PEER REVIEW HISTORY

BMJ Open publishes all reviews undertaken for accepted manuscripts. Reviewers are asked to complete a checklist review form (http://bmjopen.bmj.com/site/about/resources/checklist.pdf) and are provided with free text boxes to elaborate on their assessment. These free text comments are reproduced below.

ARTICLE DETAILS

<table>
<thead>
<tr>
<th>TITLE (PROVISIONAL)</th>
<th>The Early PREdiction of Severe Sepsis (ExPRES-Sepsis) study: protocol for an observational derivation study to discover potential leukocyte cell surface biomarkers</th>
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<tbody>
<tr>
<td>AUTHORS</td>
<td>Datta, Deepankar; Conway Morris, Andrew; Antonelli, Jean; Warner, Noel; Brown, K Alun; Wright, John; Simpson, A John; Rennie, Jillian; Hulme, Gillian; Lewis, Sion; Mare, Tracey; Weir, Christopher; Dimmick, Ian; Keenan, Jim; Rossi, Adriano; Shankar-Hari, Manu; Walsh, Tim</td>
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GENERAL COMMENTS

Novel biomarker discovery in the field of sepsis, aimed at rapid diagnosis and prognostication, has a long history and, as the authors indicate, is plagued with under-powered studies, restricted to severely ill inpatient populations in critical care and with concerns about a raft of sources of study bias. The resulting international evidence base is widely regarded as rather shambolic, with no meaningful progress from bench to bedside that has impacted on patient care to date.

Therefore, it is very refreshing to read a carefully considered planned study protocol that serves to illustrate how novel biomarker discovery in sepsis can be approached systematically alongside readily available patient populations that could, ultimately, benefit from such endeavour. This protocol design is a good example of how sepsis biomarker discovery can be de-risked while aiming to provide the best early evidence aimed at progressing the best candidates into larger patient studies for external validation. In my view, the design of this study protocol alone has important value for a growing investigator community internationally in the field of sepsis, a disease that is increasingly acknowledged as an important global challenge with considerable unmet need.

I am satisfied that all elements of this proposed study protocol have been considered carefully. I am also satisfied that a pragmatic approach to defining infection, sepsis and its complications, is understandable and translatable into current standards of care internationally. Some of these definitions are about to change (marginally) in late February 2016 – and it is my view that these will...
be a distraction to the current study design as they have not been widely considered for implementation globally and will take some years to establish. However, the investigators – given their plans for extensive recorded clinical data – will be able to re-engineer results against any subsequent adoption of new definitions as they emerge.

My only main comment to the authors relates to their communication strategy as the iterative discovery process emerges through 4 defined steps. Clearly, at this stage, it is rather difficult to define the cell surface markers in detail – but there will be important decision steps that could be communicated. Would this current study protocol be strengthened by inclusion of some of the early decisions about which markers will progress – or would a subsequent additional interim communication with BMJ Open provide real strength for your study design as it progresses into later validation steps? I also appreciate that your prestigious public funding stream with Innovate UK may also have important IP considerations with your commercial partner (by design of this funding) – so I am sure you will consider my question carefully as I would not like to see any unnecessary delays in communicating this important study protocol.

REVIEWER
B. Venkatesh
University of Qld, Australia

GENERAL COMMENTS
The morbidity and mortality associated with sepsis pose a significant personal and economical burden on affected individuals and society. One major reason for failed clinical trials for new treatments in sepsis is the lack of adequate patient stratification. Experts increasingly recognise that the heterogeneity of septic patients will require personalised treatments and stress the urgent need for effective ways of patient stratification and new adjunctive therapies. Management of septic shock patients in intensive care is hampered by lack of reliable prognostic markers for the onset, severity and duration of the disordered inflammatory responses that drive sepsis pathology.

Drs. Datta et al aim to characterise leukocyte surface markers (biomarkers) and their abnormalities in a population of patients presenting to the hospital on the basis that circulating leukocytes provide readily accessible tissues that reflect many aspects of the complex immune responses described in sepsis. They hypothesise that measuring cellular markers of immune responses by flow cytometry will enable early identification of infected patients at risk of adverse outcomes.

