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## **Profiling the dysregulated immune response in sepsis – overcoming challenges and reaching towards precision medicine**

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Each author co-wrote at least one section of this paper as part of a panel composed of three or more authors who were selected on the basis of their expertise (biochemistry, immunology, critical care, respiratory medicine, or infectious diseases). The panels (and their corresponding sections) were as follows: SC, FV, SF, IR, MW, GL, TS, BS, SBF, MAW, AT (Immunopathophysiology of sepsis); JFB-M, SC, MO, MAW, IR (Predisposing factors); FV, JCS, AC, MW, GL, TS, BS, MG, AGS, EG, MW, GM (Current and future biomarkers to profile immune status in sepsis); MK, JFB-M, BS, IML, RA, TS, MW, MB, and JMC (Broad profiling of the immune status in sepsis: from discovery to clinical application); FV, MK, JCS, GL, BS, MG, MO, AGS, MG, EG, GM, FMB (Towards precision medicine in sepsis); FV, SC, MK, JFB-M (Lessons learnt from COVID-19). All authors also served as internal reviewers for other

sections. JFB-M, RA, FV, designed and created the figures. SC, MK, JFB-M, JCS and FV wrote the abstract, introduction, key messages, and discussion. SC, MK, JFB-M, JCS and FV compiled all sections, and provided final editing of the manuscript. MK and SC managed the reference list, and SC and FV coordinated work on the paper. All authors participated in literature searches and critical analysis of published data. All authors read and approved the final manuscript. All authors are members of the European Group on Immunology of Sepsis (EGIS).

## **Abstract**

Sepsis is characterized by a dysregulated host immune response to infection. Despite recognition of its significance, immune status monitoring is not implemented in clinical practice due to the current absence of direct therapeutic implications. Technological advances in immunological profiling enhance our understanding of immune dysregulation and can facilitate integration into clinical practice. In this review, we provide an overview of the current state of immune profiling in sepsis, including its utility, challenges, and opportunities. We highlight the central role of immunological biomarkers in facilitating predictive enrichment in current and future treatment scenarios. We propose that multiple immune and non-immune-related parameters, including clinical and microbiological data, should be integrated into diagnostic and predictive “combitypes” with the aid of machine learning and artificial intelligence techniques. Ultimately, these combitypes should form the basis of workable algorithms to guide clinical decisions that make precision medicine for sepsis patients a reality and improve their outcome.

## **Key messages**

- A dysregulated immune response lacking physiological balance between hyperactivation and hyporesponsiveness is central in sepsis.
- Immune dysregulation in sepsis patients is heterogeneous and dynamic, requiring personalized treatment strategies.
- Successful immunomodulation trials in severe COVID-19 underscore the potential of therapies targeting the immune system in the management of sepsis.

- Specific immunological biomarkers and high-throughput omics-based techniques provide novel insights into immune dysregulation and allow identification of sepsis endotypes.
- An intuitive step towards achieving precision medicine in sepsis is represented by integration of various types of information, including immunological, clinical, microbiological and high-throughput omics data in so-called “combitypes” with the aid of machine learning (ML) and artificial intelligence (AI) techniques.
- The development of internationally adopted guidelines for assessment of the dysregulated host response in sepsis will lead to a better standardization and clinical utility.

### **Search strategy and selection criteria**

Literature for this review was gathered through PubMed searches using a combination of MeSH terms and free-text keywords including the terms "sepsis", "septic shock", or “covid-19”, in conjunction with at least one of the following terms: "pathophysiology", "risk factors" "immune response", "biomarker", "biomarkers", "precision medicine", “phenotypes”, “endotypes”, "immune monitoring", "immune status", “immune dysregulation”, "immunity", "immunological", “artificial intelligence”, “immunoadjuvant”, and “anti-inflammatory agents”. Filters were applied to include human studies, articles with full text available in English, and specific article types (clinical studies, reviews, and meta-analyses) published from January 2000 up to and including August 2023. Pre-print publications were excluded. The choice of topic-specific search terms and selection of articles for consideration and citation were determined by the authors responsible for each section to allow wide scrutiny of the literature. In a first step, selected articles in each section were approved for inclusion by the authors responsible of each section, but final inclusion required of approval by all authors. Inclusion criteria for articles



consisted of selection of leading articles with relevance to the aims of this paper and their contribution to a current and comprehensive understanding of the topic and a balanced perspective in the field. The reference lists of identified articles were also reviewed to ensure a thorough search and relevant articles from those lists were included if deemed appropriate. Any potential conflicts of interest were managed to maintain objectivity in our review.

## **Introduction**

In recent years, evidence-based treatment guidelines, such as the Surviving Sepsis Campaign, have significantly improved early-phase sepsis outcomes(1). Nonetheless, with an estimated 48·9 million cases worldwide and 11·0 million deaths in 2017, sepsis remains a leading cause of morbidity and mortality(2).

Extensive research has emphasized the role of dysregulated immune responses in sepsis pathophysiology, but only a few immunoadjuvant treatments have demonstrated signals of efficacy(3-6). The heterogeneity of immune dysregulation in sepsis patients largely explains the many failed attempts to target the dysregulated immune response in clinical trials(7-9). However, no individualized immune-status assessment is routinely employed and no internationally adopted guidelines provide recommendations on preferred biomarkers for sepsis research and clinical practice. In this review, we aim to emphasize the urgency of profiling the dysregulated immune response in sepsis to make precision medicine a reality and improve not only early-phase outcomes but also reduce the incidence of late sepsis complications(10). Consequently, we provide an overview of the immunopathophysiology of sepsis and discuss current and innovative strategies to characterize the immune status of patients, including recent advancements in sepsis endotyping, machine learning (ML), and artificial intelligence (AI) approaches. We also examine attempts to implement personalized medicine in sepsis,

highlighting associated challenges and proposing solutions. Finally, we draw on the lessons learnt during the COVID-19 pandemic to improve the future treatment of sepsis.

### **Immunopathophysiology of sepsis**

Our understanding of sepsis pathophysiology has substantially evolved during the past century. Currently, sepsis is portrayed as a complex and heterogeneous state characterized by concurrent pro- and anti-inflammatory responses, in which detrimental sequelae can be caused by both persistent hyperinflammation and prolonged hyporesponsiveness of the immune system (Figure 1).

The heterogeneity of the immune response can be ascribed to a variety of factors, including age (sepsis typically affects the very old and very young), different infectious etiologies, foci, host genetics, applied treatments, and rapidly changing illness' dynamics. An overview of predisposing factors for sepsis is provided in the appendix, p 1.

