cell culture comprises monocytes.

In another embodiment, the expression level of *DLL1* is measured from a tissue. Preferably, the expression level of *DLL1* is measured from a blood sample. The term
15 “blood” as used herein includes whole blood, blood plasma and blood serum. Preferably, the expression level of *DLL1* is measured from a blood plasma sample.

In a preferred embodiment, the level of expression of *DLL1* is measured and determined from a blood plasma sample taken from a patient. The term “patient” as used herein
20 includes both human and non-human mammals in risk of a severe infection or sepsis. Often, patients are humans or animals that have undergone surgery.

Any appropriate means of measuring protein expression may be used. Typically, protein expression is measured and determined using an immunoassay. Preferably, protein
25 expression is measured and determined using an enzyme-linked immunosorbent assay (ELISA) directed to a specified protein or polypeptide sequence. Protein expression levels may also be measured and determined using an immunoblot assay, such as a Western blot assay, mass spectrometry, ELISpot, flow cytometry, or immunohistochemistry.

30

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Any appropriate means of measuring nucleotide expression may be used to measure the expression level of *DLL1*. For example, expression may be measured by performing microarray analysis, polymerase chain reaction (PCR), reverse transcriptase polymerase chain reaction (RT-PCR), a Northern blot, Southern Blot, or
5 a serial analysis of gene expression (SAGE).

Severe infections are particularly evasive medical conditions, which may result in life-threatening organ failure evoked by a dysregulated immune response to infection. Examples for severe infections are sepsis, pneumonia, and meningitis.

10

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 Western blot. $CD14^+$ monocytes were isolated from blood of healthy donors and either infected *in vitro* with $1x10^6$ bacteria/mL (*Escherichia coli, Pseudomonas*
15 *aeruginosa, Klebsiella pneumoniae, Enterococcus faecalis*) or stimulated with 100 ng/mL of lipopolysaccharide (LPS). After two hours, bacteria were killed with gentamicin. Control cells (-) were left untreated. The next day cell lysates were produced. For western blot analyses equal amounts of protein lysates were blotted and probed with antibodies against DLL1 or Actin (loading control).

20

Fig. 2 shows ELISA results from 12-week old mice injected (i.p.) with either LPS (n=16) or NaCl (n=15; control group). Twenty-four hours following injection blood samples were taken and quantification of plasma concentrations were performed by mouse DLL-1 ELISAs ($p \leq 0.0001$; Mann-Whitney U test).

25

Fig. 3 shows ELISA analysis of expression levels of (A) DLL-1 or (B) DLL-4 from blood plasma samples from sepsis patients (n=50) taken immediately (t0), 24 hours (t24), 48 hours (t48), and 168 hours (t168) following the first identification of sepsis symptoms, as well as ELISA results from blood plasma samples from healthy donors
30 (healthy; n=20) and control patients following abdominal surgery (48h post-OP ("OP t2"; n=20). ***$p \leq 0.0001$; Mann-Whitney U test.

Fig. 4 shows ROC analysis of (A) leukocytes for sepsis patients at t0 versus control patients; (B) CRP levels of sepsis patients at t0 versus control patients; C) DLL-1 levels of sepsis patients at t0 versus control patients; D) DLL-1 levels of sepsis patients at t0 5 versus healthy volunteers; E) DLL-4 levels of sepsis patients at t0 versus control patients; F) DLL-4 levels of sepsis patients at t0 versus healthy volunteers. AUC = area under the curve.

Fig. 5 shows ELISA analysis of DLL-1 protein levels from blood plasma samples from 10 sepsis patients ("Sepsis"; Cohort 1: n=30, Cohort 2: n=50, Cohort 3: n=100), control patients following extensive visceral surgery ("post-OP"; Cohort 1: n=30, Cohort 2: n=20), and healthy subjects ("Healthy"; Cohort 1: n=30, Cohort 2: n=20) from three independent clinical studies (Cohort 1 (A), Cohort 2 (B), Cohort 3 (C); see Example 4 for further study details). ***p≤0.0001; Mann-Whitney U test.

15

Fig. 6 shows ELISA analysis of DLL-1 protein levels from blood plasma samples from (A) a patient cohort after severe trauma (n=38) taken immediately ("initial"), 24 hours ("24h") and 48 hours ("48h") following study inclusion (latest within 24h after onset of clinical symptoms). The same is shown for (B) a patient cohort (n=25) subjected to 20 cardiac surgery under extracorporeal circulation. Protein levels are shown for before surgery ("pre-OP"), 4 hours following extracorporeal circulation ("4h post ECC") , and 24 hours following extracorporeal circulation ("24h post ECC"). These patient cohorts showed no signs of infection at any time point.

25 **Fig. 7** shows a ROC analysis of DLL-1 protein levels from blood plasma samples (sepsis patients: n= 327; controls: n=377). AUC = area under the curve.

Fig. 8 shows $CD14^+$ monocytes that were isolated from blood of healthy donors and stimulated with 100 ng/ml LPS or infected with 10^8 *E.coli* /10^6 monocytes. After two hours bacteria were killed with gentamicin. Control cells (-) were left untreated. The 30 next day cell lysates were produced. For western blot analyses equal amounts of

protein lysates were blotted and probed with antibodies against DLL1 or Actin (loading control).

The following examples serve to further explain the invention, specifically with reference to certain embodiments and figures, which, however, are not intended to limit the present disclosure.

EXAMPLES

Example 1 – Delta-like ligand 1 detects bacterial infection *in vitro*

This example aimed to check whether the Notch ligand DLL1 could be upregulated in sepsis patients and therefore could be used as a potential biomarker. An *in vitro* infection model with sepsis-relevant bacteria is used. In the experimental setup we infected blood-derived monocytes from healthy donors for two hours with different gram negative *(Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae)* and gram positive bacteria *(Enterococcus faecalis)* and subsequently killed bacteria with antibiotics. Furthermore cells were stimulated with TLR4 agonist lipopolysaccharide (LPS), a component of gram negative cell wall which can cause septic shock when circulating in the blood stream. After overnight incubation of infected and LPS-stimulated cells lysates were produced for western blot analysis for the detection of DLL1. The experimental details are explained below.

