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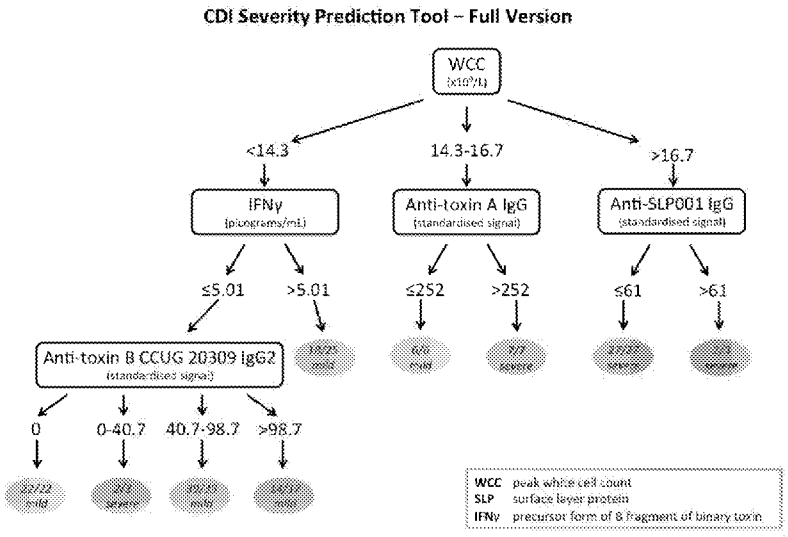

Figure 9

(57) Abstract: The present invention provides a method of determining the severity of a *Clostridium difficile* infection in a subject, the method comprising the steps of: (i) obtaining a sample from a subject having or suspected of having *Clostridium difficile* infection; (ii) detecting one or more biomarkers in the sample, wherein the one or more biomarkers are selected from the group consisting of antibodies to the *Clostridium difficile* toxin A or a fragment thereof, antibodies to the *Clostridium difficile* toxin B or a fragment thereof, antibodies to a *Clostridium difficile* surface layer protein or a fragment thereof, and IFNγ; and (iii) determining, from the results in (ii), whether the subject has a mild *Clostridium difficile* infection or a severe *Clostridium difficile* infection.

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CLOSTRIDIUM DIFFICILE BIOMARKERS AND USES THEREOF

The present invention relates to novel biomarkers that are able to distinguish between different clinical states of *Clostridium difficile* infection, and methods of using the same.

Clostridium difficile is a significant nosocomial and community-acquired pathogen and *Clostridium difficile* infection is amongst the most serious healthcare complications. *Clostridium difficile* infection causes a spectrum of clinical presentations, ranging from an asymptomatic carrier state to more severe diarrhoea and fatal fulminant colitis. *Clostridium difficile* infection is the leading worldwide cause of hospital-acquired and antibiotic-associated diarrhoea, imposing a significant financial burden on health service providers. In Europe alone, the annual estimated costs for the management of *Clostridium difficile* infection amount to €3 billion.

Management of *Clostridium difficile* infection is complicated due to antimicrobial resistance, recurring infections, and strikingly the inability to reliably differentiate between acute infection and those who may be simply carrying a toxigenic strain of *Clostridium difficile* but may have diarrhoea attributed to another cause. For example, in inflammatory bowel disease, prevalence rates of *Clostridium difficile* infection are reportedly higher but it remains unclear if *Clostridium difficile* is contributing to the inflammatory insult or whether it is acting only as a bystander. Pathogenic strains of Clostridium difficile produce two major exotoxins A and B, which are the primary virulence factors contributing to the pathogenesis of *Clostridium difficile* infection. Presently, standard diagnosis of *Clostridium difficile* infection is by detection of these toxins in stool samples. The current approach for treating most *Clostridium difficile* infection involves administration of antibiotics, typically metronidazole as a starting point and vancomycin in more severe cases of infection. Accurate diagnosis of *Clostridium difficile* infection as well as risk stratification of infected patients is critical for effective management. However, there are currently no reliable tools to determine and characterise the severity of *Clostridium difficile* infection in patients, meaning that patients are not always provided with the most suitable care and/or therapy. Ultimately this can result in increased antimicrobial resistance, recurring infections and the need for more emergency invasive treatment.

Current conventional laboratory and clinical parameters do not accurately and consistently predict clinical outcomes. No biomarkers are currently being used for predictive purposes in standard clinical practice. In fact, there are no MHRA or FDA-approved seroprognostic or other laboratory-based tests that can reliably distinguish between mild and severe infection in *Clostridium difficile* infection. The lack of evidence-based prognostication strategies limits effective patient management and use of healthcare resources.

There is thus an unmet clinical need for novel biomarkers that have prognostic value to facilitate the implementation of more targeted management strategies that could improve overall clinical outcomes, especially survival. Predicting the severity for *Clostridium difficile* infection is particularly important since treatment strategies are stratified based on disease severity. Specifically, oral metronidazole is indicated for mild *Clostridium difficile* infection, vancomycin for severe *Clostridium difficile* infection, with addition of intravenous metronidazole for severe-complicated disease. Compared with the costs of the cheapest standard-of-care antibiotic (metronidazole), vancomycin is 20 times more expensive. From a health economics perspective, this fold difference is even more striking if one considers the costs of newer treatments such as Fidaxomicin (160-fold difference) and monoclonal antitoxin antibodies such as Bezlotoxumab (greater than 300-fold difference).

It is therefore an aim of the present invention to provide biomarkers that may be used to give an indication of the prognosis of *Clostridium difficile* infection in a patient and/or to select patients for a particular therapy. Such prognostic biomarkers will allow therapies to be targeted to those most in need and those most likely to benefit. Such biomarkers will also enable stratification of patients to identify those that will respond to a particular course of treatment and those that will not. This will allow patients to be given the most appropriate treatment quickly, and avoid the administration of antibiotics which will not be effective.

Accordingly, in a first aspect of the invention there is provided a method of determining the severity of a *Clostridium difficile* infection in a subject, the method comprising the steps of:

(i) obtaining a sample from a subject having or suspected of having a *Clostridium difficile* infection;

(ii) detecting one or more biomarkers selected from the group consisting of antibodies to *Clostridium difficile* toxin A or a fragment thereof, antibodies to *Clostridium difficile* toxin B or a fragment thereof, antibodies to a *Clostridium difficile* surface layer protein or a fragment thereof, and IFNγ, in the sample; and

(iii) determining from the results in (ii) whether the subject has a mild *Clostridium difficile* infection or a severe *Clostridium difficile* infection.

In particular, the method of the invention allows a subject with mild *Clostridium difficile* infection to be distinguished from a subject with severe *Clostridium difficile* infection. In a further embodiment the method of the invention may be used to predict the likely 30 day all-cause mortality in a subject with a confirmed *Clostridium difficile* infection. Prior to undertaking the method of the invention, a subject may have been diagnosed with a severe *Clostridium difficile* infection by one or more of the following parameters, having peripheral leucocytosis $\geq 15x10^9/L$; having an increase in serum creatinine $\geq$ 1.5 x above baseline; the presence of pseudomembranous colitis; having a toxic megacolon; having an intestinal perforation; having septic shock requiring intensive care admission or the need for colectomy.

In an embodiment of the invention, in step (ii) at least one biomarker selected from the group consisting of IFNγ; antibodies to *Clostridium difficile* toxin A or a fragment thereof; and antibodies to the *Clostridium difficile* surface layer protein SLP001 or a fragment thereof; is detected. The absolute levels of the one or more biomarker may be determined, or the relative levels may be determined. If relative levels are determined these may be represented as arbitrary units, which may be denoted as standardised signals.

In a further embodiment of the invention, in step (ii) at least antibodies to the *Clostridium difficile* toxin A or a fragment thereof are screened for. The detection of antibodies to the *Clostridium difficile* toxin A or a fragment thereof may be sufficient to determine whether or not a subject has a mild or a severe *Clostridium difficile* infection. In one embodiment only antibodies to the *Clostridium difficile* toxin A or a fragment thereof are screened for.

In some embodiments of the invention, the peak white cell count (WCC) of a subject may also be utilised in a method of the invention. The method may include the step of determining the peak WCC. The peak WCC may be used to determine which subjects should be analyzed further to determine the severity of the *Clostridium difficile* infection.

In an embodiment, if the peak WCC is less than about $14x10^9$/L then a subject may be considered to have a mild *Clostridium difficile* infection and should be treated accordingly. If a subject has a peak WCC of greater than about $17x10^9$/L then a subject may be considered as having a severe *Clostridium difficile* infection, and should be treated accordingly. However, for subjects with a peak WCC between about $14x10^9$/L and about $17x10^9$/L, it is more difficult to determine if the subject has a mild *Clostridium difficile* infection or a severe *Clostridium difficile* infection, in this case the detection of antibodies to the *Clostridium difficile* toxin A or a fragment thereof in the sample may be used to determine if a subject has a mild or a severe *Clostridium difficile* infection. The cut off level for a mild infection may be a peak WCC of about $14.3x10^9$/L and below. The cut off level for a severe infection may be a WCC of about $16.7x10^9$/L and above. A peak WCC level of between about $14.3x10^9$/L and about $16.7x10^9$/L may mean that the detection of antibodies to the *Clostridium difficile* toxin A or a fragment thereof in the sample is needed to determine if a subject has a mild or a severe *Clostridium difficile* infection. The WCC may be determined per litre of blood.

In an embodiment, only antibodies to the *Clostridium difficile* surface layer protein SLP001 or a fragment thereof may be detected, or they may be detected in combination with another marker, for example in combination with the peak WCC, and this may be used to determine the severity of the *Clostridium difficile* infection.

In an embodiment, IFN-γ levels may be detected in combination with another marker, for example in combination with peak WCC, and this may be used to determine the severity of a *Clostridium difficile* infection.

In another aspect the invention provides a method to predict the 30 day all-cause mortality of individuals identified to have a *Clostridium difficile* infection, the method comprising the steps of:

(i) obtaining a sample from a subject having or suspected of having *Clostridium difficile* infection;

(ii) detecting one or more biomarkers selected from the group consisting of antibodies to the *Clostridium difficile* surface layer protein SLP001 or a fragment thereof, antibodies to the *Clostridium difficile* surface layer protein SLP002 or a fragment thereof, antibodies to the *Clostridium difficile* protein pCDTb or a fragment thereof, and antibodies to the *Clostridium difficile* toxin B or fragment thereof: and

(iii) predicting, based on the observations in (ii), whether the subject is likely to survive for 30 days or more.