In sepsis, the immune response is initially activated through sensing of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs). This in turn induces broad biological responses, such as release of cytokines and other inflammatory mediators(11) as well as induction of cell death through activation of caspases(12). Furthermore, activated granulocytes released from the bone marrow produce neutrophil extracellular traps (NETs) to capture microorganisms, which aggravates inflammation and tissue injury(13). The inflammatory response in sepsis is also characterized by neuro-endocrine alterations, activation of the complement and coagulation systems, and alterations in lipid mediators - all acting synergistically to enhance inflammation(14, 15). If systemic and/or sustained, this inflammatory cascade potently activates the endothelium, which loses its homeostatic

functions, becomes pro-coagulant, leaky, and contributes substantially to the development of shock, organ dysfunction, and ultimately death in a considerable proportion of patients(16). An important mechanism leading to imbalanced coagulation and endothelial dysfunction in septic shock is depletion of activated protein C (APC), which has important anticoagulant and anti-inflammatory effects as well as the ability to promote fibrinolysis and inhibit thrombin generation(17).

In a subset of patients, an uncontrolled hyperinflammatory response may occur, resulting in hyperferritinemic sepsis, macrophage activation-like syndrome (MALS), or in the most severe form, hemophagocytic lymphohistiocytosis (HLH). All of these conditions are associated with high mortality and typically occur early in the disease course (Figure 1)(18, 19)

However, in some sepsis patients concurrent immunoregulatory compensatory mechanisms may gain the upper hand and dictate the host response (Figure 1)(20, 21). For example, early findings revealed that circulating monocytes of septic patients display impaired pro-inflammatory cytokine secretion in response to PAMP exposure(22, 23). Similarly, innate immune cells such as dendritic cells, natural killer cells, and neutrophils also display a reduced capacity to produce mediators essential for an effective pathogen clearance(24-26). Instead, these cells secrete increased levels of anti-inflammatory or immunoregulatory molecules such as interleukin (IL)-10 that dampen antimicrobial effector mechanisms(27). These innate immune defects are explained by a i) metabolic and epigenetic reprogramming triggered by the initial (hyper)inflammatory insult(28) and/or ii) altered leukocyte differentiation/generation in the bone marrow(29). Sepsis-induced immunosuppression is also characterized by major defects in adaptive immune function(30). For instance, T-cell counts are reduced due to apoptosis while surviving T-cells frequently display an exhausted phenotype. This is, among others, characterized by and/or due to increased expression of immune checkpoint molecules as well as an impaired production of immunostimulatory cytokines such as interferon (IFN)- $\gamma$ .

Finally, a relative expansion of regulatory T-cells (T-regs) has repeatedly been reported(31-34). In most cases, hyporesponsiveness of the immune system in sepsis takes center stage in the post-acute phase, even though its molecular hallmarks are apparent very early after the onset of sepsis. This immunocompromised state can lead to serious consequences such as reactivation of latent viral infections, a phenomenon that can negatively impact recovery and overall outcome(35, 36). Furthermore, sepsis-induced immune alterations render the host highly vulnerable towards secondary infections, often with opportunistic, difficult-to-treat pathogens(37).

Sepsis-induced immune alterations may persist in survivors for years after hospital discharge, albeit at a low grade. There is compelling evidence that a significant proportion of sepsis survivors do not fully recover after sepsis but have dismal long-term functional, cognitive and physical derangements(10, 38). In these sepsis survivors, evidence for the presence of persistent low-grade inflammation, immune suppression, and lean tissue wasting was found(39). Consistently, patients with “so-called” persistent inflammation, immunosuppression and altered catabolism syndrome (PICS, Figure 1) are now recognized to constitute a specific syndrome of chronic critical illness (CCI) associated with poor long-term outcomes(40).

Taken together, pathophysiology of sepsis involves a major imbalance in the immune response to infection. Depending on the host, pathogen and sepsis phase, this may take the form of a dominant hyperinflammatory or immunosuppressive phenotype. Both are linked to poor prognosis but could necessitate contrasting immunoadjuvant therapies (Figure 2). Due to the absence of clinical signs indicative of a patient's immune status, immune monitoring is crucial in determining an appropriate treatment and therapeutic window.

### **Current and emerging biomarkers to profile the immune status in sepsis**

An overview of the significance and suggested use of currently employed biomarkers to profile the immune status in sepsis is provided in the appendix, p 3-7. Technologies to profile the immune status in sepsis that are already available in most hospitals and moving towards broad-scale clinical application are displayed in Figure 3.

#### *Routine clinical laboratory markers*

Only a few immune biomarkers are available for routine use by clinicians in the management of sepsis. Acute phase inflammatory proteins such as C-reactive protein (CRP) and more specific markers of bacterial infection including procalcitonin (PCT) are used as diagnostic markers of infection and can aid in monitoring the response to antibiotics(41), in particular in pediatrics(42, 43). They may also have prognostic value(44). Likewise, a decreased lymphocyte count was identified as a marker of poor prognosis in sepsis and pneumonia(45, 46). An increased immature granulocyte fraction (assessed through measurement of the delta neutrophil index by some blood-cell analyzers), has been proposed as a marker for early diagnosis and prognostication in sepsis(47-50). The latter also applies to alterations in the neutrophil-to-lymphocyte ratio (51). Finally, routinely-measured ferritin is typically used to identify MALS(52).

### *Soluble markers*

An early elevation in inflammatory cytokines (e.g. IL-6, IL-8) may be indicative of a hyperinflammatory phenotype(53-57). Conversely, increased levels of IL-10 and TGF- $\beta$  are associated with immunosuppression and may be causally related to the reduced expression of Human Leukocyte Antigen (HLA)–DR on monocytes (see section below)(58, 59). Activation of the complement system in sepsis leads to generation of the C5a. This anaphylatoxin has been proposed as a therapeutic target, given that C5a inhibition enhanced immune function in preclinical studies(60-62). Furthermore, complement activation also leads to consumption of complement factors such as C3, and a relationship between C3 depletion and the expansion of T-regs was identified in septic patients(63). Finally, plasma level of the soluble fraction of the immune-inhibitory receptor PD-L1 (sPD-L1) are elevated in sepsis(64, 65). Although several of these markers (e.g. some cytokines) are already routinely measured in certain hospitals, they should be broadly implemented on clinical analyzers and developed into point-of-care tests to facilitate their use in daily clinical practice.