Cell isolation and culture - Peripheral blood-derived mononucleated cells were isolated from fresh blood or buffy coat from healthy donors by means of density gradient centrifugation (Biocoll separating solution, 1.077 g/ml, Biochrom AG, Berlin, Germany). Cells were washed three times with PBS and CD14+ magnetically labeled cells were positively selected via the autoMACS separator (autoMACS, program: possel, Miltenyi Biotec, Bergisch Gladbach, Germany), supplemented with 100 IU/mL of penicillin, 100 µg/mL streptomycin containing 10% heat inactivated fetal calf

serum (FCS, Promocell, Heidelberg, Germany) at 37°C in a humidified atmosphere in the presence of 5% CO_2.

Bacterial cultures - *Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae,* 5 and *Enterococcus faecalis* were separately cultured overnight at 37°C at 5% CO2 in a humidified atmosphere. The next day, one clone from each culture was transferred into Tryptic Soy Broth media and after 2 hours under constant stirring at 37°C, bacterial suspension was adjusted by absorption measurement to a concentration of 10^8/mL RPMI.

10

In vitro infection - $1x10^6$ sorted $CD14^+$ monocytes were plated in a 24-well plate format in 1 mL RPMI/10% FCS. Cells were infected either with $1x10^6$ bacteria/mL or were stimulated with toll-like receptor 4 (TLR4) agonist lipopolysaccharide (100 ng/ml LPS). After two hours 100 ng/mL gentamicin (PAA Laboratories, Inc) was 15 added to the bacterial cultures to kill the bacteria.

After overnight incubation of infected and LPS-stimulated cells, cell lysates were produced and Western Blot analysis was performed using actin as a control.

20 Western blot assay - $2x10^6$ cells were lysed in RIPA lysis buffer containing protease inhibitors (cOmpleteTM, Roche, Mannheim, Germany) and phosphatase inhibitor (PhosSTOPTM, Roche). Cell lysates were then separated by 10% SDS-PAGE and electrotransferred to nitrocellulose membranes (Whatman Protran nitrocellulose membrane; neoLab, Heidelberg, Germany). After blocking (TBS/0.05% Tween-20/5% 25 BSA) and washing (TBS/0.05% Tween) steps, immunoblotting with antibodies against DLL-1 and beta-Actin(Cell Signaling Technology). Detection was enhanced by chemiluminescence (ECL; Perkin Elmer, Groningen, Netherlands).

Figure 1 shows that delta-like ligand 1 protein is highly detectable in all LPS- 30 stimulated and bacterial infected monocytes but not in control cells. Thus, delta-like

ligand 1 protein is suitable for detecting a cellular infection of both gram-positive and gram-negative etiology.

Example 2 – Delta-like ligand 1 is upregulated in a mouse sepsis model

5 As we had seen that activated DLL1 is highly abundant in *in vitro* bacterial infected monocytes, behaviour of the Notch ligand was examined in a mouse endotoxin sepsis model. LPS (precisely the lipid part of LPS) is also termed endotoxin and commonly used in animal models of sepsis. In critically ill patients, increased concentrations of serum endotoxin have been associated with the development of sepsis, disease

10 severity, and mortality. The theory that endotoxin plays a significant role in the pathogenesis of human sepsis is supported by the observation that antibiotic therapy can lead to a sudden release of massive amounts of endotoxin from dead bacteria and a worsening of the condition. Here 12 weeks old male mice were used for experimental purposes.

15

Twelve-week old male mice were injected intraperitoneally with either the lipid portion of LPS (n=16) or NaCl (n=15; as a control). After 24 hours, blood was drawn. Cell-free supernatants were harvested and analyzed for levels of mouse delta-like ligand 1 protein using a commercial ELISA assay (abcam) following standard protocol.

20

Figure 2 shows that delta-like ligand 1 protein is elevated in the blood of LPS-infected mice compared to that of control mice. DLL1 is upregulated in a mouse sepsis model. Thus, delta-like ligand 1 protein is suitable for detecting an infection associated with LPS in the blood of an *in vivo* animal model of sepsis.

25

Example 3 – Delta-like ligand 1 is upregulated in sepsis patients

50 patients were included in the study, all of whom showed signs of severe sepsis following abdominal surgery. Sepsis was defined according to the criteria of the

30 Surviving Sepsis Campaign. Only non-pregnant patients of at least 18 years were included. Further exclusion criteria included autoimmune diseases. After inclusion,

blood samples were drawn from septic patients directly following identification of the first signs of sepsis ("t0"), after 24 hours ("t24"), 48 hours ("t48"), and 168 hours ("t168").

5 20 control patients who had undergone abdominal surgery but presented no signs of sepsis ("OP t2") had their blood drawn 48 hours following surgery. 20 healthy volunteers ("healthy") were recruited as non-operated controls and had their blood drawn once.

10 Blood plasma was analyzed for the levels of human delta-like ligand 1 protein and additionally human delta-like ligand 4 protein using commercial ELISA assays (DLL1 - RayBio®; DLL4 - biocat) following standard protocol. Levels of proteins were statistically analyzed using the Mann-Whitney U test.

15 Figure 3A shows that delta-like ligand 1 protein is elevated in the blood of patients diagnosed with sepsis compared to both healthy controls and patients who underwent abdominal surgery, but who did not show signs of sepsis.

Figure 3B shows that delta-like ligand 4 protein is not elevated or not significantly
20 elevated in the blood of patients diagnosed with sepsis compared to both healthy controls and non-infected patients who underwent abdominal surgery.

Thus, delta-like ligand 1 protein, but not the closely related delta-like ligand 4 protein, is suitable for detecting the presence of sepsis in the blood of patients following
25 abdominal surgery and for differentiating the infected patients from both healthy controls and non-infected patients.