In the method of the invention, antibodies to the *Clostridium difficile* surface layer protein SLP001 or a fragment thereof may be screened initially. Thereafter, one or more of antibodies to the *Clostridium difficile* surface layer protein SLP002 or a fragment thereof, antibodies to the *Clostridium difficile* toxin B or a fragment thereof and antibodies to the *Clostridium difficile* protein pCDTb or a fragment thereof, may also be screened for. The results of these screens may be used in conjunction with screening for antibodies to SLP001 to predict whether the subject is likely to survive for 30 days or more.

All whole *C. difficile* surface layer proteins described herein, derived from *C. difficile* ribotypes 001, 002 and 027, may be purified from *C. difficile* anaerobic broth cultures in accordance with the methods described by Ryan et al, PLoS Pathogens 2011; 7(6): e1002076 and Lynch et al, BMC Evol Biol 2017; 17: 90. Lynch et al, also detail the GenBank accession numbers for the *slpA* gene of each ribotype.

pCDTb is a precursor form of the B fragment of the *Clostridium difficile* binary toxin. pCDTb may be produced in *Escherichia coli* from a wholly synthetic recombinant gene construct; the amino acid sequence may be based on the published sequence for 027 ribotype http:www.uniprot.org/A8DS70. The steps involved in the cloning, expression and purification of pCDTb are described in Sundriyal et al, Protein Expr Purif 2010; 74 (1): 42-8.

The method to predict the 30 day all-cause mortality may be undertaken on all individuals with *Clostridium difficile* infection.

In an embodiment, higher levels of antibodies to the *Clostridium difficile* surface layer protein SLP001 or a fragment thereof, and higher levels of antibodies to the *Clostridium difficile* surface layer protein SLP002 or a fragment thereof, and higher levels of antibodies to the *Clostridium difficile* protein pCDTb may be predictive of a 30 day or less mortality in a subject.

The method of the invention may be used to offer personalised treatments to individuals depending on the severity of the *Clostridium difficile* infection. Where a mild *Clostridium difficile* infection is indicated the therapy offered may be oral administration of metronidazole. Where a severe *Clostridium difficile* infection is indicated vancomycin, and/or fidaxomicin, may be offered, together with optionally intravenous metronidazole. Intravenous immunoglobulin (IVIG) may also be administered if a severe infection is identified.

The method of the invention may be used, for example, for any one or more of the following: to advise on the prognosis for a subject with a *Clostridium difficile* infection; to monitor infection progression; to advise on treatment options; and to monitor effectiveness or response of a subject to a treatment for *Clostridium difficile* infection.

The sample may be a bodily fluid, such as, blood, serum, plasma, urine, spinal fluid, synovial fluid, sputum, mucus or lymph. The sample is preferably a blood sample, for example a whole blood sample, blood serum sample, or blood plasma sample.

The method of the invention may include the step of taking the sample from the subject. Alternatively, the method of the invention may not include in step of taking the sample, but instead the sample may be provided after it has been taken from the subject.

In one embodiment, the subject may already have been diagnosed with *Clostridium difficile* infection before the method of the invention is undertaken. The diagnosis of

the *Clostridium difficile* infection may be based on an assessment of one or more of clinical presentation, pathology and other biomarker expression levels, for example the presence of *Clostridium difficile* toxin proteins in a stool sample.

The subject may be a human or a non-human animal. In one embodiment, the subject is a human. A non-human animal may include dogs, cats, horses, cows, pigs, sheep and non-human primates.

The level of a specific biomarker in a sample, be it an antibody or a protein, may be determined by any suitable method, for example, by immunohistochemistry, spectrometry, ELISA, immunoprecipitation, western blot, or dot blot assay, protein microarray, radioimmunoassay (RIA), fluoroimmunoassay, an immunoassay using an avidin-biotin or streptoavidin-biotin system, and combinations thereof. These methods are well known to the person skilled in the art and other similar methods may also be used.

The level of one or more biomarkers may be determined using targeted tandem mass spectrometry (MS) methods. Examples of such methods include the: accurate inclusion mass spectrometry (AIMS), and quantitative selection reaction monitoring (Q-SRM).

The antibodies detected in a method of the invention may be total IgA antibodies (and IgA subclasses, including IgA1 and IgA2), total IgG antibodies (and IgG subclasses including IgG1, IgG2, IgG3, IgG4), total IgM antibodies, or combinations thereof.

The anti-toxin A antibodies detected in a sample may be IgA antibodies (and IgA subclasses), IgG antibodies (and IgG subclasses), IgM antibodies, or combinations thereof. In one embodiment, the anti-toxin A antibodies detected are IgG antibodies.

The anti-toxin B antibodies detected in a sample may be IgA antibodies (or IgA sublasses) or IgG antibodies (or IgG subclasses).

The anti-SLP001 antibodies detected may be IgG antibodies (or IgG subclasses).

The anti-SLP002 antibodies detected may be IgG antibodies (or IgG subclasses).

The anti-pCDTb antibodies detected may be IgG antibodies, for example IgG2 antibodies or other IgG subclasses.

The one or more antibodies may be detected, for example, by using a protein array, wherein the array carries the antibody target and by detecting the level of binding to the array at a particular position (corresponding to the antibody target) an arbitrary level of a particular antibody can be assigned to a particular sample. Whilst the level assigned may be arbitrary (denoted as a standardised signal), it is comparable with other targets in the array and between samples which have used the same array under the same conditions.

The method of the invention may be carried out in vitro.

According to a further aspect, the invention provides a panel of biomarkers, or a panel of agents for detecting a panel of biomarkers, for use in determining the severity of a *Clostridium difficile* infection in a subject, the panel of biomarkers comprising at least one of:

(i) antibodies to the *Clostridium difficile* toxin A or a fragment thereof;

(ii) antibodies to the *Clostridium difficile* toxin B or a fragment thereof,

(iii) IFNγ;

(iv) antibodies to a *Clostridium difficile* surface layer protein or a fragment thereof; and

(v) antibodies to the *Clostridium difficile* protein pCDTb or a fragment thereof.

In order to predict the severity of a *Clostridium difficile* infection in a subject the panel preferably comprises at least antibodies to the *Clostridium difficile* toxin A or fragment thereof.

In order to predict the 30 day all-cause mortality in a subject with a severe *Clostridium difficile* infection, the panel preferably comprises at least antibodies to the *Clostridium difficile* surface layer protein SLP001 or a fragment thereof, and optionally one or more of antibodies to the *Clostridium difficile* surface layer protein

SLP002 or a fragment thereof, and antibodies to the *Clostridium difficile* protein pCDTb or a fragment thereof.

In another aspect, the invention provides means to detect the biomarkers in the biomarker panel of the invention. This means that a panel of proteins or peptide targets may be used to detect the levels of antibodies in a sample which recognize these proteins or peptides. By determining the level of the biomarkers in a sample, either there absolute or relative levels, the severity or 30 day all-cause mortality in a subject with a *Clostridium difficile* infection can be determined.

In another aspect of the invention, there is provided a use of a method of the invention to detect biomarkers in a sample to determine the prognosis for a subject with a *Clostridium difficile* infection.

According to another aspect to the invention there is provided a method of choosing the most appropriate treatment for a subject with a *Clostridium difficile* infection by performing the method of the invention on a sample from the subject and choosing, and optionally administering, treatment based on the biomarkers detected in the sample.

In another aspect of the invention, there is provided a method of treating a *Clostridium difficile* infection, the method comprising the steps of:

(i) detecting anti-toxin A antibody in a sample from a subject; and

(ii) administering treatment for the *Clostridium difficile* infection based on the anti-toxin A antibody levels.

The skilled person will appreciate that preferred features of any one embodiment and/or aspect of the invention may be applied to all other embodiments and/or aspects of the invention.

There now follows, by way of example only, a detailed description of the present invention with reference to the accompanying figures, in which:

Figure 1 – is a representative analysis of *Clostridium difficile* antigens using silver stain. Antigens were run on 4-20% precast polyacrylamide gels with a

broad range of protein markers. The 7 antigens were then silver stained to check for purity and showed bands at the expected molecular sizes.

Figure 2 - lists the immunoglobulins used in this study.

Figure 3 – lists the cytokines used in this study.

Figure 4 – is a univariate analysis for severe *Clostridium difficile* infection.

Figure 5 – is a univariate analysis for 30-day all-cause mortality.

Figure 6 – is a regression tree for *Clostridium difficile* infection severity.

Figure 7 – is a regression tree for *Clostridium difficile* infection 30 day all-cause mortality.

Figure 8 – shows performance metrics for severity and 30-day all-cause mortality.

Figure 9 – is an 'Easy read' version of the severity decision tree.

Figure 10 – is an 'Easy read' version of the 30-day all-cause mortality decision tree

Figure 11 – a simplified clinical version of the severity decision tree.

Figure 12 – a simplified clinical version of the 30-day all-cause mortality decision tree.

MATERIALS AND METHODS

Data from a cohort of inpatients with *Clostridium difficile* infection at Nottingham University Hospitals NHS Trust was analysed from 2009 to 2013. All patients with *Clostridium difficile* infection had diarrhoea (defined as a change in bowel habit with 3 or more unformed stools per day for at least 48h) and a positive stool *Clostridium*

difficile (enzyme immunoassay) toxin test. Severe *Clostridium difficile* infection was defined as peripheral leukocytosis $\geq 15x10^9$/L or an increase in serum creatinine $\geq$ 1.5 times above baseline, or pseudomembranous colitis, megacolon, intestinal perforation, need for colectomy or septic shock requiring intensive care unit admission.