### *Phenotypic cellular markers*

HLA-DR is part of the major histocompatibility class II complex (MHC-II) expressed by antigen-presenting cells at the interface between innate and adaptive immunity. Its expression is generally measured by flow cytometry, although mRNA expression has been used as well(66, 67). Several studies have demonstrated an association between decreased monocyte HLA-DR expression (mHLA-DR) and functional alterations of monocytes such as reduced pro-inflammatory cytokine release and diminished antigen presentation capacity(68, 69). Most importantly, low mHLA-DR expression was shown to be independently associated with an increased risk of secondary infections and death in critically ill patients(70). Recent work identified clusters of septic shock patients exhibiting specific mHLA-DR trajectories related to

unfavorable outcomes(71, 72). A wide consensus in the scientific community therefore claims that a low mHLA-DR expression constitutes a surrogate marker of sepsis-induced immunosuppression(21, 73).

More complex cellular phenotyping by flow cytometry can provide detailed information on cellular activation/exhaustion, maturation and function in septic patients. Concerning neutrophils, low surface expression of CD16 and CD10 is indicative of low-density immature granulocytes and increased abundance of these immunosuppressive myeloid-derived suppressor cells (MDSC)(74-76) is associated with an increased risk of death and secondary infections in sepsis(74, 77). T-cell exhaustion is defined by three interrelated features: (1) impaired effector function, (2) sustained increase in cell-surface expression of inhibitory immune checkpoint molecules such as programmed cell death 1 (PD-1) on T-cells and/or its ligand PD-L1 on antigen-presenting cells, and (3) distinct transcriptional state that impairs development of functional T-cell memory. Increased expression of PD-1 and PD-L1 is associated with progression of infection to sepsis(78), risk of nosocomial infections(79), and a more severe disease state(80). In aged patients with sepsis, especially in non-survivors, a relative abundance of the immunocompetent CD28<sup>+</sup> subset of CD4<sup>+</sup> T-cells decreased, whereas that of the immunosuppressive PD-1<sup>+</sup> T-cells and CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T-regs increased(81). Regarding the latter, an increased proportion of T-regs has been repeatedly observed in sepsis(31-34), and is related to a decreased lymphoproliferative response(31). The CD4/CD8 cell ratio is another potential marker of immune functionality. For example, in elderly patients with sepsis, the mean CD4/CD8 T-cell ratio is significantly lower in non-survivors than in survivors(82, 83). Finally, an increasing number of studies have demonstrated the feasibility of using dedicated flow cytometry panels in multicentric and/or longitudinal clinical studies. This analytical capacity illustrates its potential for clinical application(78, 84, 85).

### *Functional tests*

Functional testing potentially represents an attractive method to identify immune deficiencies in sepsis, because it directly assesses the *ex vivo* capacity of a cell population to respond to an immune challenge by measuring its cytokine production, oxidative burst capacity, proliferation, and/or activation status. Low Tumor Necrosis Factor (TNF) production by *ex vivo*-stimulated whole blood was used as an inclusion criterion to apply immunostimulatory therapy in pediatric patients with sepsis(86). Although commercial easy-to-use products are emerging(87), routinely available assays remain to be developed in a standardized format, similar to the assay for interferon (IFN- $\gamma$ ) release in response to tuberculosis antigens.

### *Monitoring of viral reactivation*

There is growing evidence of reactivation of latent viruses in sepsis patients(35, 88, 89). Compared to other critically ill patients (burns, trauma), septic patients show elevated herpes virus titers (mostly determined as DNAemia via PCR assays available in most hospitals)(90, 91) that correlate with transcriptomic(36) or humoral(36) features of immunosuppression and are associated with disease severity and mortality(90-92). While viral reactivation may be pathogenic and contribute to disease severity(36, 93, 94), viral titers primarily represent a potentially useful metric to gauge immunocompetence.

### **Broad profiling of the immune status in sepsis: from discovery to clinical application**

The emergence of high-throughput “omics” technologies combined with advanced data analysis facilitates gaining a broader picture of the immune status in sepsis. This approach has been coined “sepsis systems immunology”(95).

### *Transcriptomics*



Whole blood (i.e. leukocyte) gene expression profiling has contributed extensively to the derivation of transcriptomic panels that aid rapid diagnosis of sepsis and identification of different sepsis subclasses, also known as “endotypes”, e.g. specific patterns of gene expression that are related to the host response in sepsis(96-107). It was demonstrated that endotypes characterized by a reduced expression of genes involved in lymphocyte signaling and antigen presentation pathways, are indicative of impaired innate/adaptive immune responses and identify poor prognosis patients. These are the so-called “sepsis response signature 1” (SRS1)(100) and the Mars1 endotype in adults(99), as well as the A endotype in children(108). These findings suggest that impaired immunity is a common signature conferring severity to sepsis. The identification of a cluster of sepsis patients characterized by an overall activation of adaptive immunity and improved survival supports this notion(97). Furthermore, endotypes have been shown to evolve during hospitalization in parallel to disease severity, and the persistence of pro-inflammatory or “coagulopathic” endotypes predicts worse outcomes(109). Progressing towards the overarching goal of stratification of sepsis patients into endotypes of clinical utility will nevertheless require an international collaboration to establish a consensus sepsis endotype model including variability stemming from geographically diverse patient populations. In addition, although the blood transcriptomics have been central to increasing our understanding of the host response during sepsis, most studies have focused on samples obtained at ICU admission, i.e. acute sepsis. Recent work, supportive of the PICS endotype, demonstrated that blood transcriptional patterns of surgical sepsis patients who developed CCI were partly distinct at both 1 and 14 days post-sepsis relative to sepsis survivors who rapidly recovered(110). Further work should clarify whether sepsis survivors exhibit *bona fide* transcriptomic alterations in connection with these endotypes(39).

A major evolution in transcriptomic analysis is the development of single-cell RNA sequencing (scRNA-seq) technologies, which permits the analysis of transcriptomic profiles at the level of

single cells. So far, a few small studies limited to peripheral blood mononuclear cells have employed scRNAseq in the context of sepsis(111-115). Despite their limitations, those studies have uncovered potentially important cellular features, for example, identification of a novel CD14<sup>+</sup> monocyte subset (termed MS1), which was expanded in bacterial sepsis patients(114). Furthermore, among survivors who develop CCI, scRNA-seq analysis (in combination with cellular indexing of transcriptomes and epitopes (CITE)-seq) revealed lymphocytes characterized by gene expression profiles attuned to simultaneous immunosuppressive and low-grade pro-inflammatory states(113). Another study reported that myeloid-derived suppressor cells maintain a transcriptomic profile reflective of an immunosuppressive state in patients with late sepsis(115), while recently, scRNAseq of circulating hematopoietic stem and progenitor cells revealed altered granulopoiesis in patients with sepsis and poor outcome(116). These studies illustrate that scRNA-seq is a powerful tool that has potential in resolving the diversity in patients' immune status. Large population studies that profile millions of single cells, including the challenging granulocytes are nevertheless necessary to confirm, refine and operationalize scRNA-seq.