The collected data for delta-like ligand 1 protein and delta-like ligand 4 protein were analyzed using the Receiver Operating Characteristic (ROC) Curve. The area under the
30 ROC curve (AUC) was calculated and the data were compared with the established clinically relevant markers of leukocyte count and CRP. In detail the area under the

curve (AUC) of ROC curve t0 versus Op t2 was 0,511 (cutoff 20.73/nl) (Fig. 4A) for leucocytes and 0,795 (cutoff 175.1mg/ml) for CRP (Fig. 4B). The AUC of DLL1 Sepsis t0 versus OP t2 was 0.991 (cutoff 36331 pg/ml) and 1.0 (cutoff 25269 pg/ml) Sepsis t0 versus healthy. Hence the prediction of infection by DLL-1 is far more reliable than
5 the routinely used biomarkers CRP and leucocyte count. The AUC of the DLL4 ROC analysis was 0,696 (cutoff 1084 pg/ml) when compared with OP t2 (Fig. 4E) and 0,655 (cutoff 639.3 pg/ml) when compared with healthy controls (Fig. 4F).

Example 4 – Delta-like ligand 1 is upregulated in sepsis patients

10

Plasma samples collected within various studies were secondarily analyzed for the concentration of Delta-like ligand 1 (DLL1) by ELISA. Overall, 180 adult patients with sepsis (see "Sepsis" in Fig. 5) were analyzed from three independent cohorts as outlined below using the scheme: "[German Clinical Trials Register reference
15 number]/[ethics vote reference number] (responsible committee)":

- Cohort 1: [DRKS00012446] / [S-200/2017] (Heidelberg, Germany), n=30 (see Fig. 5A)
- Cohort 2: [DRKS00005463] / [S-097/2013] (Heidelberg, Germany), n=50 (see
20 Fig. 5B).
- Cohort 3: [DRKS00008090] / [S-247/2014] (Heidelberg, Germany), n=100 (see Fig. 5C).

Samples were taken on the time of study inclusion ("initial) for all three cohorts as
25 well as 24h ("24h") and 48h ("48h") after study inclusion for cohorts 1 and 2. All patients were recruited either according to the Sepsis-2 (2 ≥ SIRS criteria in combination with a clinically suspected or proven infection; Cohorts 1 and 2) or Sepsis-3 (change in SOFA score of 2 ≥ points and clinically suspected or proven infection; Cohort 3) consensus criteria.

30

In addition, Cohorts 1 and 2 enrolled patients after extensive visceral surgery who showed no signs of infection at any time point during the study (see "post-OP" in Fig. 5; Cohort 1: n=30, Cohort 2: n=20) and healthy volunteers ("Healthy"; Cohort 1: n=30, Cohort 2: n=20).

5

Cohort 2 corresponds to the same cohort as described under Example 3. Thus, the data values in Fig. 5 B are identical to the data values in Fig. 3A.

Further controls included a cohort of 38 patients after severe trauma

10 ([DRKS00010991]/ [164/14] (Giessen, Germany); see Fig. 6A) and a cohort of 25 patients subjected to cardiac surgery under extracorporeal circulation ([S-112/2018] (Heidelberg, Germany); see Fig. 6B).

Data analysis was performed with GraphPad Prism (version 6.0, GraphPad Software

15 Inc.). Scatter plots were used for visualization. For group comparisons, non-parametric Mann-Whitney U test was used and a p-value < 0.05 was assumed significant; **** marks p-values < 0.0001.

For the evaluation of the diagnostic value of DLL1, all samples from patients with

20 sepsis (all time points, n=327 samples from 180 patients) were combined and compared to all samples of the control group (all time points, n=377 samples; 327 samples from 113 control patients and 50 samples from 50 healthy volunteers) using ROC analysis. The optimal cut-off was calculated by the Youden index procedure ((Sensitivity + Specificity)-100)).

25

The number of 327 (Sepsis) respectively 377 (Control) samples given above and used in the combined ROC analysis results from patient loss over the observation period (mainly due to death for patients with sepsis and hospital discharge for patients after surgery or severe trauma). Sample numbers are in detail:

30

<u>Cohort 1</u>

Sepsis: 30 / 30 / 27 (initial / 24h / 48h)

Post-OP: 30 / 29 / 28 (initial / 24h / 48h)

Healthy: 30

5 Cohort 2

Sepsis: 50 / 46 /44 (initial / 24h / 48h)

Post-OP: 20 / 20 / 20 (initial / 24h / 48h)

Healthy: 20

10 Cohort 3

Sepsis: 100 (initial)

Severe trauma

38 / 36 / 31 (initial / 24h / 48h)

15

Cardiac surgery

25 / 25 / 25 (pre-OP / 6h / 24h post ECC)

Initially after recruitment, mean plasma concentrations of patients with sepsis in
20 Cohorts 1, 2 and 3 were 60,292 pg/ml (95% CI: 47,820 – 72,765; n=30), 106,126
pg/ml (95% CI: 90,102 – 122,149; n=50) and 56,064 pg/ml (95% CI: 49,494 – 62,634;
n=100), respectively (see Figs. 5A-C). Mean plasma concentrations of patients with
sepsis in Cohorts 1 and 2 at t=24h were 53,027 pg/ml (95% CI: 41,824 – 64,229;
n=30) and 104,944 pg/ml (95% CI: 89,786 – 120,101; n=46), respectively. Mean
25 plasma concentrations of patients with sepsis in Cohorts 1 and 2 at t=48h were 49,485
pg/ml (95% CI: 39,766 – 59,205; n=27) and 88,999 pg/ml (95% CI: 75,490 – 102,508;
n=44), respectively. Levels differed highly significant between sepsis patients and
controls (i.e. control patients and healthy subjects) at the corresponding time points
after surgery.

30

DLL1 concentrations of the cohorts of control trauma patients (see "post-OP" in Figs. 5A and 5B and all data in Fig. 6A), as well as patients before and after cardiac surgery (see Fig. 6B), were remarkably lower compared to patients with sepsis at all time points measured.

5

Mean plasma concentrations of the control surgical patients ("post-OP") in Cohort 1 at t=initial, t=24h, and t=48h were 14,193 pg/ml (95% CI: 12,385 – 16,001; n=30), 19,550 pg/ml (95% CI: 14,536 – 24,565; n=29), and 17,721 pg/ml (95% CI: 15,498 – 19,944; n=28), respectively.

10

Mean plasma concentrations of the control surgical patients ("post-OP") in Cohort 2 at t=initial, t=24h, and t=48h were 13,548 pg/ml (95% CI: 11,275 – 15,821; n=20), 16,187 pg/ml (95% CI: 13,409 – 18,964; n=20), and 19,287 pg/ml (95% CI: 13,618 – 24,955; n=20), respectively.