Demographics, co-morbidities (including Charlson co-morbidity index), history, laboratory data [peak white cell count (WCC), eosinophil count, C-reactive protein (CRP), minimum albumin], imaging and endoscopy data, previous history of *Clostridium difficile* infection and 30-day all-cause mortality were recorded. Serum subclass and strain-specific antitoxin and anti-surface layer protein (SLP) antibody responses were determined by an established and validated *Clostridium difficile* antigen-specific protein microarray. Anti-toxin A and B neutralising antibodies (NAb) were assessed using a Caco-2 cell-based neutralisation assay. An antibody microarray assay was used for the simultaneous quantification of serum interleukin-(IL-) 1-α, IL-1β, IL-6, IL-8, IL-12/23, IL-27, tumour necrosis factor-α (TNF α)-, granulocyte-macrophage colony stimulating factor (GM-CSF), interferon-gamma (IFN-γ), and transforming growth factor-β (TGF- β).

Antigen Microarray

A panel of seven *Clostridium difficile* antigens were included in this study: highly purified *Clostridium difficile* whole toxins A and B (toxinotype 0, strain VPI 10463, ribotype 087), toxin B from a *Clostridium difficile* toxin-B only expressing strain (CCUG 20309), precursor form of B fragment of binary toxin, pCDTb (produced from a wholly synthetic recombinant gene construct) and purified native whole ribotype-specific (001, 002, 027) surface layer proteins (SLPs). In addition, two antigens of common pathogens as positive control targets were also included: tetanus toxoid (National Institute for Biological Standards and Control (NIBSC)) and lysates from Candida albicans (Jena Bioscience). Moreover, 10-point two-fold serial dilutions of purified human immunoglobulin matching the tested antibody isotype were printed in each array as a standard curve, 4 types were used; IgG (Sigma Aldrich, UK), IgG2, IgA, IgA1 (Athens Research & Technology, Inc, USA).

Assessment of the quality of the antigens

The quality of the *Clostridium difficile* antigens was assessed by electrophoresis and silver stain. For electrophoresis, an equal volume of NovexTm 2x SDS Tris-Glycine

Loading buffer with 40mM DTT was added to each antigen (1ug) and following heating at 90°C for 5min, antigens were separated on Novex 4–20% Tris glycine polyacrylamide gels (Invitrogen Life Technologies, USA). Silver stain was performed as stated in the Pierce' Silver Stain Kit manufacturer's instructions (Thermo Scientific, UK). Briefly, the gel was fixed twice using 2 x 25mL of a 30% Ethanol, 10% Glacial Acetic Acid and 60% De-ionised water for 15min each, then washed twice in 2 x 25mL of a 10% ethanol solution for 5min each followed by washing twice in ultrapure water for 5 minutes each. After 1min incubation in a sensitizer working solution, the gel was washed twice in ultrapure water for 1 min each. A stain working solution was added for 30 min, followed by washing twice in ultrapure water for 20 seconds each. The developer working solution was added immediately for 2-3min and replaced with the stop solution after the first band appeared (Figure 1).

Microarray procedure

The applicants have previously established and validated a *Clostridium difficile* multiplex protein microarray system to measure the antibodies in patient sera to specific *Clostridium difficile* antigens (Negm et al, Clinical Exp Immunology, 2017, Negm et al, Clin Vaccine Immunol, 2015, 22(9): 1033-1039) . Each antigen was diluted in PBS-Tween-Trehalose (50mM) at the optimum concentration which was tested before running the patient sera; toxins A (200 µg/mL) and B (100 µg/mL), toxin B CCUG 20309 (90 µg/mL), pCDTb (200 µg/mL) and purified SLPs (200 µg/ml); Candida and Tetanus (100 µg/mL). Diluted antigens were loaded onto a 384 well plate (Genetix, UK), and printed in quadruplicate onto aminosialine-coated glass slides (Schott, Germany) using a contact arrayer (MicroGridII; Digilab, Marlborough, MA, USA) and a silicon contact pin (Parallel Synthesis Technologies, USA). Printed slides were blocked with fresh 5% BSA for one hour and washed three times with 0.05% PBS-Tween washing buffer. Serum samples were appropriately diluted in antibody diluent according to the immunoglobulin used (Figure 2) for 1 h with shaking. Following three times washing with PBS-Tween 0.05%, slides were incubated with the relevant biotinylated secondary antibodies for one hour with shaking. Finally, slides were incubated with streptavidin-conjugated cy5 diluted 1:2000 in fresh 5% BSA (E-Biosciences, UK). Probed slides were scanned by GenePix scanner at 635nm and 400 PMT. The images were processed with Genepix Pro-6 Microarray Image Analysis software (Molecular Devices Inc.). Protein signals were determined after background subtraction though customized modules in the R statistical language to generate

general mean of signal levels. Specific isotype responses were interpolated against the internal isotype standard curve for each sample.

Cytokine/Antibody microarray

Cytokines were analysed in the serum samples from *Clostridium difficile* patients by antibody microarray. DuoSet paired antibody kits (R&D Systems, Minneapolis, MN) were used for the detection of 12 cytokines (Figure 3): Human IL-27, BAFF/TNFSF13, Human IL-β/IL-1F2, Human IL-6, Human IL-α/IL-1F1, Human IFN-Y, Human TNF-α, Human GM-CSF, Human CXCL8/IL-8, Human TGF-β1, Human APRIL/TNFSF13 and Human IL-12/IL-23 P40. The array-based methodology has been previously established (Selvarajah et al, Mediators Inflamm, 2014, 2014:820304) Briefly, 100 μg/mL of capture antibodies were diluted in PBS-50 mM Trehalose and printed onto aminosialine-coated glass slides (Schott, Germany) using a contact arrayer (MicroGridII; Digilab, Marlborough, MA, USA) and a silicon contact pin (Parallel Synthesis Technologies, USA). The slides were blocked with 5% BSA blocking buffer for one hour at room temperature with shaking. After washing the slides three times with PBS-Tween 0.05%, a cocktail of recombinant protein standards was prepared containing each cytokine at the maximum recommended concentration for standard curve generation and diluted twofold across eight dilutions. The standards and samples were added to the relevant blocks for one hour at room temperature with frequent shaking followed by washing with PBST three times. A mixture of biotinylated detection antibodies was added to each block for 1 h with shaking. Reaction was detected by streptavidin-conjugated cy5 diluted 1:1000 in fresh 5% BSA (E-Biosciences, UK). The slides were scanned at 635 nm with a Genepix 4200AL scanner (Axon GenePix®) and fluorescence intensities were quantified using Axon Genepix Pro-6 Microarray Image Analysis software. The concentration of each cytokine in different samples was extrapolated from the standard curve values and presented in pictograms per mL (pg/mL).

Toxin neutralisation assay

A Caco-2 cell-based assay for anti-toxin A and anti-toxin B neutralising antibodies was used as previously published. Briefly, Caco-2 cells (HTB-37; ATCC) were maintained in minimal essential medium (MEM) plus 20% fetal calf serum, 2mM

glutamine and non-essential amino acids at 37°C. Serum samples were diluted in the assay medium at three dilutions (1:10, 1:100 and 1:1000), then premixed with toxin A or B (at a 50% lethal dose [LD50]) for 1 h at 37°C before 50 µL of this mixture was transferred to the cells and incubated for 96 h. Following aspiration of the medium, 50 µL methylene blue (0.5% [wt/vol] dissolved in 50% [vol/vol] ethanol) was added to the cell culture and incubated for 1 h at room temperature. Then, the cells were washed gently with tap water (to remove excess stain) and air dried. The cells were then lysed by adding 100 µL 1% (vol/vol) N-lauryl-sarcosine and incubated on a shaker for 15 min at room temperature. The cell biomass was determined by measuring the absorbance of each well on a BioTek Synergy2 (BioTek, USA) plate reader at 405nm. Toxin activity and working LD50 concentrations were defined empirically in preliminary experiments and for each individual batch/lot of toxin used.

Statistical analysis

Descriptive statistics for demographics and outcomes are reported as median (range) or frequency (percent). Univariate and multivariate logistical regression models examined a set of clinical, demographic and immunological variables. These models were used to assess the association of the candidate variables with severe outcomes and 30-day mortality.

A machine-learning approach (regression Tree Model) was used for constructing decision tree algorithms tailored to protein microarray and clinical data sets to predict factors associated with severe *Clostridium difficile* infection and all-cause 30-day mortality. The same variables evaluated by decision tree analysis were also entered as potential predictors of severity and 30-day all-cause mortality in separate multivariable logistic regression models. Covariates were selected using correlation matrix among predictor variables for possible interactions. A cut-off value of 0.05 was used to include/exclude covariates in the binary multivariable logistic regression models.

The performance of both models was assessed by the following metrics: sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), diagnostic accuracy and the Receiver operating Curve (ROC) value. The chi-square test compared the performance metrics between the models.

The version of decision tree used was CHAID (Chi-squared Automatic Interaction Detection). CHAID chooses the independent (predictor) variable that has the strongest interaction with the dependent variable. Categories of each predictor are merged if they are not significantly different with respect to the dependent variable.

The performance of the decision tree models were internally validated using the k-fold cross-validation (also named one versus all) approach. Using this statistical technique, the original data is randomly partitioned into k subsamples of equal sizes. Of the k subsamples, k-1 samples are used for model training while one subsample is retained for model validation. The whole process is repeated for each of the k-folds, with each of the k subsamples used exactly once as the validation data. Finally, the results from different k folds can be merged to produce a single estimation. Discrimination of the original and cross-validated models was evaluated through the generation of receiver operating characteristic (ROC) curves and calculation of C-statistics in R.

RESULTS

Of 151 subjects (52.3% female, median age 72 y, range 19-98 y), severe *Clostridium difficile* infection was present in 32% and 30-day all-cause mortality was 8.6%, respectively. In univariate analyses, factors independently associated with severe *Clostridium difficile* infection were: peak WCC, minimum albumin, and CRP (Figure 4); for 30-d all-cause mortality: peak WCC, SLP001IgG (Figure 5).

Separate regression tree algorithms were constructed for *Clostridium difficile* infection severity and 30-day all-cause mortality. These are illustrated in Figures 6 and 7.

For severity (Figure 6), the first splitting parameter is peak white cell count (10^9/L); partitioned into 3 categories. Anti-toxin A IgG is partitioned into 2 categories. Using these two factors alone, a clinical prediction rule can be produced which correctly categorized 93.3% of the cohort into mild or severe infection. Accuracy can be further improved by examining IFN-γ, anti-SLP001 IgG and anti-toxin B IgG2.