### *Proteomics*

Liquid chromatography-based separation methods coupled with mass spectrometry has contributed to identifying up to 3000 proteins involved in sepsis pathology, for instance in the inflammatory response, induction of oxidative stress and mitochondrial dysfunction(117). Other simpler tests to profile immunological proteins are the bead-based multiplex assays, extensively used for simultaneous quantification of cytokines, chemokines and neutrophil degranulation markers in sepsis(118, 119). A relatively new technology, Proximity Extension Assay, combines a targeted immunoassay with PCR to provide multiplex quantification of hundreds of proteins in minimal volumes of any biological fluid(120). This technology has been used to identify new biomarkers in sepsis and COVID-19(121, 122).

### *Metabolomics*

Metabolic pathways are crucial in regulating immune responses(123). An integrated metabolomics and proteomics analysis of septic patients' plasma revealed a dysregulation of amino acid metabolism related to inflammation and immunity(124). For example, tryptophan degradation and kynurenine generation via the indoleamine-2,3-dioxygenase (IDO-1) pathway is a driver of sepsis-associated immunosuppression and is associated with increased T-cell apoptosis, decreased T-cell proliferation, and generation of T-regs(125). Furthermore, metabolomic profiling of neutrophils of septic patients revealed that inhibition of glycolysis contributed to the immunosuppressed phenotype of those cells<sup>(126)</sup>. Also, leukocytes from septic patients exhibit generalized metabolic defects at the level of both glycolysis and oxidative metabolism, which are restored in recovered patients and when exposed to *ex vivo* treatment with IFN- $\gamma$  or IL-7<sup>(28, 127)</sup>. Finally, recent work demonstrates that metabolomics may have value in identifying subphenotypes of sepsis-induced ARDS(128).

### *Cytomics*

The limitations of traditional flow cytometry related to resolution and number of fluorescence channels do not apply to two novel cytomics technologies: i) mass cytometry by time of flight (CyTOF)(129) and ii) spectral cytometry(130). Both technologies enable simultaneous analysis of more than 40 markers. In sepsis, CyTOF identified shifts in B-cell subpopulations and novel subsets of myeloid cells(131). Furthermore, a barcoding method for standardized immunophenotyping to derive immune trajectories in critically ill patients has been recently reported(132).

### *Emerging tools to rapidly profile the immune status in sepsis*

While these omics-based technologies are helpful to identify immune profiles associated with sepsis diagnosis and prognosis, their application to clinical practice remains difficult given their complexity, labor-intensity and lack of standardization. Emerging technologies have the potential to solve this problem. For example, new reverse transcription amplification technologies allow profiling of gene expression signatures within minutes(133, 134). Such methodologies have already shown value for rapid diagnosis of sepsis(133, 135). Furthermore, an automated multiplex-quantitative PCR of several targeted genes on whole blood samples (results within 60 min) identified septic patients with low mHLA-DR expression. The same technology was recently employed to calculate a transcriptomic score which identified patients at risk of ICU-acquired infections(136). New microfluidics-based platforms are able to quantify immunological and endothelial proteins in a multiplex format in less than two hours(137). Finally, biosensors are emerging to rapidly profile host response proteins, cell surface markers and even functional parameters such as neutrophil motility(138, 139).

#### *Machine learning and artificial intelligence approaches*

An intuitive step towards establishing precision medicine in sepsis is integration of various types of information including immunological, clinical, microbiological and high-throughput “omics” data in meaningful combinations: so-called “combitypes”. ML and AI algorithms have a potential to facilitate implementation of combinatorial strategies by assimilating large datasets to learn key patterns that could deliver timely and accurate information on the status of a patient and guide treatment decisions(140).

Examples of AI approaches used in sepsis research includes a natural language processor enabled AI algorithm that was trained on clinician’s free-text and electronic medical records for an early detection and diagnosis of sepsis(141). Additionally, unsupervised k-means clustering (a type of ML) was used to derive clinical sub-phenotypes of sepsis ( $\alpha$ ,  $\beta$ ,  $\gamma$  and

$\delta$ (142)) and another AI method trained on a 29-gene signature was able to discriminate acute bacterial and viral infections(143). While most studies have focused on investigating different data modalities in isolation, combinatorial strategies using ML/AI-based methods should receive more attention given their efficacy. For example, a ML algorithm was able to classify all-cause sepsis patients as blood transcriptomic endotypes (Mars1-4), which when combined with APACHE IV scores improved on mortality prediction compared to APACHE IV scores in isolation(144). A retrospective analysis of the PROWESS study (testing recombinant human APC) reported that patient assignment to  $\alpha$ ,  $\beta$ ,  $\gamma$  and  $\delta$  sub-phenotypes in the context of the treatment effect, was significantly modified by integrating clinical microbiological data in a host-pathogen model versus host-only model(145). Recently, an ML approach analyzing blood transcriptional profiles and metagenomic data identified a multi-omic signature that accurately distinguishes sepsis from non-infectious critical illness(146). Ultimately, combitypes should be integrated into workable algorithms/decision trees that can be effectively utilized by clinicians. One example is the pediatric PERSEVERE model, which combines soluble markers of immune and endothelial responses, platelet counts, and mRNA expression to generate a prediction model and serve as a prognostic enrichment tool(147-150).

Another emerging and futuristic data-driven approach is represented by the creation of virtual replicas of a patient's physiology. This concept, known as “digital twinning”, can facilitate an even more refined personalized treatment plan based on a patient's unique physiological characteristics(151-153). As such, an “AI clinician”(154) could utilize data from a patient's digital twin to identify the most effective treatment strategies, considering their individual response to different medications and interventions.

Table 1 lists (other) examples of emerging applications of immunological profiling in sepsis.