15

Mean plasma concentrations of the control severe trauma patients in Fig. 6A at t=initial, t=24h, and t=48h were 19,119 pg/ml (95% CI: 16,892 – 21,345; n=38), 19,224 pg/ml (95% CI: 17,184 – 21,263; n=36), and 20,409 pg/ml (95% CI: 16,351 – 24,468; n=31), respectively.

20

Mean plasma concentrations of the control cardiac surgery patients in Fig. 6B at t=initial, t=24h, and t=48h were 13,846 pg/ml (95% CI: 10,633 – 17,059; n=25), 14,603 pg/ml (95% CI: 11,356 – 17,850; n=25), and 22,194 pg/ml (95% CI: 18,497 – 25,891; n=25), respectively.

25

DLL1 concentrations of healthy controls (see "Healthy" in Figs. 5A and 5B), were remarkably lower compared to patients with sepsis at all time points measured. There were no significant differences between healthy controls and control patients. Mean plasma concentrations of healthy controls measured in Cohorts 1 and 2 were 11.928

30 pg/ml (95% CI: 10,645 – 13,211; n=30), and 16,737 pg/ml (95% CI: 14,542 – 18,932; n=20), respectively.

Receiver-operator curve (ROC) analysis of all available samples grouped to sepsis (n=327) or controls (n=377) yielded an area-under-curve (AUC) of 0.9555 (95% CI: 0.9401 – 0.9710; see Fig. 7). An optimal diagnostic cut-off of 29,538 pg/ml was found,
5 yielding a sensitivity of 88.7% and specificity of 93.4%.

Example 5 – Delta-like ligand 1 cleavage products detect bacterial infection *in vitro*

Upon binding to its receptor the transmembrane protein DLL1 is cleaved. The
10 extracellular domain is released into the environment. The transmembrane (TM) domain and the intracellular (IC) domain (TMIC-DLL1) remain linked inside of the cell. Further cleavage events can release the IC-DLL1 that will migrate to the nucleus. To investigate, whether TMIC-DLL1 can be used to prove infections the cleavage product was detected in *in vitro* infected primary monocytes by western blot analyses.
15

Primary monocytes isolated from blood of healthy donors were infected with gram-negative *Escherichia coli (E.coli)* or were stimulated with LPS, the main component of Gramgram negative outer membranes that activates TLR4 signaling. Bacteria were killed by gentamicin 2 h after infection. The next day infected/LPS-treated cells were
20 lysed and analyzed.

Isolation of primary human monocytes - PBMCs were isolated from fresh blood or buffy coat from healthy donors by density gradient centrifugation (Biocoll separating solution, 1.077 g/ml, Biochrom AG, Berlin, Germany). $CD14^{+}$ cells were magnetically
25 labeled with beads (MiltenyiBiotec) and selected via the autoMACS separator (autoMACS, program: possel, Miltenyi Biotec, Bergisch Gladbach, Germany) twice. Purified monocytes (1×10^{6} cells/ml) were cultured in RPMI 1640 (Sigma-Aldrich, Taufkirchen, Germany) supplemented with 100 IU/ml of penicillin, 100 μg/ml streptomycin and 10% heat inactivated fetal calf serum (Promocell, Heidelberg,
30 Germany) at 37°C in a humidified atmosphere in the presence of 5% CO2.

Bacterial cultures - *Escherichia coli (*ATCC25922) were cultured overnight on Columbia blood sheep agar at 37°C at 5% CO_2 in a humidified atmosphere. The next day 1 colony of each culture was transferred into TSB (Tryptic Soy Broth) media and cultured at constant shaking at 200 rpm/37°C until mid-log phase.

5

In vitro infection - 1 × 10^6 sorted $CD14^+$ monocytes were plated in 24-well plate format in 1 ml RPMI/10% FCS. Cells were infected with 10^8 *E.coli* /10^6 monocytes. After 2 h gentamicin was added to a final concentration of 100 ng/ml to kill the bacteria. The next day cells were lysed analyzed.

10

Western blot assay - 2 × 10^6 cells were harvested and washed with PBS. For whole cell lysates monocytes were lysed in 50 µl RIPA buffer (50 mM Tris-HCl, pH7.4; 1% Igepal; 0.25% sodium deoxycholate; 150 mM NaCl; 1 mM EDTA; 1 mM PMSF; 1 mg/ml each aprotinin, leupeptin, and pepstatin; 1 mM Na3VO4; and 1 mM NaF). Samples were
15 vortexed and incubated 30 min on ice. Lysates were then cleared via centrifugation at 14,000 × g for 20 min. Equal amounts of lysates were used for separation by SDS-PAGE (12.5%). After semi-dry transfer onto nitrocellulose membranes (Whatman Protran nitrocellulose membrane; neoLab, Heidelberg, Germany), the latter were blocked with 5% (w/v) BSA in TBS/0.1% (v/v) Tween-20 for 2 h at RT. Probing was
20 performed with antibodies: anti-DLL1, anti-β actin (Cell Signaling Technology, Danvers, MA, USA) Detection was based on enhanced chemiluminescence (ECL; Perkin Elmer, Groningen, Netherlands).

Figure 8 shows that TMIC (transmembrane domain linked to intracellular domain)
25 cleavage product of the delta-like ligand 1 protein is highly detectable in LPS-stimulated and *E. coli* infected monocytes, but not in control cells. Thus, transmembrane domain and intracellular domain DLL1 cleavage products are suitable for detecting an infection, in particular that of gram-negative etiology.

30

Claims

1. Use of delta-like ligand 1 (DLL1) protein or a nucleotide sequence coding for delta-like ligand 1 protein as a biomarker for testing in vitro in a biological sample and diagnosis of a severe infection; wherein detection of an elevated expression level of delta-like ligand 1 protein or the nucleotide sequence encoding delta-like ligand 1 protein is indicative for the diagnosis of the severe infection.

2. The use according to claim 1, wherein the delta-like ligand 1 protein is encoded by the nucleotide sequence SEQ ID NO: 1 or a nucleotide sequence being at least 80% identical to SEQ ID NO: 1.