For 30-d all-cause mortality (Figure 7), anti-SLP001 IgG, anti-SLP002 IgG and anti-pCDTb IgG2 were each portioned into 2 categories. Using these 3 factors, a predictive tool for 30-day all-cause mortality was able to correctly categorize 92.1% of the

cohort. Predictive accuracy can be further improved by examining anti-toxin B IgG, anti-toxin B IgA and anti-SLP001 IgA.

The corresponding performance metrics (sensitivity, specificity, accuracy, positive predictive value, negative predictive value and ROC value) for severity and 30-day all-cause mortality are presented in Figure 8.

Two easy-read versions of the above algorithms (Figures 9 and 10) were produced as well as simplified prediction algorithms or tools (Figure 11 and 12) for predicting disease severity and 30-d all-cause mortality based on the Decision Tree Model analysis.

Following implementation of the binary multivariable logistic regression model, the performance metrics for predicting severity of *Clostridium difficile* infection was as follows: Sensitivity = 75.8%, specificity = 97.4%, Accuracy = 90.9%, Positive prediction value (PPV) = 92.6%, negative prediction value (NPV) = 90.4%.

CONCLUSIONS

Presented herein are two novel microarray-based clinical risk stratification tools for patients with a *Clostridium difficile* infection who may benefit from receiving early, more aggressive and personalized treatment interventions. These decision tree prediction models correctly categorize severity as well as 30-day all-cause mortality in 91.9%, 95.4% of this patient cohort respectively.

CLAIMS

1. A method of determining the severity of a *Clostridium difficile* infection in a subject, the method comprising the steps of:

(i) obtaining a sample from a subject having or suspected of having *Clostridium difficile* infection;

(ii) detecting one or more biomarkers in the sample, wherein the one or more biomarkers are selected from the group consisting of antibodies to the *Clostridium difficile* toxin A or a fragment thereof, antibodies to the *Clostridium difficile* toxin B or a fragment thereof, antibodies to a *Clostridium difficile* surface layer protein or a fragment thereof, and IFNγ; and

(iii) determining, from the results in (ii), whether the subject has a mild *Clostridium difficile* infection or a severe *Clostridium difficile* infection.

2. The method of claim 1 wherein the method allows a subject with a mild *Clostridium difficile* infection to be distinguished from a subject with a severe *Clostridium difficile* infection.

3. The method of claim 1 or 2 which is further able to predict the likely 30 day all-cause mortality in a subject with a confirmed *Clostridium difficile* infection.

4. The method of any preceding claim wherein in step (ii) at least one biomarker selected from the group consisting of IFNγ; antibodies to *Clostridium difficile* toxin A or a fragment thereof; and antibodies to the *Clostridium difficile* surface layer protein SLP001 or a fragment thereof; is detected.

5. The method of any preceding claim wherein in step (ii) at least antibodies to the *Clostridium difficile* toxin A or a fragment thereof are screened for.

6. The method of any preceding claim further comprising the step of determining the peak WCC.

7. The method of claim 6 wherein a peak WCC of less than about $14x10^9$/L is diagnostic of a mild *Clostridium difficile* infection

8. The method of claim 6 wherein a peak WCC of greater than about $17x10^9$/L is diagnostic of a severe *Clostridium difficile* infection

9. The method of claim 6 wherein both the peak WCC is determined and antibodies to the *Clostridium difficile* toxin A or a fragment thereof are screened for.

10. The method of any of claims 1 to 4 wherein only antibodies to the *Clostridium difficile* surface layer protein SLP001 are screened for.

11. The method of any of claims 1 to 9 wherein IFN-γ levels are screened for in combination with another marker.

12. A method to predict the 30 day all-cause mortality of individuals identified to have a *Clostridium difficile* infection, the method comprising the steps of:

(i) obtaining a sample from a subject having or suspected of having *Clostridium difficile* infection;

(ii) detecting one or more biomarkers selected from the group consisting of antibodies to the *Clostridium difficile* surface layer protein SLP001 or a fragment thereof, antibodies to the *Clostridium difficile* surface layer protein SLP002 or a fragment thereof, antibodies to the *Clostridium difficile* protein pCDTb or a fragment thereof, and antibodies to the *Clostridium difficile* toxin B or fragment thereof: and

(iii) predicting based on the observations in (ii) whether the subject is likely to survive for 30 days or more.

13. A method of choosing the most appropriate treatment for a subject with a *Clostridium difficile* infection by performing the method of any of claims 1 to 12 on a sample from the subject and choosing, and optionally administering, treatment based on the biomarkers detected in the sample.

14. A method of treating a *Clostridium difficile* infection, the method comprising the steps of:

(i) detecting anti-toxin A antibody in a sample from a subject; and

(ii) administering treatment for the *Clostridium difficile* infection based on the anti-toxin A antibody levels.

15. The method of any preceding claim wherein the sample is blood, serum, plasma, urine, spinal fluid, synovial fluid, sputum, mucus or lymph.

16. The method of any preceding claim wherein the subject is a human.

17. The method of any preceding claim wherein the one or more antibodies are detected by using a protein array.

18. A panel of biomarkers for use in determining the severity of a *Clostridium difficile* infection in a subject, the panel comprising at least one of:

(i) antibodies to the *Clostridium difficile* toxin A or fragment thereof;
antibodies to the *Clostridium difficile* toxin B or fragment thereof,

(ii) IFNγ;

(iii) antibodies to a *Clostridium difficile* surface layer protein or fragment thereof; and

(iv) antibodies to the *Clostridium difficile* protein pCDTb or a fragment thereof.

19. The panel of claim 18 comprising antibodies to the *Clostridium difficile* toxin A or fragment thereof and at least one of (ii), (iii) or (iv).

20. The panel of claim 18 comprising antibodies to the *Clostridium difficile* surface layer protein SLP001 or a fragment thereof, and optionally one or more of antibodies to the *Clostridium difficile* surface layer protein SLP002 or a fragment thereof, and antibodies to the *Clostridium difficile* protein pCDTb or a fragment thereof.

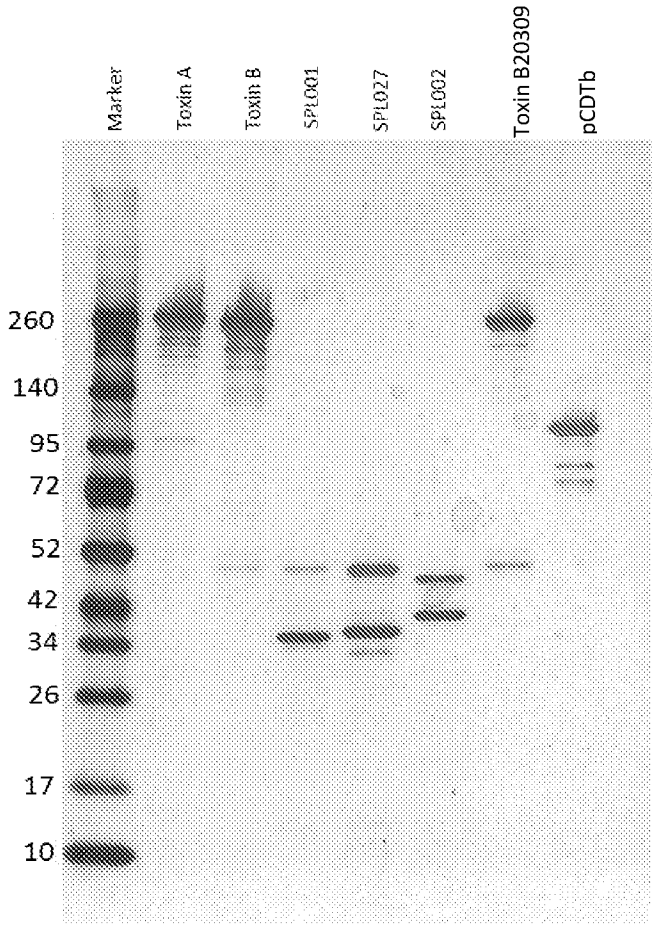
Marker
Toxin A
Toxin B
SPL001
SPL027
SPL002
Toxin B20309
pCDTb
260
140
95
72
52
42
34
26
17
10

Figure 1

	Purified protein concentration	Serum dilution	Secondary antibodies
IgG	50 µg/ml	1/500	1/20000
IgG2	200 µg/ml	1/100	1/10000
IgA	50 µg/ml	1/100	1/5000
IgA1	200 µg/ml	1/100	1/10000

Figure 2

Cytokine	Detection	Standard
Human IL-27	**100 ng/ml**	**10000-156 pg/ml**
Human BAFF/TNFSF13B	**10 ng/ml**	**2500-39.1 pg/ml**
Human IL-beta/IL-1F2	**200 ng/ml**	**500-7.8 pg/ml**
Human IL-6	**50 ng/ml**	**600-9.3 pg/ml**
Human IL-alpha/IL-1F1	**50 ng/ml**	**500-7.8 pg/ml**
Human IFN-gamma	**200 ng/ml**	**1000-15.6 pg/ml**
Human TNF-alpha	**50 ng/ml**	**1000-15.6 pg/ml**
Human GM-CSF	**10 ng/ml**	**1000-15.6 pg/ml**
Human CXCL8/IL-8	**20 ng/ml**	**2000-31.2pg/ml**
Human TGF-beta 1	**300 ng/ml**	**2000-31.2pg/ml**
Human APRIL/TNFSF13	**1ug/ml**	**2000-31.2pg/ml**
Human IL-12/IL-23 P40	**50 ng/ml**	**8000-125 pg/ml**