## **Towards precision medicine in sepsis**

Precision medicine constitutes a new concept embracing individual patient characteristics in clinical decision-making, thus abandoning a “one-size-fits-all” approach(155, 156). This personalized approach was first conceptualized in oncology and has become standard-of-care for the treatment of many cancers(157). For precision medicine to succeed, population-scale heterogeneity needs to be reduced by applying an “enrichment” strategy(156). The enrichment can be subdivided into two, not mutually exclusive approaches: i) “prognostic” and/or ii) “predictive” enrichment. In prognostic enrichment, a subgroup of patients is selected from a larger, and heterogeneous patient population who are more likely to meet clinically defined endpoints/outcomes (e.g., mortality)(158, 159). Predictive enrichment refers to a selection of a subgroup of patients from a diverse population who are more likely to respond to a specific biologically driven therapeutic intervention(158, 159). In contrast to oncology, enrichment strategies in sepsis, particularly predictive enrichment, are challenging. This is due to the highly dynamic nature of sepsis and incomplete knowledge on the pathophysiological mechanisms of a given sepsis phenotype. There is consensus among researchers and clinicians that a successful application of precision medicine in sepsis requires a balanced application of both prognostic and predictive enrichment strategies(159, 160).

Although limited, we present an overview of clinical studies evaluating adjunctive immunotherapy in sepsis using precision medicine approaches. Unlike numerous failed trials with immunomodulatory therapies in unselected sepsis patients, these studies show promising results, suggesting that precision medicine is likely the path forward.

### *Anti-inflammatory treatments*

Methylprednisolone was investigated in a multicenter, double-blind randomized clinical trial (RCT) performed in patients with sepsis due to severe community-acquired pneumonia and pronounced inflammation (CRP >150 mg/L at admission)(161). The methylprednisolone treatment group exhibited significantly less late treatment failure and a reduced mortality trend. These results are in line with those of a recent multicenter prospective cohort study, in which glucocorticoids significantly reduced 30-day mortality in the subgroup of patients with septic shock and/or requiring mechanical ventilation and a CRP >150 mg/L(162).

The impact of the anti-TNF monoclonal antibody afelimomab on survival was studied in the RAMSES RCT conducted in septic patients with elevated serum IL-6 concentration (>1000 pg/mL)(163). Although high IL-6 levels identified a sepsis subgroup with higher mortality, the study was terminated prematurely, as the modest mortality reduction in the afelimomab-treated group was unlikely to achieve statistical significance even with more enrolled patients. A few years later, in the larger MONARCS RCT that used the same IL-6-based stratification, treatment with afelimomab demonstrated a covariate-adjusted 5.8% reduction in the risk of death.(164). Furthermore, afelimomab significantly reduced circulating TNF and IL-6 levels and more rapidly improved organ failure scores compared with placebo.

Anakinra is a recombinant, engineered variant of the IL-1 receptor antagonist (IL-1RA) blocking activity of both IL-1 $\alpha$  and IL-1 $\beta$ . Two previous phase III RCTs showed no efficacy of anakinra treatment in an unstratified severe sepsis patient population(165, 166). Interestingly, a re-analysis of the first trial demonstrated a mortality benefit of anakinra in patients with an initial plasma IL-1RA level above an empirical threshold of 2071 pg/mL(167). Moreover, a subgroup analysis of the second trial reported an absolute 30% reduction of 28-day mortality in anakinra-treated patients with MALS (identified by hepatobiliary dysfunction and disseminated intravascular coagulation)(168). The recently published PROVIDE trial stratified patients with septic shock into three endotypes based on

circulating ferritin concentrations and mHLA-DR(169). Patients with MALS (i.e. ferritin > 4420 ng/mL) were randomized into intravenous treatment with placebo or anakinra. Anakinra treatment led to a significant improvement in SOFA score, absolute lymphocyte counts, and international normalized ratio (INR) during the first week.

### *Immunostimulatory therapies*

The use of IFN- $\gamma$  to restore immune function in sepsis was first reported in 1997, in a case series of nine septic patients with low mHLA-DR expression(22). IFN- $\gamma$  restored mHLA-DR expression, enhanced TNF secretion by monocytes *ex vivo* stimulated with LPS, and resulted in clearance of sepsis in eight patients. Several case reports/series demonstrating similar results in sepsis patients with signs of overt immunosuppression were published in the following decades(170-172), the most recent example was the use of IFN- $\gamma$  in five critically ill COVID-19 patients with impaired cellular immunity(173). The PROVIDE study is currently the only RCT on IFN- $\gamma$  therapy in sepsis. Patients with low mHLA-DR expression (<5000 mAb/cell) and no hyperinflammation (ferritin <4420 ng/mL) received either IFN- $\gamma$  or placebo(169) but due to the low enrollment (two patients with immunoparalysis) no conclusions can be drawn.

An RCT with limited sample size showed efficacy of granulocyte-macrophage colony-stimulating factor (GM-CSF) to restore mHLA-DR in patients with persisting sepsis-associated immunosuppression (mHLA-DR <8000 Ab/cell)(174). Furthermore, although not powered for that clinical endpoint, GM-CSF treatment decreased the duration of mechanical ventilation(174). A double-blind RCT in surgical ICU patients showed that treatment with GM-CSF in patients with postoperative immunosuppression was safe and effective in restoring mHLA-DR, and reduced duration of infection(175).

IL-7 is a non-redundant potent cytokine involved in T-cell development, survival and proliferation. As such, recombinant human (rh)IL-7 has primarily been developed to treat



lymphopenia-associated disorders(176-178), but is currently under investigation in sepsis and COVID-19. Importantly, all published clinical reports on rhIL-7 use (phase II RCT and clinical cases) were guided by an absolute lymphocyte count of  $<900$  cells/ $\mu$ L or lower(179-183). Preliminary results showed the efficacy of rhIL-7 to enhance lymphocyte counts in septic patients in the absence of any severe side effects(179-183).

Immune checkpoints inhibitors targeting among others PD-1, PD-L1, CTLA-4 are revolutionizing cancer treatments. A double-blind, phase Ib RCT evaluated anti-PD-1 treatment nivolumab in adult septic patients stratified by absolute lymphocyte count (i.e.  $\leq 1100$  cells/ $\mu$ L). This therapy was safe, without signs of a “cytokine storm” and was associated with restoration of mHLA-DR(184). Similarly, anti-PD-L1 therapy was well tolerated in a small group of sepsis patients with absolute lymphocyte counts  $\leq 1100$  cells/ $\mu$ L, with no evidence of drug-induced hypercytokinemia(185). Furthermore, at higher dosages, anti-PD-L1 treatment increased mHLA-DR expression, an effect that persisted beyond 28 days. In two case reports, nivolumab combined with IFN- $\gamma$  conferred a clinical benefit in immunosuppressed ICU patients with invasive bacterial and fungal infections based on the monitoring of PD-1 expression on T-cells and mHLA-DR(186, 187).