The study is a prospective multi-center study and they will study 3 patient populations
1) Patients with suspected sepsis presenting to ED
2) To assess test performance characteristics they will also study 2 other cohorts of n=100. (critically patients with sepsis and non-septic patients requiring hospitalization)

My comments are as follows:

The morbidity and mortality associated with sepsis pose a significant personal and economical burden on affected individuals and society. One major reason for failed clinical trials for new treatments in sepsis is the lack of adequate patient stratification. Experts increasingly recognise that the heterogeneity of septic patients will
require personalised treatments and stress the urgent need for effective ways of patient stratification and new adjunctive therapies. Management of septic shock patients in intensive care is hampered by lack of reliable prognostic markers for the onset, severity and duration of the disordered inflammatory responses that drive sepsis pathology. From that perspective, the approach to identify a new biomarker that will identify subsequent deterioration is logical and laudable. However, I have a number of comments and questions in relation to the proposal and the manuscript.

1) The background does not go into the discussion of which leukocyte marker is responsible for organ dysfunction. Although a hypothesis is stated, there is no discussion of the biological basis for the derivation of the hypothesis.

2) This same issue is noted in the Methods Section – Which leukocyte marker are they going to choose? A number have been reported in the literature. This is a major criticism of the study. It would appear that they are going to depend on the antibody panel available through the BD system. Although they state that “Antibodies selected for cell surface staining have either shown previous association with differential expression in inflammation, and therefore noted to be of potential in the development of a clinical test, or are used to select leukocyte subtypes of interest.” It is unclear how many and what antibodies are going to be studied.

3) It appears to me that at a meeting in April 2016, the results will be reviewed and an expert consensus will determine selection of biomarkers to be used for further analysis. Does this mean that this study has already commenced for them to complete enrolment of the 300 patients in phase 1? This appears to be at variance from what has been stated about the ethics approval.

4) It is unclear from the Methods if ethics approval has been obtained. They use the words “favourable response”.

5) They also make the assumption that the event rate in Cohort 1 will be 5-10%. This may be an overestimate. From the study by Majuran et al (Emerg Med J 2008;25:11-14), of the 123 with uncomplicated sepsis only 1 patient required ICU admission (0.8%). In the study by Kruger et al (Am J Respir Crit Care Med. 2011 Mar 15;183(6):774-81.), of those already in hospital with sepsis, the admission rate to ICU was only 16%. I suspect that the event rate of 5-10% may be an overestimate. This will have implications for sample size.

6) They are also planning to compare this with CRP and PCT measurements as predictors of sepsis and organ dysfunction. It is well known that the time course of response of these mediators during sepsis is variable and that they may also vary depending on the aetiology – bacteria vs viral etc. How will they account for this?

7) It is possible that patients may harbor symptoms for 2-3 days before presentation to ED and therefore the peak of the leukocyte marker may be missed.

8) Similar considerations apply to cohort 2. If they have been in hospital for a few days before deterioration and ICU admission, this will impact on the plasma concentrations and profiles of these markers.

9) In critically ill patients, there is evidence that plasma IL-6 may fluctuate over a 24 hour period (Robinson, Clin Endocrinol (Oxf). 2013 Dec;79(6):892-8). Will a random measurement of this leukocyte marker be indicative of the 24 hour profile?

10) Why is pregnancy an exclusion?

11) Why is trial registered with clinical trials.gov? Is an intervention being planned?
**GENERAL COMMENTS**

Well written protocol, detailed but yet concise. The protocol is clear and scientifically sound.

Comment:
1) Page 11 lines 3, 12 and 22: Please change "(3) clinical suspicion of sepsis" to "(3) clinical suspicion of infection".

2) Page 11 line 57 - page 12 line 16: Does this study include patients with virus, parasite or fungal infection for cohorts 1 and 2? If it does, how will the infections be confirmed?

3) Page 11 lines 35-41: I suggest to exclude patients with acute cholecystitis, patients on long term inhaled corticosteroids, autoimmune diseases and patients who have taken antibiotics 72 hours before the enrollment. Reasons as below:

- **Acute cholecystitis**
  Acute cholecystitis is initially a chemical inflammation, but regularly complicated by bacterial invasion from the gut. Bactibilia occurs in at least 60% of the early stage of acute cholecystitis and is particularly prevalent in the elderly. Bactibilia occur in 2 third of cases and 1 third are usually sterile. It is similar to acute pancreatitis, and will lead to bias during data analysis.

- **Inhaled Corticosteroids**
  Only a fraction of the dose, approximately 10% to 40% depending on the delivery device, is deposited in the lungs; the rest is swallowed. This swallowed corticosteroid enters the gastrointestinal tract in which, during a prolonged period, it can have undesired local effects. In addition, the swallowed corticosteroid is itself absorbed from the gastrointestinal tract and enters the systemic circulation.