Figure 3

Univariate Analyses for Patient Severity (Mild / Severe)				
Covariates	Mean (SD) with 95% Confidence interval			
		Mild	Severe	P-Value
Age	67.77(17.08) 65.05 - 70.49	66.44(1.78) 62.95 - 69.92	71.9(2.07) 67.83 - 75.96	0.175
PeakWCC	12.52(5.76) 11.59 - 13.46	9.92(0.35) 9.24 - 10.6	17.96(0.85) 16.29 - 19.64	**0.001**
MinAlb	24.95(6.96) 23.78 - 26.12	26.5(0.72) 25.08 - 27.92	21.91(0.91) 20.14 - 23.69	**0.014**
PeakCr	100.66(70.95) 89.23 - 112.09	98.71(7.63) 83.75 - 113.67	104.73(8.48) 88.11 - 121.35	0.481
EosinophilCount	2.77(15.87) 0.25 - 5.3	1.13(0.98) -0.78 - 3.05	0.21(0.06) 0.09 - 0.33	0.837
CRP	76.95(88.81) 61.38 - 92.52	48.33(5.74) 37.08 - 59.59	124.45(16.8) 91.53 - 157.37	**0.001**
NAbA	5.28(13.91) 2.99 - 7.57	5.99(1.53) 2.99 - 8.98	3.48(1.73) 0.09 - 6.87	0.750
NAbB	12.19(24.84) 8.12 - 16.26	12.47(2.49) 7.6 - 17.35	12.12(3.96) 4.36 - 19.89	0.987
C.difficileToxinAIgAHN	59.17(76.12) 46.73 - 71.6	65.3(8.29) 49.06 - 81.55	49.52(9.69) 30.52 - 68.51	0.267
C.difficileToxinBIgAHN	380.99(883.43) 240.08 - 521.9	463.5(94.81) 277.67 - 649.33	228.99(107.28) 18.72 - 439.25	0.264
ToxinB94660IgAHN	281.01(733.25) 162.47 - 399.55	325.99(77.78) 173.53 - 478.44	202.56(99) 8.51 - 396.61	0.272
pCDTbIgAHN	21.67(35.51) 15.87 - 27.47	20.08(3.45) 13.32 - 26.83	26.65(5.95) 14.98 - 38.32	0.868
SLP001IgAHN	21.03(51.12) 12.71 - 29.35	22.37(5.39) 11.79 - 32.94	19.47(7.2) 5.36 - 33.58	0.942
SLP027IgAHN	16.14(41.79) 9.27 - 23.01	15.63(4.17) 7.45 - 23.81	18.4(6.92) 4.84 - 31.96	0.995
SLP002IgAHN	12.54(30.93) 7.52 - 17.56	13.14(3.25) 6.78 - 19.5	11.12(4.34) 2.62 - 19.62	1.000
C.difficileToxinAIgG	418.67(663.37) 309.56 - 527.78	447.04(73.22) 303.53 - 590.55	339.73(77.15) 188.51 - 490.95	0.437
C.difficileToxinBIgG	5425.5(7681.91) 4179.41 - 6671.58	5585.68(813.87) 3990.49 - 7180.88	5288.98(1065.48) 3200.64 - 7377.32	0.755
ToxinB94660IgG	4071.32(5631.85) 3154.63 - 4988.01	4112.24(583.75) 2968.1 - 5256.38	4221.82(827.79) 2599.36 - 5844.28	0.805

Figure 4

pCDTbIgG_OKay	1567.46(2041.11) 1228.13 - 1906.78	1445.18(197.41) 1058.25 - 1832.11	1771.66(362.65) 1060.87 - 2482.45	0.787
SLP001IgG_OKay	618.78(739.81) 497.09 - 740.46	538.25(70.51) 400.06 - 676.44	763.76(124.3) 520.14 - 1007.38	0.113
SLP027IgG_OKay	932.17(1147.85) 745.34 - 1119.01	769.11(102.91) 567.4 - 970.82	1161.68(189.03) 791.18 - 1532.18	0.131
SLP002IgG	783(829.81) 646.99 - 919.01	700(82.59) 538.13 - 861.87	928.25(128.1) 677.17 - 1179.33	0.085
C.difficileToxinAIgG2	92.47(122.25) 72.43 - 112.51	96.89(12.79) 71.82 - 121.96	88.12(17.93) 52.97 - 123.27	0.430
C.difficileToxinBIgG2	84.45(106.79) 66.95 - 101.95	82.26(10.14) 62.38 - 102.13	92.69(18.8) 55.85 - 129.53	0.973
ToxinB94660IgG2	79.1(90.79) 64.27 - 93.93	78.78(9.06) 61.03 - 96.54	84.09(14.55) 55.58 - 112.6	0.642
pCDTbIgG2	53.45(87.28) 39.04 - 67.86	52.19(8.8) 34.95 - 69.43	58.25(14.39) 30.04 - 86.45	0.900
SLP001IgG2	60.87(99.54) 44.62 - 77.13	58.4(8.53) 41.68 - 75.13	66.25(19.47) 28.08 - 104.42	0.271
SLP027IgG2	34.12(56.45) 24.9 - 43.34	33.03(5.35) 22.54 - 43.52	37.74(9.83) 18.48 - 57	0.894
SLP002IgG2	58.06(66.57) 47.15 - 68.97	56.64(6) 44.89 - 68.39	62.47(12.53) 37.92 - 87.02	0.992
HumanIL27Cytokines	891.8(1417.58) 658.64 - 1124.97	852.31(132.65) 592.31 - 1112.3	886.4(232.36) 430.97 - 1341.83	0.994

Figure 4 continued

BAFFTNFSF13Cytokines	643.65(517.53) 560.27 - 727.03	592.49(46.85) 500.66 - 684.33	730.97(89.61) 555.33 - 906.61	0.407
HumanILbetaIL1F2Cytokines	29.22(43.16) 22.15 - 36.3	30.32(4.54) 21.43 - 39.21	25.46(5.75) 14.2 - 36.73	0.867
IL6Cytokines	24.78(34.76) 19.14 - 30.42	23.69(3.63) 16.58 - 30.8	26.04(4.66) 16.9 - 35.17	0.361
HumanILALPHAIL1F1Cytokines	8.47(16.02) 5.88 - 11.06	8.43(1.67) 5.17 - 11.7	8.3(2.28) 3.82 - 12.77	0.988
HumanIFNgammaCytokines	11.02(26.8) 6.64 - 15.4	7.95(2.37) 3.3 - 12.59	13.88(4.36) 5.34 - 22.42	0.242
HumanTNFalphaCytokines	50.84(110.38) 33.12 - 68.57	48.83(10.46) 28.33 - 69.34	57.34(18.44) 21.2 - 93.49	0.949
HumanGMCSFCytokines	38.64(94.13) 23.53 - 53.76	36.33(8.45) 19.78 - 52.89	45.97(16.82) 13.01 - 78.94	0.990
HumanCXCL8IL8Cytokines	24.67(45.9) 17.3 - 32.04	20.23(3.15) 14.04 - 26.41	34(9.81) 14.77 - 53.22	0.803
HumanTGFbeta1Cytokines	161.25(126.52) 140.93 - 181.56	168.4(12.53) 143.84 - 192.96	143.08(19.23) 105.39 - 180.77	0.218
HumanAPRILTNFSF13Cytokines	4163.75(3860.79) 3541.73 - 4785.77	3768.02(349.59) 3082.81 - 4453.22	4602.01(644.04) 3339.7 - 5864.32	0.663
HumanIL12IL23P40Cytokines	260.66(468.26) 183.09 - 338.23	255.1(49.91) 157.28 - 352.93	251.95(60.28) 133.8 - 370.1	0.684

Figure 4 continued

Univariate Analyses for Patient 30 Days (Survived / Died)							
	Total		Survived		Died		
	Mean(SD)	95% confidence interval	Mean(SD)	95% confidence interval	Mean(SD)	95% confidence interval	P-Value
Age	67.77(1.39)	65.05-70.49	67.7(1.47)	64.82-70.59	70.62(3.71)	63.34-77.89	0.887
PeakWCC	12.52(0.48)	11.59-13.46	12.25(0.48)	11.3-13.19	15.62(2.12)	11.46-19.77	0.042
MinAlb	24.95(0.6)	23.78-26.12	25.49(0.6)	24.32-26.66	18.82(2.23)	14.45-23.18	0.067
PeakCr	100.66(5.83)	89.23-112.09	98.73(6.07)	86.82-110.63	120.77(20.68)	80.24-161.3	0.08
EosinophilCount	2.77(1.29)	0.25-5.3	2.33(1.23)	-0.08-4.74	0.09(0.03)	0.03-0.16	0.622
CRP	76.95(7.94)	61.38-92.52	80.05(8.62)	63.16-96.95	50.23(16.75)	17.4-83.06	0.229
NAbA	5.28(1.17)	2.99-7.57	5.4(1.28)	2.9-7.9	4.1(1.46)	1.23-6.97	0.402
NAbB	12.19(2.08)	8.12-16.26	12.63(2.27)	8.18-17.07	7.84(2.68)	2.59-13.09	0.643
C.difficileToxinAIgAHN	59.17(6.34)	46.73-71.6	60.76(6.81)	47.41-74.1	46.08(17.36)	12.06-80.11	0.151
C.difficileToxinBIgAHN	380.99(71.89)	240.08-521.9	411.51(78.7)	257.25-565.77	87.39(41.97)	5.13-169.64	0.665
ToxinB94660IgAHN	281.01(60.48)	162.47-399.55	303.95(66.45)	173.71-434.19	67.89(39.26)	-9.05-144.83	0.302
pCDTbIgAHN	21.67(2.96)	15.87-27.47	22.01(3.14)	15.85-28.16	19.75(9.53)	1.07-38.44	0.69
SLP001IgAHN	21.03(4.24)	12.71-29.35	21.71(4.64)	12.62-30.8	15.3(5.1)	5.29-25.3	0.109
SLP027IgAHN	16.14(3.51)	9.27-23.01	16.77(3.84)	9.24-24.31	10.69(3.45)	3.93-17.45	0.169
SLP002IgAHN	12.54(2.56)	7.52-17.56	12.69(2.78)	7.25-18.14	8.37(4)	0.54-16.2	0.999
C.difficileToxinAIgG_OKay	418.67(55.67)	309.56-527.78	404.4(54.17)	298.22-510.58	606.98(313.35)	-7.19-1221.14	0.978
C.difficileToxinBIgG_OKay	5425.5(635.76)	4179.41-6671.58	5502.67(678.32)	4173.17-6832.17	4990.8(1922.54)	1222.63-8758.98	0.465
ToxinB94660IgG_OKay	4071.32(467.7)	3154.63-4988.01	4097.52(491.07)	3135.01-5060.02	4104.47(1689.88)	792.31-7416.63	0.813
pCDTbIgG_OKay	1567.46(173.12)	1228.13-1906.78	1592.22(186.39)	1226.89-1957.55	1348.25(450.84)	464.61-2231.89	0.999
SLP001IgG_OKay	618.78(62.08)	497.09-740.46	564.71(61.23)	444.7-684.72	1149.3(290.51)	579.9-1718.69	0.04
SLP027IgG_OKay	932.17(95.32)	745.34-1119.01	850.68(95.29)	663.91-1037.45	1762.18(416.83)	945.19-2579.17	0.033