In the appendix p 2, we discuss additional biomarker-guided immunoadjuvant treatments using extracorporeal therapy and immunoglobulin supplementation.

### **Lessons learnt from COVID-19**

COVID-19, in its most severe form, fulfills sepsis criteria. However, it is much less heterogeneous than “classic” sepsis due to pathogen etiology and focus. The treatment strategies tested in COVID-19 have resurrected the notion that anti-inflammatory therapies could improve survival in sepsis(188-190), especially when applied to selected patients. For instance, in the SAVE-MORE trial, enrollment was guided by a biomarker (suPAR) that acts

as a danger-associated molecular pattern(191). In that enriched patient population, treatment with anakinra improved survival(191). Furthermore, both dexamethasone and the IL-6 receptor antagonist tocilizumab were found to be most effective in patients with high CRP levels(192, 193), and there was even evidence of harm from tocilizumab in patients with low CRP(193). The results obtained with IL-6 receptor antagonists and Janus kinase-inhibitors have renewed interest in those therapies for biomarker-guided treatment of non-COVID septic patients with acute lung injury and hypercytokinemia(194, 195). The fact that the general COVID-19 patient population is relatively homogenous is a likely reason why the above therapies were frequently effective in relatively unselected COVID-19 patient populations. This observation might underscore the notion that targeted application of immunomodulatory therapies in classic sepsis is promising following characterization of the immune status. Nonetheless, an important consideration for future intervention studies involving anti-inflammatory drugs in bacterial sepsis is the potential impact on resolving ongoing infections and the risk of developing secondary infections. In this respect, a relatively low risk for protracted and secondary infections may also play a role in the beneficial effects of anti-inflammatory therapies in severe COVID-19(188, 189). For example, COVID-19 patients exhibit less pronounced immune suppression (measured by mHLA-DR expression) compared to bacterial sepsis patients(196). Additionally, peak viral shedding typically occurs early in the course of COVID-19(197), whereas anti-inflammatory agents were shown to improve outcome of hospitalized patients who progressed to severe disease(188, 190, 198). Unlike influenza, where high-dose corticosteroids increase mortality(199) and bacterial superinfections are common and contribute to poor clinical outcomes, bacterial co-infection upon admission is relatively rare in COVID-19(200-202). As a result, the potential negative impact on pathogen clearance from the use of anti-inflammatory drugs in COVID-19 trials in severe and critical COVID-19 may have been minimal. These aspects underscore the potential value of the aforementioned combitypes in

future studies investigating anti-inflammatory agents in bacterial sepsis. Using such an approach, patients may be characterized according to their immunological endotype enriched by clinical and microbiological features. The pandemic has also highlighted the importance of sex-based differences in the dysregulated immune response to infection, as males with COVID-19 are at a higher risk of worse outcomes(203). This underscores the necessity of considering sex as an important factor in sepsis-immunology research.

## **Discussion**

In this review, we present the current status and future clinical perspectives concerning profiling of the dysregulated host response in sepsis. Although targeted immunotherapies have the potential to transform care for sepsis patients, current challenges include: i) a poor performance of routinely available biomarkers, ii) limited access in routine clinical care to more specific immune-related biomarkers, iii) uncertainty of when and how often immune biomarkers should be measured, iv) current lack of clear therapeutic implications, and v) failure of single biomarkers to profile the full complexity of the dysregulated host response.

First, routinely used biomarkers including CRP, PCT, and lymphocyte counts do not reflect the functionality of the immune system. These markers have a limited specificity and are insufficient to guide individual treatments and/or monitor effects of immunotherapy. Nevertheless, they have shown value in enrichment strategies in preliminary RCTs evaluating immuno-adjuvant therapies in sepsis and COVID-19. An increased use of these markers in clinical practice will likely expand clinicians' empirical knowledge of the dysregulated immune responses in sepsis patients. This can spark new ideas on how to utilize these (and related) biomarkers to improve patient outcomes. Additionally, it will enhance possibilities to conduct

large-scale sepsis studies based on retrospective data, taking into account at least some form of immunological profiling.

Second, this review also highlights potential of more specific markers (i.e. mHLA-DR, viral reactivation, multi-cytokine panels, and/or functional tests) to distinguish patients with overriding hyperinflammation from those with persisting immunosuppression. These markers, primarily available in specialized and large centers, are increasingly used to guide individual patient treatments and as enrichment strategies in clinical trials. For example, detection of immunosuppression may facilitate a more rapid identification of secondary infections and viral reactivation through increased preparedness for specific nosocomial/opportunistic pathogens. This knowledge should further motivate clinicians to combine immunological markers with therapeutic drug monitoring, as it is crucial to ensure an appropriate antibiotic administration in patients whose immune responses fail to efficiently resolve infections. With ongoing technical improvements and development of point-of-care devices, we anticipate that such immune monitoring markers will become broadly available and integrated in routine care within the next decades.

Third, optimal timing of immune monitoring is challenging, because sepsis is highly dynamic. Consequently, immune monitoring should start on the first day of ICU admission to identify patients who may benefit most from early interventions (e.g. targeting hyperinflammation). In addition, to distinguish between homeostatic physiological responses and pathogenic immunosuppression, markers related to sepsis-induced immunoregulatory mechanisms should not be interpreted based on a single measurement obtained at ICU admission. Instead, repeated measurements over time or assessment of levels after a few days of ICU admission can inform about persisting immunosuppression and help to identify patients who fail to recover/return to immune homeostasis.

Fourth, a major reason why immune-related biomarkers are sparsely used is the lack of high-grade evidence proving the efficacy of biomarker-guided immune-adjuvant treatments in sepsis. As a result, there are no internationally defined guidelines for biomarker use in sepsis patients. However, several small and/or retrospective studies in sepsis and COVID-19 suggest that a biomarker-driven approach is feasible and associated with beneficial outcomes. COVID-19 studies have clearly demonstrated that anti-inflammatory therapies can improve outcomes in subgroups of “hyperinflamed” patients. However, there are significant disparities between severe COVID-19 and bacterial sepsis regarding the risk of impaired pathogen clearance upon the use of anti-inflammatory agents. Therefore, future stratification strategies in non-COVID sepsis patients should be simultaneously based on: i) a hyperinflammatory phenotype and ii) clinical and microbiological features. The latter element would be indicative of the presence of infections that are amenable (or not) to effective antimicrobial treatment. In other words, anti-inflammatory treatments should be withheld when the causative pathogen is known to cause complicated infections. In this context, the recently published CAP-COD trial provides valuable insights, as it demonstrated that low-dose hydrocortisone improved survival in patients with severe community-acquired pneumonia that were predominantly caused by *S. pneumoniae*, a relatively easy-to-treat pathogen(3). Recent findings further support the important role of microbial etiology in treatment responses and derivation of subphenotypes of sepsis(9, 145)

Fifth, considering the complexity and diversity of mechanisms involved in immunological dysregulation in sepsis, combinations of multiple immune and non-immune-related parameters such as clinical information and microbiology data (see the previous point) are key in making the true precision medicine a reality. Such combitypes will be facilitated by the rapidly evolving development of high-throughput omics-based techniques, advanced point-of-care tests that measure multiple (panels of) biomarkers simultaneously and are subjected to the ML/AI

analytical scrutiny. In the final operationalization step, the most precise combitypes will be integrated into simple workable algorithms to guide complex clinical decisions.