3. The use according to claim 1 or claim 2, wherein the delta-like ligand 1 protein is a protein having at least 90% identity with the amino acid sequence of SEQ ID NOs: 2 or 3.

4. The use according to any one of claims 1 to 3, wherein the delta-like ligand 1 protein is a cleavage product of the delta-like ligand 1 protein.

5. The use according to claim 4, wherein the cleavage product is a protein having an amino acid sequence of SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6 and/or SEQ ID NO: 7.

6. The use according to any one of claims 1 to 5, wherein the severe infection is sepsis.

7. The use according to claim 6, wherein the biological sample is selected from the group consisting of whole blood, buffy coat, plasma, serum, peripheral blood mononucleated cells (PBMCS), neutrophils, monocytes, T cells, urine, spinal fluid, lymph fluid, external secretions of the skin, tears, and saliva.

8. The use according to any one of claims 1 to 7, wherein the level of expression is detected in a biological sample taken from a patient following a surgery.

9. The use according to claim 8, wherein the surgery is an abdominal surgery.

10. The use according to any one of claims 1 to 9, as guidance of an antibiotic therapy.

11. The use according to any one of claims 1 to 10, wherein the DLL1 protein expression is measured and detected using an enzyme-linked immunosorbent assay (ELISA), an immunoblot assay, mass spectrometry, ELISpot, flow cytometry, or immunohistochemistry.

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12. The use according to any one of claims 1 to 10, wherein the DLL1 nucleotide expression is measured and detected using microarray analysis, polymerase chain reaction (PCR), reverse transcriptase polymerase chain reaction (RT-PCR), Northern blot, Southern Blot, or a serial analysis of gene expression (SAGE).

13. A method for in vitro diagnosis of a severe infection comprising detecting delta-like ligand 1 (DLL1) protein or a nucleotide sequence coding for delta-like ligand 1 protein in a biological sample wherein detection of an elevated level of expression of the delta-like ligand 1 protein or the nucleotide sequence coding for delta-like ligand 1 protein is indicative of the severe infection.

14. The method according to claim 13, wherein the delta-like ligand 1 protein is encoded by the nucleotide sequence SEQ ID NO: 1 or a nucleotide sequence being at least 80% identical to SEQ ID NO: 1.

15. The method according to claim 13, wherein the delta-like ligand 1 protein has an amino acid sequence that is at least 90% identical to SEQ ID NOs: 2 or 3.

16. The method according to any one of claims 13 to 15, wherein the delta-like ligand 1 protein is a cleavage product of the delta-like ligand 1 protein.

17. The method according to claim 16, wherein the cleavage product is a protein having an amino acid sequence of SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6 and/or SEQ ID NO: 7.

18. The method according to any one of claims 13 to 17, wherein the infection is sepsis.

19. The method according to any one of claims 13 to 18, wherein the biological sample is selected from the group consisting of whole blood, buffy coat, plasma, serum, peripheral blood mononucleated cells (PBMCS), neutrophils, monocytes, T cells, urine, spinal fluid, lymph fluid, external secretions of the skin, tears, and saliva.

20. The method according to any one of claims 13 to 19, wherein the level of expression is determined in a biological sample taken from a patient following a surgery.

21. The method according to claim 20, wherein the surgery is an abdominal surgery.

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22. The method according to any one of claims 13 to 21, wherein the DLL1 protein expression is measured and detected using an enzyme-linked immunosorbent assay (ELISA), an immunoblot assay, mass spectrometry, ELISpot, flow cytometry, or immunohistochemistry.

23. The method according to any one of claims 13 to 21, wherein the DLL1 nucleotide expression is measured and detected using microarray analysis, polymerase chain reaction (PCR), reverse transcriptase polymerase chain reaction (RT-PCR), a Northern blot, Southern Blot, or a serial analysis of gene expression (SAGE).

24. The method according to any one of claims 13 to 23, as guidance of an antibiotic therapy.

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FIG. 1

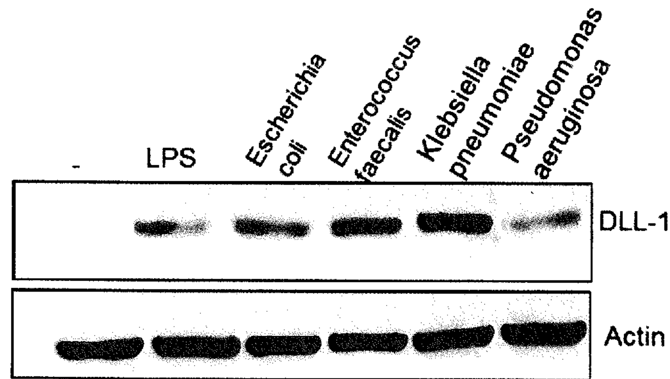
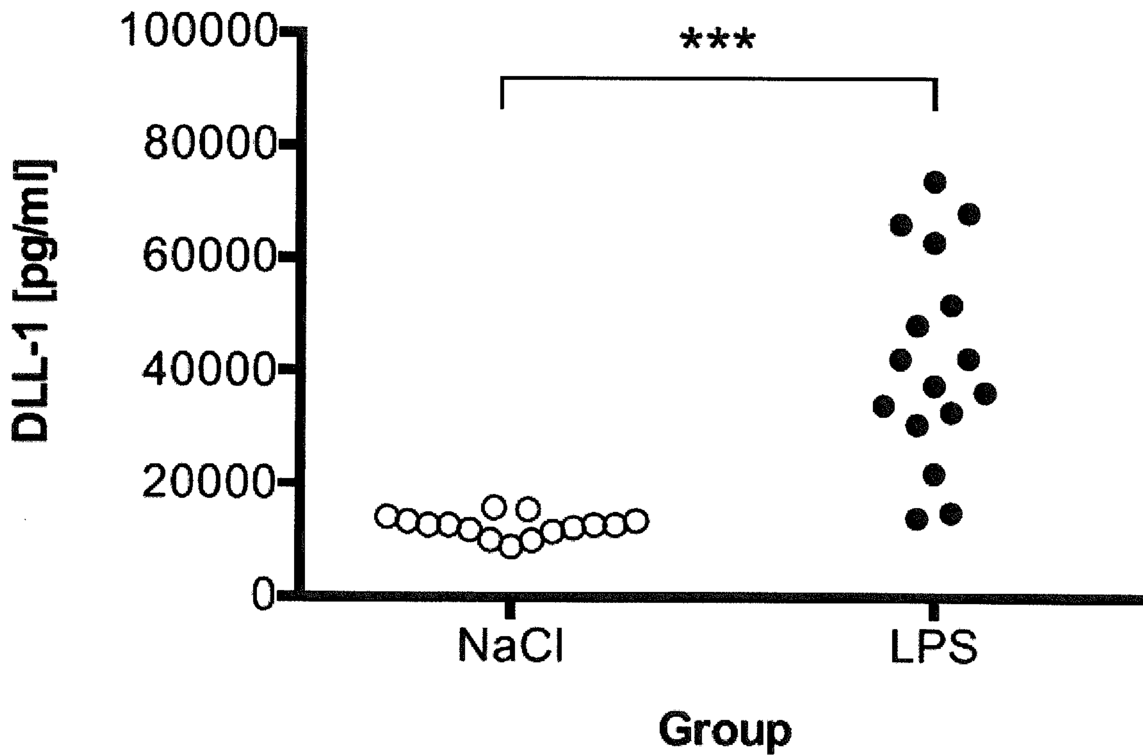