Figure 5 (Part 1)

SLP002IgG	783(69.39)	646.99- 919.01	759.19(73.39)	615.34- 903.05	1020.66(224.18)	581.26- 1460.06	0.397
C.difficileToxinAIgG 2	92.47(10.22)	72.43- 112.51	89.62(10.31)	69.41- 109.82	127.89(46.8)	36.16- 219.62	0.919
C.difficileToxinBIgG 2	84.45(8.93)	66.95- 101.95	87.63(9.69)	68.63- 106.63	57.05(14.08)	29.45- 84.65	0.681
ToxinB94660IgG2	79.1(7.57)	64.27- 93.93	81.51(8.16)	65.52- 97.51	59.32(15.71)	28.52- 90.12	0.958
pCDTbIgG2	53.45(7.35)	39.04- 67.86	55.7(7.94)	40.13- 71.26	31.98(12.36)	7.75- 56.21	0.951
SLP001IgG2	60.87(8.3)	44.62- 77.13	62.97(9.07)	45.19- 80.74	39.9(14.29)	11.9- 67.9	0.977
SLP027IgG2	34.12(4.7)	24.9- 43.34	34.27(5.05)	24.37- 44.18	35.19(12.99)	9.73- 60.65	0.775
SLP002IgG2	58.06(5.57)	47.15- 68.97	59.62(6.01)	47.85- 71.4	45.95(11.91)	22.61- 69.29	0.933
HumanIL27Cytokine s	891.8(118.96)	658.64- 1124.97	855.75(126.86)	607.1- 1104.39	1283.74(352.15)	593.53- 1973.94	0.338
BAFFTNFSF13Cytoki nes	643.65(42.54)	560.27- 727.03	649.72(45.57)	560.4- 739.05	588.98(121.53)	350.78- 827.19	0.891
HumanILbetaIL1F2C ytokines	29.22(3.61)	22.15- 36.3	29.31(3.79)	21.89- 36.73	30.03(13.26)	4.04- 56.01	0.983
IL6Cytokines	24.78(2.88)	19.14- 30.42	25.02(3)	19.14- 30.89	24.33(11.11)	2.56- 46.09	0.571
HumanILALPHAIL1F 1Cytokines	8.47(1.32)	5.88- 11.06	8.08(1.28)	5.57- 10.59	12.83(7.33)	-1.53- 27.19	0.238
HumanIFNgammaCy tokines	11.02(2.23)	6.64- 15.4	11.63(2.45)	6.82- 16.43	2.65(0.82)	1.04- 4.26	0.853
HumanTNFalphaCyt okines	50.84(9.04)	33.12- 68.57	54.29(9.9)	34.89- 73.69	18.59(9.26)	0.43- 36.74	0.54
HumanGMCSFCytok ines_104ZEROs	38.64(7.71)	23.53- 53.76	41.56(8.44)	25- 58.11	11.36(7.67)	-3.68- 26.39	0.911
HumanCXCL8IL8Cyt okines	24.67(3.76)	17.3- 32.04	24.21(4.01)	16.34- 32.08	27.61(11.2)	5.66- 49.57	0.898
HumanTGFbeta1Cyt okines_BIGRAPH	161.25(10.37)	140.93- 181.56	164.17(10.87)	142.86- 185.48	135.74(37.28)	62.67- 208.81	0.685
HumanAPRILTNFSF1 3Cytokines	4163.75(317.36)	3541.73- 4785.77	4320.51(340.93)	3652.29- 4988.73	2115.84(440.45)	1252.55- 2979.13	**0.056**
HumanIL12IL23P40 Cytokines	260.66(39.58)	183.09- 338.23	277.54(43.16)	192.94- 362.13	117.12(70.12)	-20.32- 254.57	0.533

Figure 5 (Part 2)

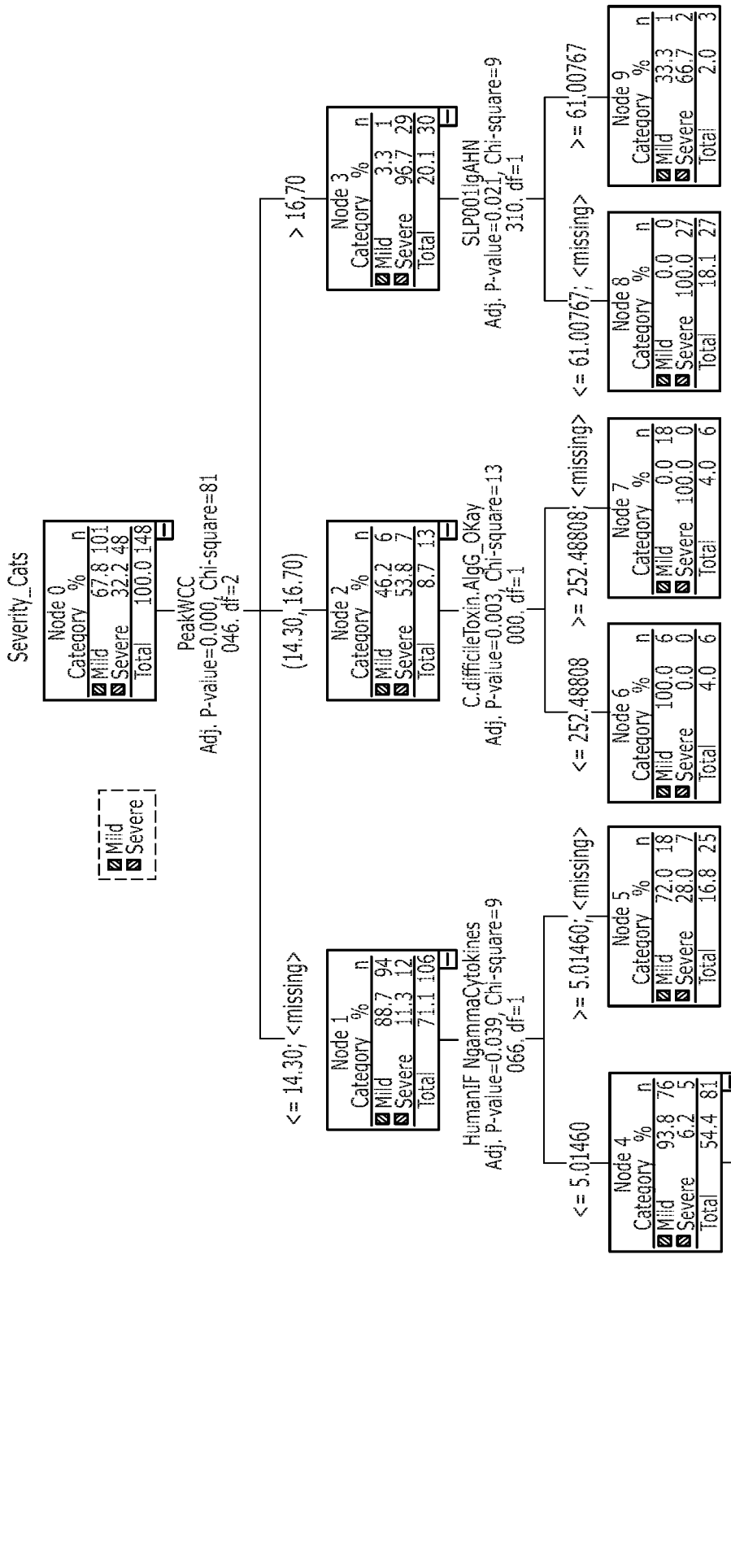
Severity_Cats
Mild
Severe
Node 0
Category % n
Mild 67.8 101
Severe 32.2 48
Total 100.0 148
PeakWCC
Adj. P-value=0.000, Chi-square=81
046, df=2
<= 14.30; <missing>
(14.30, 16.70)
> 16.70
Node 1
Category % n
Mild 88.7 94
Severe 11.3 12
Total 71.1 106
Node 2
Category % n
Mild 46.2 6
Severe 53.8 7
Total 8.7 13
Node 3
Category % n
Mild 3.3 1
Severe 96.7 29
Total 20.1 30
HumanIF NgammaCytokines
Adj. P-value=0.039, Chi-square=9
066, df=1
C.difficileToxin.AlgG_OKay
Adj. P-value=0.003, Chi-square=13
000, df=1
SLP001lgAHN
Adj. P-value=0.021, Chi-square=9
310, df=1
<= 5.01460
>= 5.01460; <missing>
<= 252.48808
>= 252.48808; <missing>
<= 61.00767; <missing>
>= 61.00767
Node 4
Category % n
Mild 93.8 76
Severe 6.2 5
Total 54.4 81
Node 5
Category % n
Mild 72.0 18
Severe 28.0 7
Total 16.8 25
Node 6
Category % n
Mild 100.0 6
Severe 0.0 0
Total 4.0 6
Node 7
Category % n
Mild 0.0 18
Severe 100.0 0
Total 4.0 6
Node 8
Category % n
Mild 0.0 0
Severe 100.0 27
Total 18.1 27
Node 9
Category % n
Mild 33.3 1
Severe 66.7 2
Total 2.0 3
ToxinB9466OlgG2
Adj. P-value=0.002, Chi-square=26
833, df=3
<= 0.00000; <missing>
(0.00000, 40.77403]
(40.77403, 98.70831]
> 98.70831
Node 9
Category % n
Mild 100.0 22
Severe 0.0 0
Total 14.8 22
Node 11
Category % n
Mild 33.3 1
Severe 66.7 2
Total 2.0 3
Node 12
Category % n
Mild 100.0 39
Severe 0.0 0
Total 26.2 39
Node 13
Category % n
Mild 82.4 14
Severe 17.6 3
Total 11.4 17