- **Autoimmune Diseases**
  Patients with autoimmune disease may present with systemic inflammation which mimics sepsis. It is a confounding factor for C-reactive protein analysis.

  Patients who have taken antibiotics 72 hours prior enrollment

  Bacterial culture results will be affected for these patients.

4) Page 14 line 51: Please change "discharge home with 72 hours" to "discharge home within 72 hours"

5) Page 14 line 51: The phrase "(3) discharge to home, or in hospital with no organ failure, within 72 hours" is unclear. It sounds similar to statement "(2) discharge home with 72 hours". Please elaborate & explain the difference or rephrase the sentence.
Page 15 lines 12-15: The phrase "A further outcome of discharge to home, or in hospital with no organ failure, within 72 hours will identify the group of patients admitted to hospital but unsuitable for discharge due to non-medical reasons." is again unclear. Please explain or rephrase this.

**VERSION 1 – AUTHOR RESPONSE**

Reviewer 1: Paul Dark
We thank the reviewer for his generally positive comments about our study design and manuscript. We note the following comment:

Would this current study protocol be strengthened by inclusion of some of the early decisions about which markers will progress – or would a subsequent additional interim communication with BMJ Open provide real strength for your study design as it progresses into later validation steps?

We believe the protocol itself should be separated from the actual data analysis of the markers which will progress into the different phases of the study. All stages in the analysis and biomarker selection have been defined in this protocol paper, and the analyses are currently ongoing. We have not currently made a plan to report interim communications about these analysis stages, and it is more likely that we will report all analyses together in a single manuscript to ensure clarity and adequate reporting of biomarker that either lacked sufficient reliability and/or sufficient predictive value to explore in the final stage of the analysis. As the reviewer notes there are potential intellectual property issues with regards to the public funding stream and our commercial partner, and we plan to report data when all analyses have been completed. We have clarified this on page 11 of the manuscript.

Reviewer 2: B. Venkatesh
We thank this reviewer for the supportive comments, and for highlighting the potential importance of our area of research. In response to his specific comments:

1. "The background does not go into the discussion of which leukocyte marker is responsible for organ dysfunction. Although a hypothesis is stated, there is no discussion of the biological basis for the derivation of the hypothesis."

The immune response to infection is recognised to be complex, and the relationship between immune cell dysfunction and the development of organ dysfunction is incompletely understood. Despite extensive previous research into the immune response to sepsis there has been no biomarker that has been shown to have good predictive ability for subsequent clinical deterioration. It seems unlikely that a single leukocyte marker is responsible for organ dysfunction, and these may be part of complex pathways involving multiple aspects of the immune response. Our approach has been to select a range of cell surface biomarkers that indicate the types of immune and inflammatory cells activated in the circulation, and use an “empiric” deductive approach to explore their association with subsequent clinical severity of disease. In this sense our hypothesis is “general”, namely that identifying an immune cell type/or status can predict subsequent severity of organ dysfunction before it has occurred. We believe avoiding limiting hypotheses to specific cell markers is a strength of our approach. We believe the section “rationale for study” with its associated references presents the biological basis for the hypothesis in a succinct fashion that is suitable for a protocol paper, rather than a review paper. We have added an additional statement of hypothesis to the end of this section on page 6.

2. “This same issue is noted in the Methods Section – Which leukocyte marker are they going to choose? A number have been reported in the literature. This is a major criticism of the study. It would appear that they are going to depend on the antibody panel available through the BD system. Although they state that “Antibodies selected for cell surface staining have either shown previous association with differential expression in inflammation, and therefore noted to be of potential in the development of a clinical test, or are used to select leukocyte subtypes of interest.” It is unclear how many and what antibodies are going to be studied.”
The panels of antibodies used were selected using the methodology set out in the manuscript, and were the decision of the consortium based on our reading of the existing literature and pilot data. We are unable to reveal the final antibody panels at present, as they constitute novel IP. The final manuscript will contain a full description of the antibody clones, conjugated fluorophores and concentrations used. A sentence clarifying this has been added to the manuscript (page 11).

3. “It appears to me that at a meeting in April 2016, the results will be reviewed and an expert consensus will determine selection of biomarkers to be used for further analysis. Does this mean that this study has already commenced for them to complete enrollment of the 300 patients in phase 1? This appears to be at variance from what has been stated about the ethics approval.”