FIG. 2



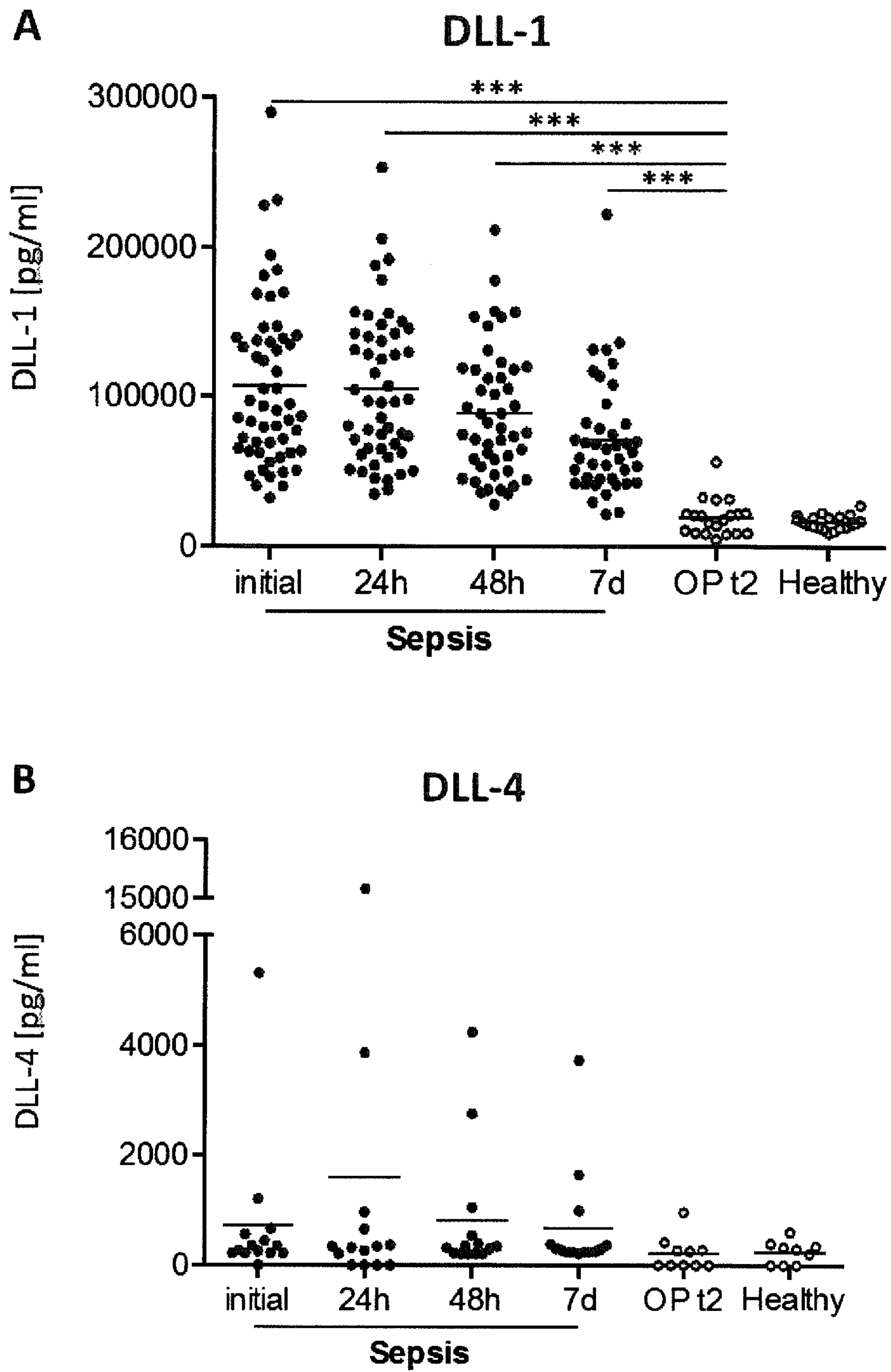
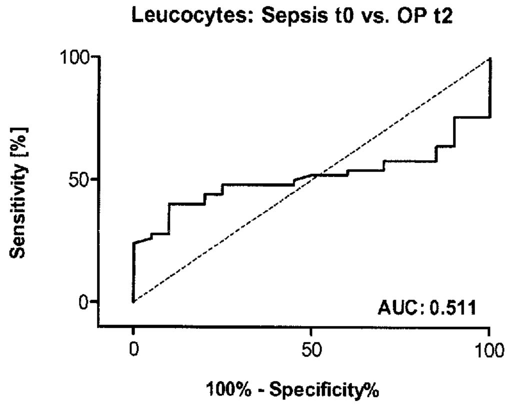
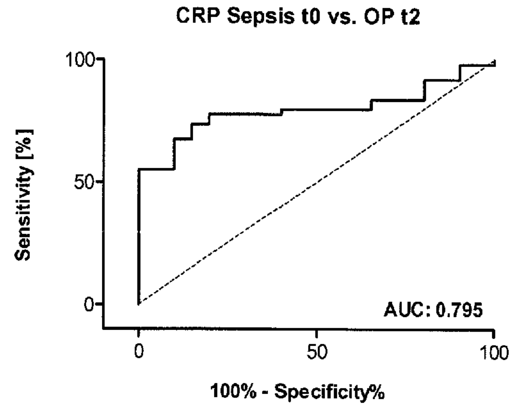
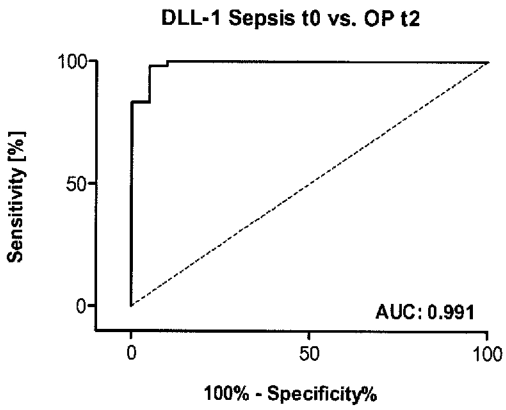