Fig. 6

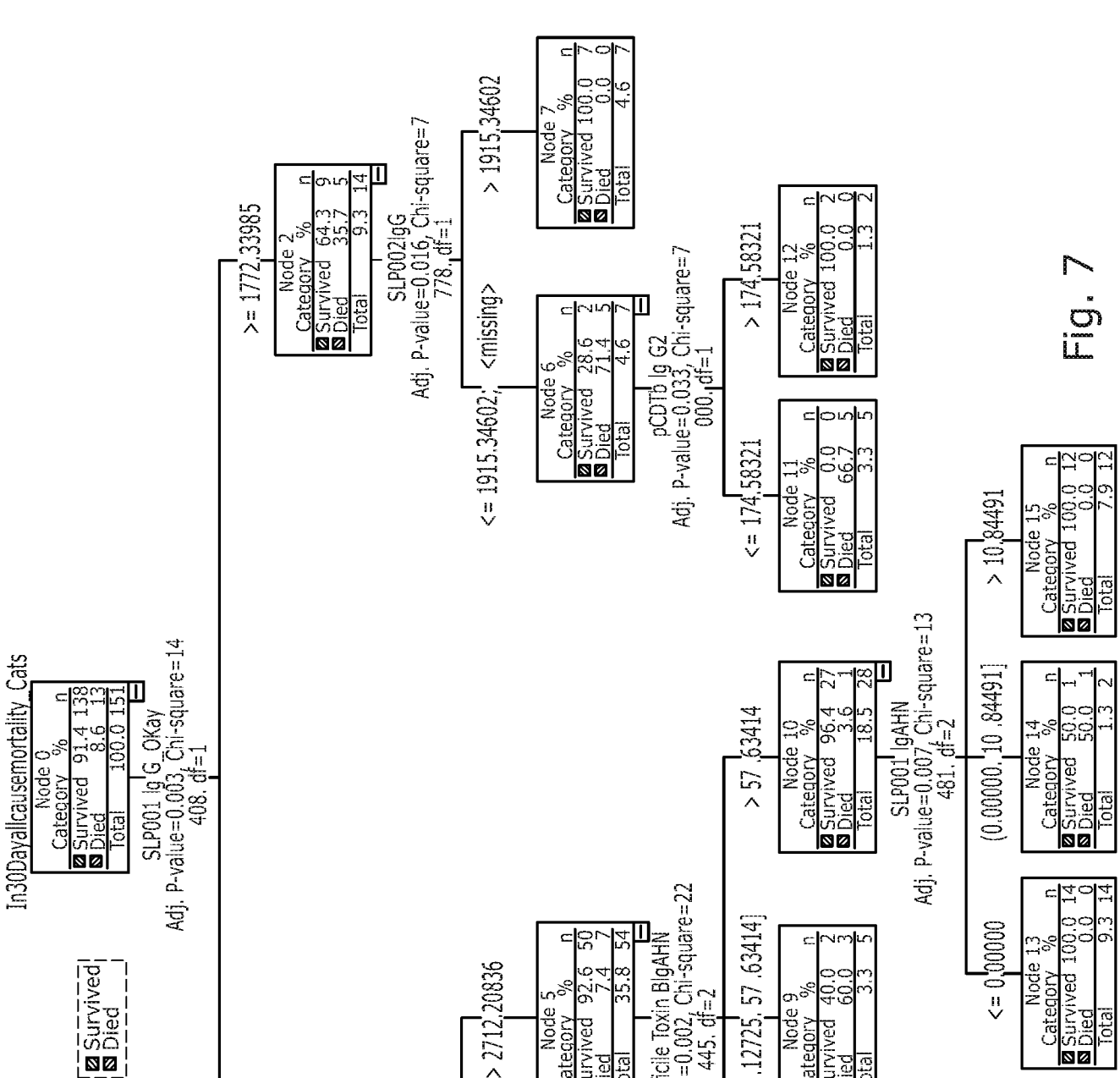
In30Dayallcausemortality_Cats
Survived
Died
Node 0
Category % n
Survived 91.4 138
Died 8.6 13
Total 100.0 151
SLP001 lg G_OKay
Adj. P-value=0.003, Chi-square=14
408, df=1
<= 1772.33985; <missing>
>= 1772.33985
Node 1
Category % n
Survived 94.2 129
Died 5.8 8
Total 90.7 137
Node 2
Category % n
Survived 64.3 9
Died 35.7 5
Total 9.3 14
C .difficile Toxin Blg G_OKay
Adj. P-value=0.008, Chi-square=19
277, df=2
SLP002lgG
Adj. P-value=0.016, Chi-square=7
778, df=1
<= 558.08674
(558.08674, 2712.20836]; <missing>
> 2712.20836
<= 1915.34602; <missing>
> 1915.34602
Node 3
Category % n
Survived 69.2 9
Died 30.8 4
Total 8.6 13
Node 4
Category % n
Survived 100.0 70
Died 0.0 0
Total 46.4 70
Node 5
Category % n
Survived 92.6 50
Died 7.4 7
Total 35.8 54
Node 6
Category % n
Survived 28.6 2
Died 71.4 5
Total 4.6 7
Node 7
Category % n
Survived 100.0 7
Died 0.0 0
Total 4.6 7
C .difficile Toxin BlgAHN
Adj. P-value=0.002, Chi-square=22
445, df=2
pCDTb lg G2
Adj. P-value=0.033, Chi-square=7
000, df=1
<= 37.12725; <missing>
(37.12725, 57.63414]
> 57.63414
<= 174.58321
> 174.58321
Node 8
Category % n
Survived 100.0 21
Died 0.0 0
Total 13.9 21
Node 9
Category % n
Survived 40.0 2
Died 60.0 3
Total 3.3 5
Node 10
Category % n
Survived 96.4 27
Died 3.6 1
Total 18.5 28
Node 11
Category % n
Survived 0.0 0
Died 66.7 5
Total 3.3 5
Node 12
Category % n
Survived 100.0 2
Died 0.0 0
Total 1.3 2
SLP001 lgAHN
Adj. P-value=0.007, Chi-square=13
481, df=2
<= 0.00000
(0.00000, 10.84491]
> 10.84491
Node 13
Category % n
Survived 100.0 14
Died 0.0 0
Total 9.3 14
Node 14
Category % n
Survived 50.0 1
Died 50.0 1
Total 1.3 2
Node 15
Category % n
Survived 100.0 12
Died 0.0 0
Total 7.9 12

Fig. 7

Performance Metrics for Disease Severity using Decision Tree Model	
Sensitivity	80%
Specificity	98.2%
Accuracy	91.9%
Positive Predictive Value (PPV)	95.1%
Negative Predictive Value (NPV)	90.8%
ROC curve	0.812 (using the Youden index *J*)

Performance Metrics for 30-d mortality using Decision Tree Model	
Sensitivity	61.5%
Specificity	98.6%
Accuracy	95.4%
Positive Predictive Value (PPV)	96.5%
Negative Predictive Value (NPV)	80%
ROC curve	0.601 (using the Youden index *J*)