We have clarified the start date for recruitment to the study in the “duration of study” section (page 15). Since we submitted our manuscript we have completed recruitment, and the staged analysis is underway as described in the protocol. We do not have any results regarding the predictive properties of any of the biomarkers at the present time.

4. “It is unclear from the Methods if ethics approval has been obtained. They use the words “favourable response”.”

We have clarified the wording to explicitly state that ethics approval has been obtained on page 15.

5. “They also make the assumption that the event rate in Cohort 1 will be 5-10%. This may be an overestimate. From the study by Majuran et al (Emerg Med J 2008;25:11-14 ), of the 123 with uncomplicated sepsis only 1 patient required ICU admission (0,8%). In the study by Kruger et al (Am J Respir Crit Care Med. 2011 Mar 15;183(6):774-81.), of those already in hospital with sepsis, the admission rate to ICU was only 16%. I suspect that the event rate of 5-10% may be an overestimate. This will have implications for sample size.”

The assumption of the event rate that we used when designing the study was based on several studies that recruited in the ED, including a study conducted on a local population (Emerg Med J 2013;30:397-401). This study noted that 16.4% of patients with sepsis met the criteria for severe sepsis or sepsis shock by the time they had left the emergency department. This was the basis for the sample size calculations.

However, the reviewer is correct that the event rate was uncertain, and could have been overestimated. We reviewed clinical event rates in early 2015 and found that rates of septic shock were much lower than anticipated. At this time we decided to change the outcome of interest to sepsis with significant organ dysfunction, together with the clinical outcomes relating to critical care admission or discharge home. A protocol amendment was completed at that time, although the data collected and study procedures were unaltered within cohort 1. Since the publication of the new international sepsis definitions in March 2016 we have finalised our proposed analysis plan and exact cut-offs for organ failure severity to be used. We have extensively changed the manuscript in the revision to acknowledge and clarify all of these points, and ensure that the protocol manuscript published is accurate at the time of publication in relation to protocol versions and the analysis plan.

“They are also planning to compare this with CRP and PCT measurements as predictors of sepsis and organ dysfunction. It is well known that the time course of response of these mediators during sepsis is variable and that they may also vary depending on the aetiology- bacteria vs viral etc. How will they account for this?”

We thank the reviewer for this important comment. We agree that the response of any marker will fluctuate with time. However, our study has been designed to develop a pragmatic predictive test in a specific population and a specific point in the health care system, namely patients with possible sepsis in the emergency department. Therefore, consistent with agreed best practice for evaluating new diagnostic tests, we are comparing performance characteristics in this setting, and exploring whether performance is any better than existing markers.

6. “It is possible that patients may harbor symptoms for 2-3 days before presentation to ED and therefore the peak of the leukocyte marker may be missed.”

We agree that this phenomenon may be present, and considered this issue on planning the study. However, as per the previous comment this project has been designed to potentially deliver a
pragmatic test to front-line clinicians who would also not be aware of the true onset time of illness. We have stated in the manuscript that this initial study is exploratory.

7. “Similar considerations apply to cohort 2. If they have been in hospital for a few days before deterioration and ICU admission, this will impact on the plasma concentrations and profiles of these markers.”

Cohort 2 patients are recruited within 72 hours of presentation to hospital as per the inclusion criteria. The reason for including cohort 2 is to have a different clinical phenotype to compare to cohort 3 (severe sepsis positive versus negative) and with the cohort in whom we want to explore prediction by the leukocyte biomarkers (cohort 1). As explained in the manuscript (page 13) the rationale for the three cohorts is to provide very different clinical phenotypes to explore which biomarkers are clearly different, and therefore might have predictive value within a population of interest. The differences between the extreme phenotypes of cohorts 2 (established sepsis with organ dysfunction) and cohort 3 (matched non-septic patients without acute organ dysfunction) will be important for this discovery phase. We would argue that the issue of timing is not relevant to this part of the analysis.

8. “In critically ill patients, there is evidence that plasma IL-6 may fluctuate over a 24 hour period (Robinson, Clin Endocrinol (Oxf). 2013 Dec;79(6):892-8). Will a random measurement of this leukocyte marker be indicative of the 24 hour profile?”

As noted previously we are trying to develop a pragmatic test – in reality most tests ordered by clinicians for the initial management of patients are random measurements.

9. “Why is pregnancy an exclusion?”

Pregnancy has been identified as a state of immune suppression, as are some of the other exclusion criteria.