Considering the challenges outlined in this review, Table 2 delineates our specific recommendations for future research.

In conclusion, we are convinced that accurate profiling of the dysregulated host response will be essential to take the next step in optimization of care for septic patients. Several candidate biomarkers have shown potential for patient enrichment in trials investigating immunomodulatory therapies. Nevertheless, additional promising approaches to assess the immune status that are combinatorial in nature should be developed and/or refined for clinical operationalization. Such a multi-directional research effort will pave the way for improved disease conceptualization, sepsis diagnosis, disease prediction, patient enrichment, and superior therapy guidance. Ultimately, refined clinical trials confirming clinical benefits of biomarker-guided strategies are an indispensable step to reach the widely desired goal of personalized medicine in sepsis.

### **Declaration of interests**

SC report personal fees from Pfizer, AstraZeneca, Swedish Orphan Biovitrum (SOBI), GlaxoSmithKline (GSK), and Merck Sharp & Dohme. MK reports personal fees from ARTCLINE, Atriva, AOP pharma, Inflammatix, and 4TEEN4, and discloses institutional funding from MediSieve, 4TEEN4, Adrenomed, Spinghotec, Cytosorbents, and Inflammatix. JBM report personal fees from GSK, PROFARMA program, Ministry of Industry Spain, GSK-MODUS program and Inflammatix, and has patents on the following biomarkers related to management of severe infections: PCT/EP2018/064363 “MMP-8 as a marker for identifying

infectious disease” PCT/EP2018/052499 “Pro-ADM as marker indicating an adverse event” WO2020030745 “Pro-ADM for prognosing the risk of a medical condition requiring hospitalization in patients with symptoms of infectious disease” EP20383140.9 “In vitro method for predicting mortality in COVID-19 patients” (based on detecting N-antigenemia of SARS-CoV-2 in plasma) JCS disclose institutional funding from Orion Pharma, Abbott Nutrition International, B. Braun Medical AG, CSEM AG, Edwards Lifesciences Services GmbH, Kenta Biotech Ltd, Maquet Critical Care AB, Omnicare Clinical Research AG, Nestle, Pierre Fabre Pharma AG, Pfizer, Bard Medica S.A., Abbott AG, Anandic Medical Systems, Pan Gas AG Healthcare, Bracco, Hamilton Medical AG, Fresenius Kabi, Getinge Group Maquet AG, Dräger AG, Teleflex Medical GmbH, Glaxo Smith Kline, Merck Sharp and Dohme AG, Eli Lilly and Company, Baxter, Astellas, Astra Zeneca, CSL Behring, Novartis, Covidien, Philips Medical, Prolong Pharmaceuticals and Nycomed, Phagenesis, and Cytel, outside of the submitted work. JCS is Chair of the Clinical Advisory Board of Hemotune (no financial compensation). MSW has received unrestricted funding from Sartorius and report personal fees from GRIFOLS, Gilead, and Amomed. GL reports personal fees from SOBI. MAW reports personal fees from MSD, Gilead, Pfizer, Shionogi, Eumedica, and Coulter, Biotest, Sedana, SOBI, Böhringer, and has patent on EPA17198330 “Delta-Like Ligand 1 for diagnosing severe infections”. IML report personal fees from MSD, Pfizer, and Gilead. EG disclose institutional funding from Abbott CH, bioMérieux Inc, InflaRx GmbH, Johnson & Johnson, MSD, SOBI and XBiotech Inc, Horizon2020 Marie Skłodowska-Curie International Training Network “the European Sepsis Academy”, Horizon 2020 European Grants ImmunoSep and RISCinCOVID, Horizon Health European Grants EPIC-CROWN-2. MO is President of the European Shock Society and Deputy editor in Intensive Care Medicine Experimental. All other authors declare no competing interests.

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**Table 1. Emerging applications of immunological profiling in sepsis.**

<b>Application</b>	<b>Description</b>
<b>Predicting the risk of sepsis</b>	A profoundly altered peripheral adaptive immune compartment after critical injury is identified as a potential biomarker to identify individuals at a high risk of developing sepsis (measured by flow cytometry in PBMCs)(204).
<b>Identification of pre-sepsis/early diagnosis of sepsis</b>	Host biomarker signatures may be able to identify postoperative infection or sepsis up to three days in advance of clinical recognition(205). Distinct immune signatures precede the onset of severe sepsis and lethality, providing a method to triage early sepsis patients(103).
<b>Combination strategies to improve diagnosis and severity stratification</b>	Combining host response and microbial signatures improves sepsis diagnosis(146). Combining transcriptomic, lipidomic and targeted proteomics facilitates early detection of neonatal sepsis(206). Combining gene expression in leukocytes and inflammatory mediators improves infection diagnosis (SeptiCyte LAB+ CRP)(207). Combining gene expression and protein quantification improves severity stratification (PERSEVERE-XP)(149).
<b>Adding immune signatures to clinical scores to improve outcome prediction</b>	29-mRNA host response whole-blood signature + qSOFA improves mortality prediction(208).
<b>Improving differential diagnosis of sepsis of bacterial or viral origin using rapid transcriptomic tests in whole blood</b>	The HR-B/V host gene expression test rapidly and accurately discriminates bacterial and viral infection (better than procalcitonin)(209). The TriVerity test uses two algorithms (IMX-BVN and IMX-SEV) to produce three separate scores that determine the likelihood of bacterial infection, viral infection, and requirement of organ supportive therapy(133, 210).
<b>Use of leukocyte transcriptomics to improve diagnosis of fungal infections</b>	Transcriptional analysis of circulating leukocytes differentiates candidemia, viral, and bacterial infection(211).
<b>Understanding the interaction between the immune response and the coagulation system</b>	A prolonged PT is associated with stronger anomalies in pathways implicated in the pathogenesis of sepsis, suggesting that activation of coagulation impacts other host response mechanisms(212). Advanced computational techniques on transcriptomic datasets identify Inflammopathic, Adaptive, and Coagulopathic Clusters(97).
<b>Profiling endotypes to predict response to steroids</b>	Secondary analysis of the VANISH Randomized Trial: The immunocompetent SRS2 endotype is associated with a significantly higher mortality when treated with corticosteroids compared with placebo(213).
<b>Understanding the impact of sepsis-associated immunosuppression on antibiotic stewardship</b>	The upcoming RISC-sepsis trial will provide insights into the impact of sepsis-associated immunosuppression on a biomarker-guided antibiotic duration intervention. The primary outcome measures are monocyte human leucocyte antigen-DR; neutrophil CD88; programmed cell death-1 on monocytes, neutrophils and T lymphocytes and the percentage of regulatory T cells(214).
<b>Understanding long-term consequences of sepsis</b>	Profiling of Long-term Host Immune Response Trajectories: Persistent elevation of inflammation and immunosuppression biomarkers occurred in two-thirds of patients who survived a hospitalization for sepsis and was associated with worse long-term outcomes(64).