Figure 8

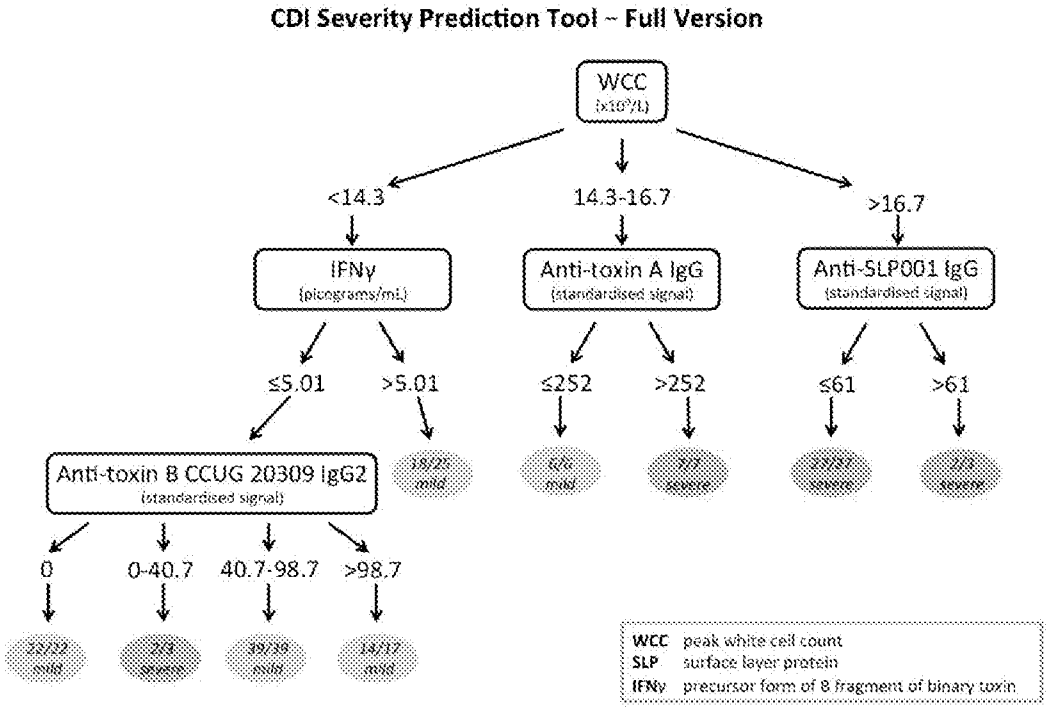

Figure 9

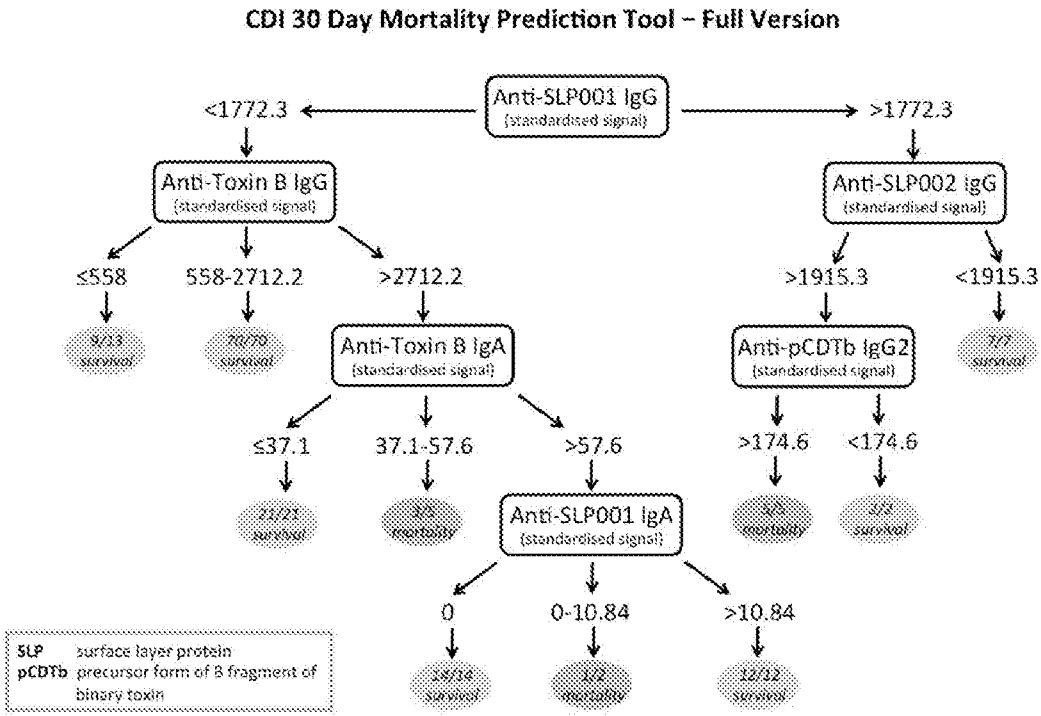

Figure 10

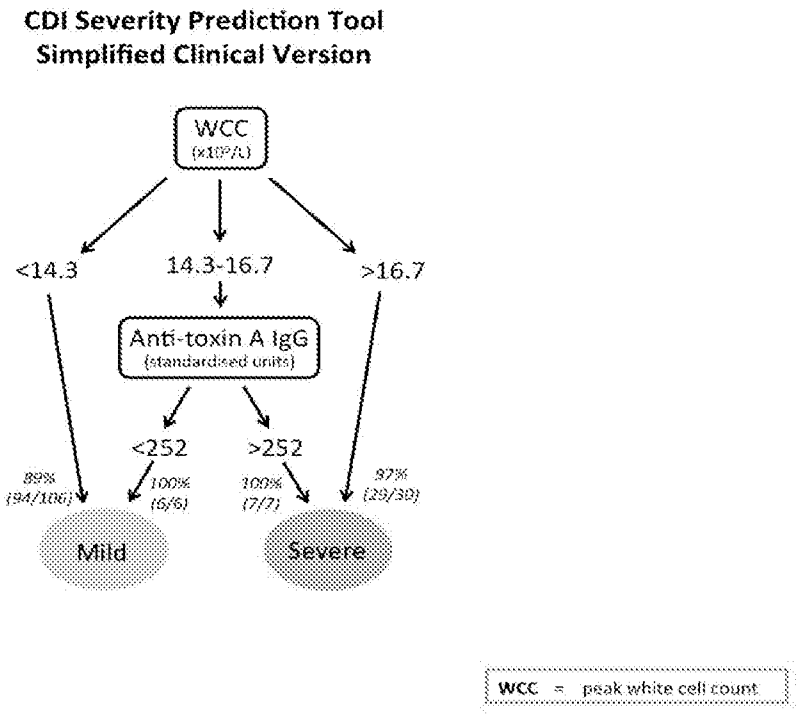

Figure 11

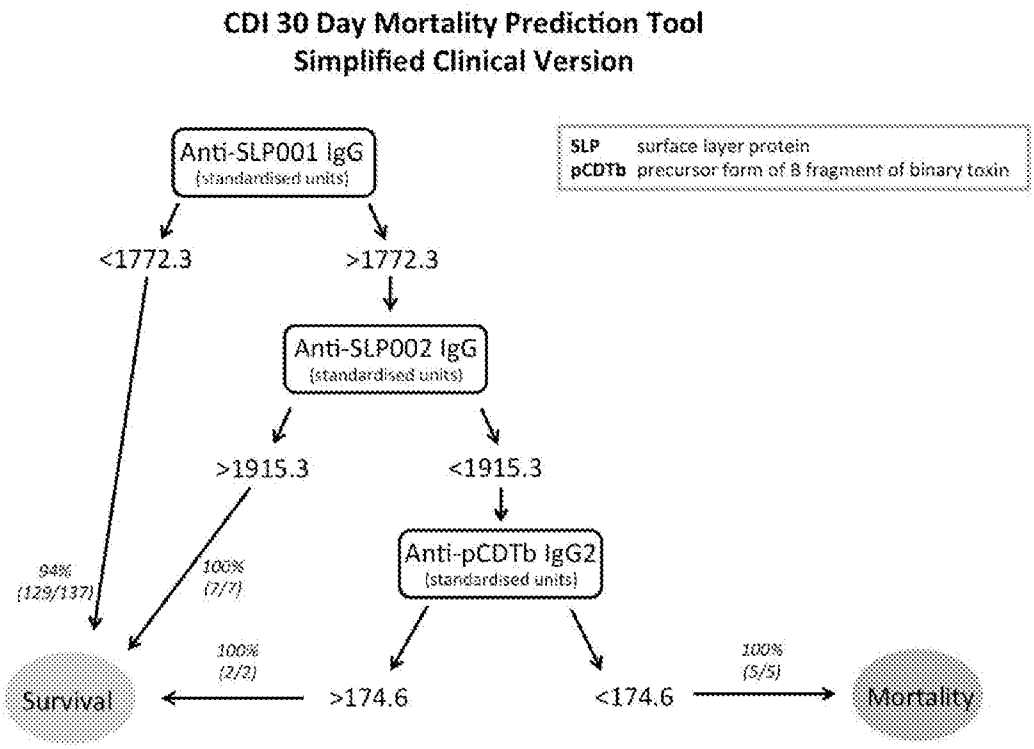

Figure 12

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2018/051090

A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N33/569
ADD.

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

B. FIELDS SEARCHED

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

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

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

EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	LAURIE R ARCHBALD-PANNONE: "Citation: Archbald-Pannone LR. Quantitative Fecal Lactoferrin as a Biomarker for Severe Clostridium difficile Infection in Hospitalized Patients Quantitative Fecal Lactoferrin as a Biomarker for Severe Clostridium difficile Infection in Hospitalized Patients", J GERIATRICS PALLIATIVE CARE, vol. 2, no. 1, 1 March 2014 (2014-03-01), pages 1-3, XP055485467, the whole document abstract ----- -/--	1-9,11, 13-19

[X] Further documents are listed in the continuation of Box C.

[ ] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 June 2018	29/08/2018

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Jenkins, Gareth

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2018/051090

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	KRISHNA RAO ET AL: "Elevated fecal calprotectin associates with adverse outcomes from Clostridium difficile infection in older adults", INFECTIOUS DISEASES, vol. 48, no. 9, 20 May 2016 (2016-05-20), pages 663-669, XP055485468, ISSN: 2374-4235, DOI: 10.1080/23744235.2016.1186832 the whole document abstract	1-9,11, 13-19
A	DATABASE BIOSIS [Online] BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US; January 2010 (2010-01), PANT CHAITANYA ET AL: "Laboratory Markers as Predictors of Mortality in Patients With Clostridium difficile Infection", XP002782159, Database accession no. PREV201000316156 abstract & PANT CHAITANYA ET AL: "Laboratory Markers as Predictors of Mortality in Patients With Clostridium difficile Infection", JOURNAL OF INVESTIGATIVE MEDICINE, vol. 58, no. 1, January 2010 (2010-01), pages 43-45, ISSN: 1081-5589	1-9,11, 13-19
X	K. SOLOMON ET AL: "Mortality in patients with Clostridium difficile infection correlates with host pro-inflammatory and humoral immune responses", JOURNAL OF MEDICAL MICROBIOLOGY, vol. 62, no. Pt_9, 30 May 2013 (2013-05-30), pages 1453-1460, XP055485470, ISSN: 0022-2615, DOI: 10.1099/jmm.0.058479-0 the whole document abstract page 1455, columns 1-2, bridging section page 1457, column 2, section "Risk factors for ..."	1-9,11, 13-16, 18,19

-/--

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2018/051090

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	M Warny ET AL: "Human antibody response to Clostridium difficile toxin A in relation to clinical course of infection", Infection and Immunity, vol. 62, no. 2 1 February 1994 (1994-02-01), pages 384-389, XP055191277, UNITED STATES Retrieved from the Internet: URL:http://iai.asm.org/content/62/2/384.abstract the whole document abstract figure 3	1-9,11, 13-19
X	L ORRAINE ET AL: "ASYMPTOMATIC CARRIAGE OF CLOSTRIDIUM DIFFICILE AND SERUM LEVELS OF IgG ANTIBODY AGAINST TOXIN A A BSTRACT Background Clostridium difficile infection can re", THE NEW ENGLAND JOURNAL OF MEDICINE, vol. 342, no. 6, 10 February 2000 (2000-02-10), pages 390-396, XP055485484, the whole document abstract figure 2	1-9,11, 13-19
X,P	DATABASE EMBASE [Online] ELSEVIER SCIENCE PUBLISHERS, AMSTERDAM, NL; 1 July 2017 (2017-07-01), MONAGHAN T M: "A protein microarray assay for predicting severe outcomes in clostridium difficile infection", XP002782160, Database accession no. EMB-621902846 abstract & MONAGHAN T M: "A protein microarray assay for predicting severe outcomes in clostridium difficile infection", GUT 20170701 BMJ PUBLISHING GROUP NLD, vol. 66, no. Supplement 2, 1 July 2017 (2017-07-01), pages A31 CONF 20170619 to 20170622 Manchester-British S, ISSN: 1468-3288	1-9,11, 13-19

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB2018/051090

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

5, 9, 14, 19(completely); 1-4, 6-8, 11, 13, 15-18(partially)

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 5, 9, 14, 19(completely); 1-4, 6-8, 11, 13, 15-18(partially)

The claims in so far as they relate to the biomarker antibodies to toxin A.

2-5. claims: 10, 12, 20(completely); 1-4, 6-8, 11, 13, 15-18(partially)

The claims in so far as they relate to the biomarkers antibodies to toxin B, antibodies to SLP001, antibodies to SLP002, and IFNgamma, corresponding to inventions 2-5 respectively.