10. “Why is trial registered with clinical trials.gov? Is an intervention being planned?”

No intervention has been carried out. Clinicaltrials.gov allows the registration of observational studies. The study was registered with clinicaltrials.gov to allow external observers to demonstrate the ethical robustness of the study protocol, and was mandated by the ethics committee.

Reviewer 3: TOH LEONG TAN

1. “Page 11 lines 3, 12 and 22: Please change “(3) clinical suspicion of sepsis” to “(3) clinical suspicion of infection”.”

We note the semantics of the term infection and sepsis, with respect to the fact the first inclusion criteria were that patients were SIRS positive. We have left the phrase as “clinical suspicion of sepsis” to reflect both the protocol, and that we wished clinical staff to help identify patients not just with infection but were physiologically altered to be SIRS positive. This study was conceived and designed before the new Sepsis III definitions, when ‘sepsis’ was still defined as ‘infection with evidence of systemic inflammation’ (i.e. infection with 2/4 SIRS criteria). We therefore believe that our terminology is a more accurate description of our inclusion criteria.

2. “Page 11 line 57 - page 12 line 16: Does this study include patients with virus, parasite or fungal infection for cohorts 1 and 2? If it does, how will the infections be confirmed?”

The study was designed to be pragmatic, to allow us to recruit patients that are identified and treated as infection prospectively in hospital. The patients recruited may have viral, parasitic or fungal infection. The further microbiological testing of these patients was carried out by the admitting medical team, and any test recorded to confirm or refute these diagnoses was recorded in the patients’ clinical recording form. Diagnostic criteria for infections were set out in the study protocol, with criteria for clinical and microbiological definitions of infection. As it is often not clear what aetiological agent is responsible for an infection at time of presentation we took the pragmatic decision to enrol all comers, which we believe appropriate when evaluating a potential diagnostic test. For those patients in whom an aetiological agent is confirmed, we will explore the relationship between our markers and organism as a secondary, exploratory outcome.

3. “Page 11 lines 35-41: I suggest to exclude patients with acute cholecystitis, patients on long term inhaled corticosteroids, autoimmune diseases and patients who have taken antibiotics 72 hours
before the enrolment. Reasons as below

We appreciate the suggestion made by this reviewer. However, the study has already started so changes to enrolment criteria are not possible. Furthermore the aim of study is to design a pragmatic test that can be used in any admitting hospital facility. There is a balance between selectivity and generalizability, as a pragmatic study we opted to not exclude patients on long term corticosteroids, autoimmune diseases, and recent antibiotics. We will report the actual diagnoses of patients that were enrolled in the study when it is reported.

4. “Page 14 line 51: Please change "discharge home with 72 hours" to "discharge home within 72 hours””

We thank the reviewer for his observation: we have made the change.

5. “Page 14 line 51: The phrase "(3) discharge to home, or in hospital with no organ failure, within 72 hours" is unclear. It sounds similar to statement "(2) discharge home with 72 hours". Please elaborate & explain the difference or rephrase the sentence.”

The phrase has been changed to “discharge to home within 72 hours, or in hospital with no organ failure at 72 hours”. The difference between outcome 2 and 3 is explained in the next section “Justification and rationale for outcomes of interest”.

6. “Page 15 lines 12-15: The phrase "A further outcome of discharge to home, or in hospital with no organ failure, within 72 hours will identify the group of patients admitted to hospital but unsuitable for discharge due to non-medical reasons." is again unclear. Please explain or rephrase this.”

We have made a clarification to the statement.

VERSION 2 – REVIEW

<table>
<thead>
<tr>
<th>REVIEWER</th>
<th>Paul Dark</th>
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<tr>
<td>University of Manchester, UK</td>
<td></td>
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<tr>
<td>REVIEW RETURNED</td>
<td>26-May-2016</td>
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| GENERAL COMMENTS | In my view, the authors have addressed all of my concerns and the concerns of the other reviewers. |

<table>
<thead>
<tr>
<th>REVIEWER</th>
<th>Tan Toh Leong</th>
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<tbody>
<tr>
<td>Universiti Kebangsaan Malaysia, Malaysia</td>
<td></td>
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<tr>
<td>REVIEW RETURNED</td>
<td>13-May-2016</td>
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| GENERAL COMMENTS | The article has been significantly improved and is much easier to comprehend. All the comments have been addressed. I recommend the article for publication in BMJ Open. |