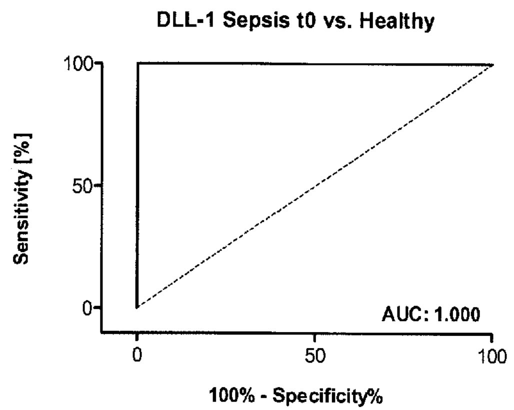
FIG. 3
A
DLL-1
DLL-1 [pg/ml]
300000
200000
100000
0
***
***
***
***
initial
24h
48h
7d
OP t2
Healthy
Sepsis
B
DLL-4
DLL-4 [pg/ml]
16000
15000
6000
4000
2000
0
initial
24h
48h
7d
OP t2
Healthy
Sepsis

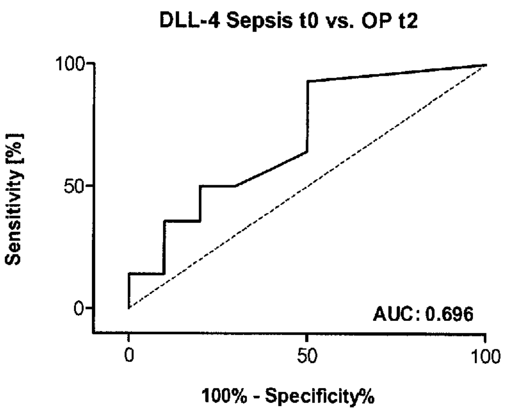
FIG. 4











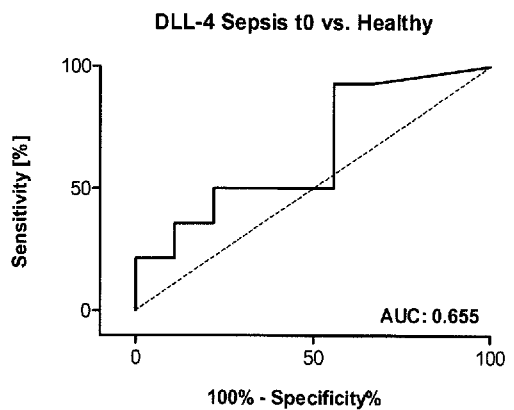


FIG. 5

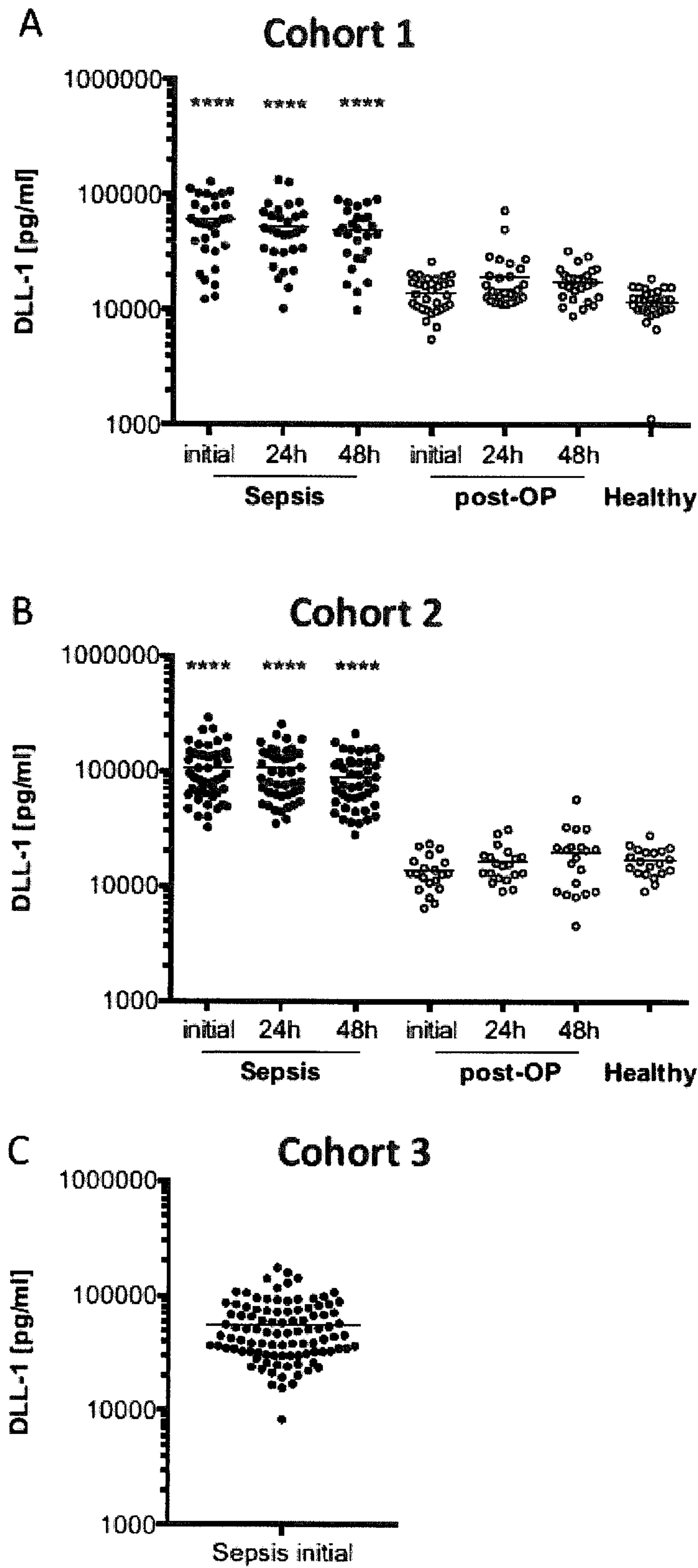


FIG. 6

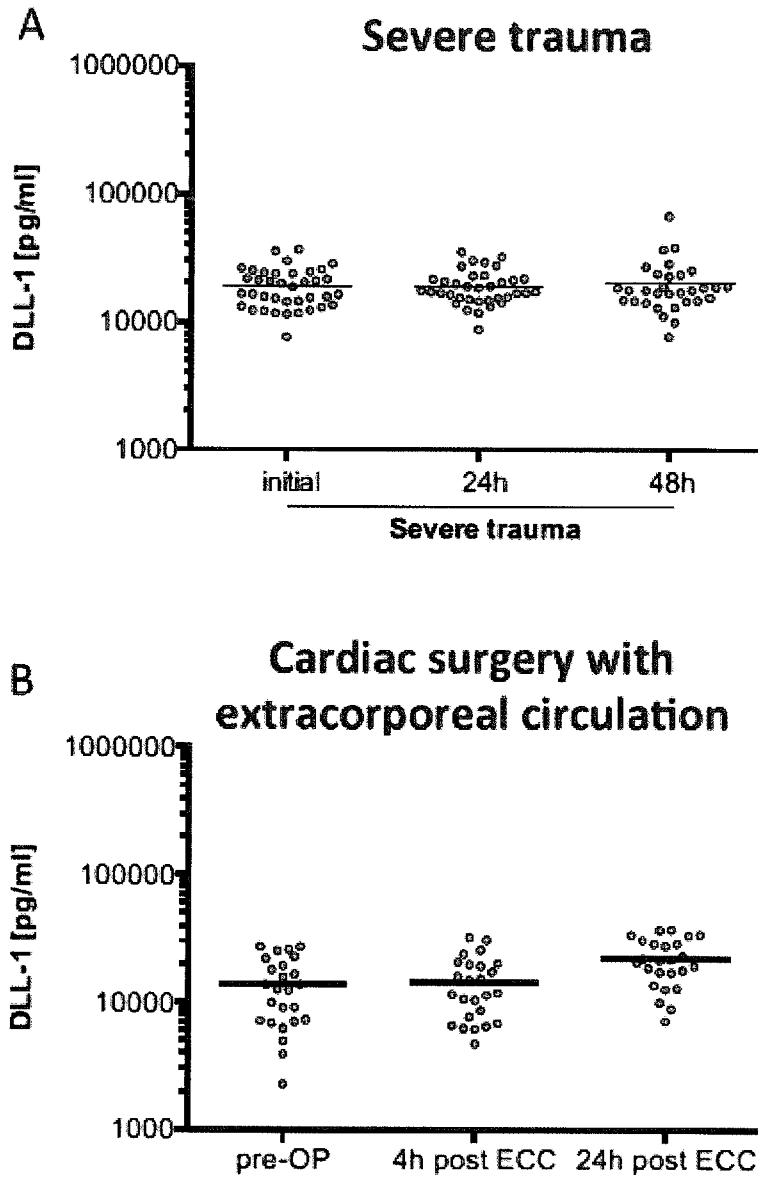
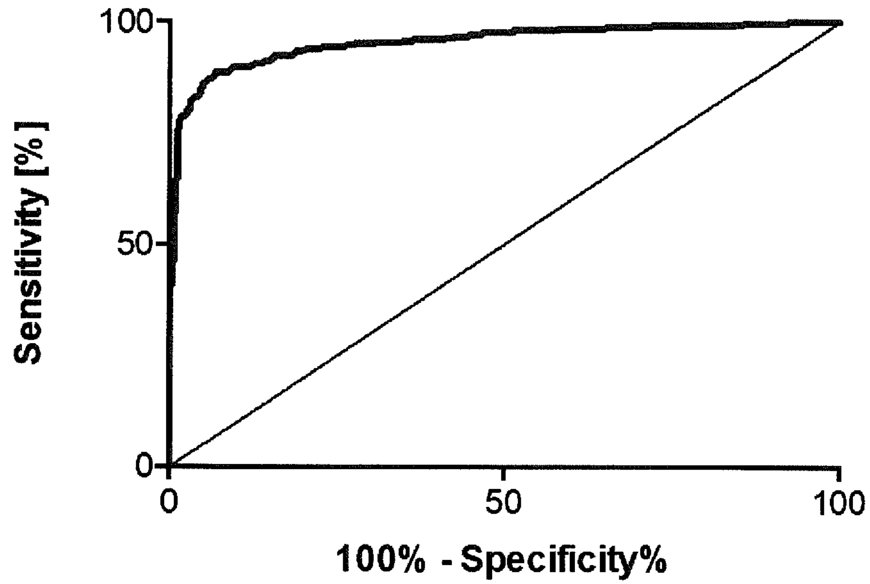


FIG. 7

Sepsis (all time points) vs. Controls (all time points)

Sepsis: 327 samples
Controls: 377 samples



AUC: 0.9555 (0.9401 – 0.9710)

FIG. 8