**Table 2. Recommendations for Research**

<b>Recommendations for Research</b>
<p><b>Pathophysiology</b></p> <p>Improve understanding of the immune response during sepsis using systems immunology, specifically</p> <ol style="list-style-type: none"><li>1/ Local vs. systemic responses: identify shared / specific immunological alterations leading to the failure of specific organs in sepsis and identify direct or indirect markers of immune failure in different body compartments.</li><li>2/ Pathogen-specific responses: develop tests to assess specific immunological responses against different kinds of infecting microbes, to assess the likelihood that a patient is able to clear the infection (immunobiogram).</li><li>3/ Subpopulation specific responses (children, sex/gender, elderly, immunocompromised etc.)</li><li>4/ Compare with and learn from immune responses in non-infectious conditions.</li><li>5/ Describe evolution over time, including long-term consequences.</li></ol>
<p><b>Basic Immune monitoring</b></p> <p>Current standardised tests profile mostly non-specific markers of systemic inflammation, which are also elevated in other non-infectious conditions. Make better use of already available / basic markers:</p> <ol style="list-style-type: none"><li>1/ Because features of immune dysfunction change over the course of sepsis, repeated measurements of these markers will be more informative than single measurements.</li><li>2/ International guidelines defining the optimal list of immunological markers to be followed in patients and their measurement kinetic are required.</li><li>3/ A list of markers, which should be systematically provided in observational / clinical studies focused on the immune response in sepsis (minimal reported informations) should be defined.</li><li>4/ To reach this goal, a large effort is necessary to standardise measurements of cellular and soluble biomarkers of immune failure in sepsis, so they can be compared between studies, going beyond enumeration to also address functionality.</li><li>5/ Furthermore, microbiology data should be used more extensively, as: persistence of positive results in microbiological cultures and/or, the presence of high microbial burden in blood / other samples represent indirect markers on the existence of impaired immunity in sepsis.</li></ol>

### **Development of new (combinations of) markers**

Studies on the impact of endotypes derived from “omics” analysis combined with routinely assessed immunological biomarkers on outcome and response to treatment are warranted.

Initiate an effort to standardise Omics technologies to transfer them from research labs to routine labs, by:

- 1/ Identifying consensual panels
- 2/ Performing cross validation
- 3/ Transferring panels to simpler and standardizable assays/technologies
- 4/ Combine these markers with other parameters (microbiological data / clinical and demographic data) into combitypes.
- 5/ Clinical validation of newly developed panels/combitypes.
- 6/ Use validated panels / combitypes to create simple workable algorithms that can guide clinical decisions or patient enrichment in trials.

### **Precision medicine**

Provide the rationale for a precision medicine approach in sepsis by performing biomarker-guided RCTs with a high likelihood of a positive outcome, specifically:

- 1/ Define the appropriate design, target population, endpoint, treatment, marker / panel / combitype selection and measurement techniques.
- 2/ Demonstrate the safety of this approach (monitoring of side effects).
- 3/ Demonstrate its efficacy on clinically relevant endpoints.

## Figure legends

**Figure 1: Sequence of immune dysregulation in sepsis:** Many patients with sepsis have predisposing factors such as aging and/or comorbidities which are known to contribute to immune dysregulation (see appendix p 1), thereby impairing their ability to mount an effective response against an infecting microbe. An impaired initial response typically translates into poor microbial control, leading to excessive activation of the innate immune response, acute-on-chronic endothelial dysfunction and/or activation of coagulation. This hyperinflammatory signature is paralleled by the emergence of an immunosuppressive phenotype, exemplified by uncontrolled migration of leukocytes to the extravascular space, increased apoptosis and impaired function of lymphocytes, defective expression of molecules required for antigen presentation such as HLA-DR as well as relative expansion of suppressor cells such as T-regulatory cells (T-regs), Myeloid-derived suppressor cells (MDSC), and immature granulocytes (IGs). This frequently leads to “sepsis-associated immunosuppression”, which renders the host unable to clear the primary infection, provokes reactivation of dormant viruses, and renders the host vulnerable to secondary infections, culminating in a vicious pathogenic circle. These events are compounded by the induction of microbial and immunological-driven tissue injury, leading to organ dysfunction. Many sepsis survivors face long-term clinical consequences, including the so called “Persistent Inflammation, Immunosuppression and Catabolism Syndrome” (PICS) frequently accompanied by cardiovascular, neurological complications and cancer, representing chronic immunological sequelae.

**Figure 2. Potential immunological phenotypes in sepsis.** Patients with sepsis can present with different features of immunological dysfunction and related endothelial and coagulation disturbances. These multi-system derangements can coexist, present with different magnitudes over the disease course and are linked to varying degrees of organ failure. Accurate longitudinal

profiling of the type and dimension of these features can guide appropriate interventions, not only with immunomodulatory drugs, but also with therapeutics targeting specific elements of the endothelial and /or coagulation derangements.

**Figure 3: Technologies to profile the immune status in sepsis.** This figure summarizes technologies that can be used to profile the immune status in sepsis. Presented technologies are either already available in most hospital settings or moving towards broad-scale clinical application. The Figure was partly generated using Servier Medical Art, provided by Servier, licensed under a Creative Commons Attribution 3.0 unported license.

Figure 1

Figure 1

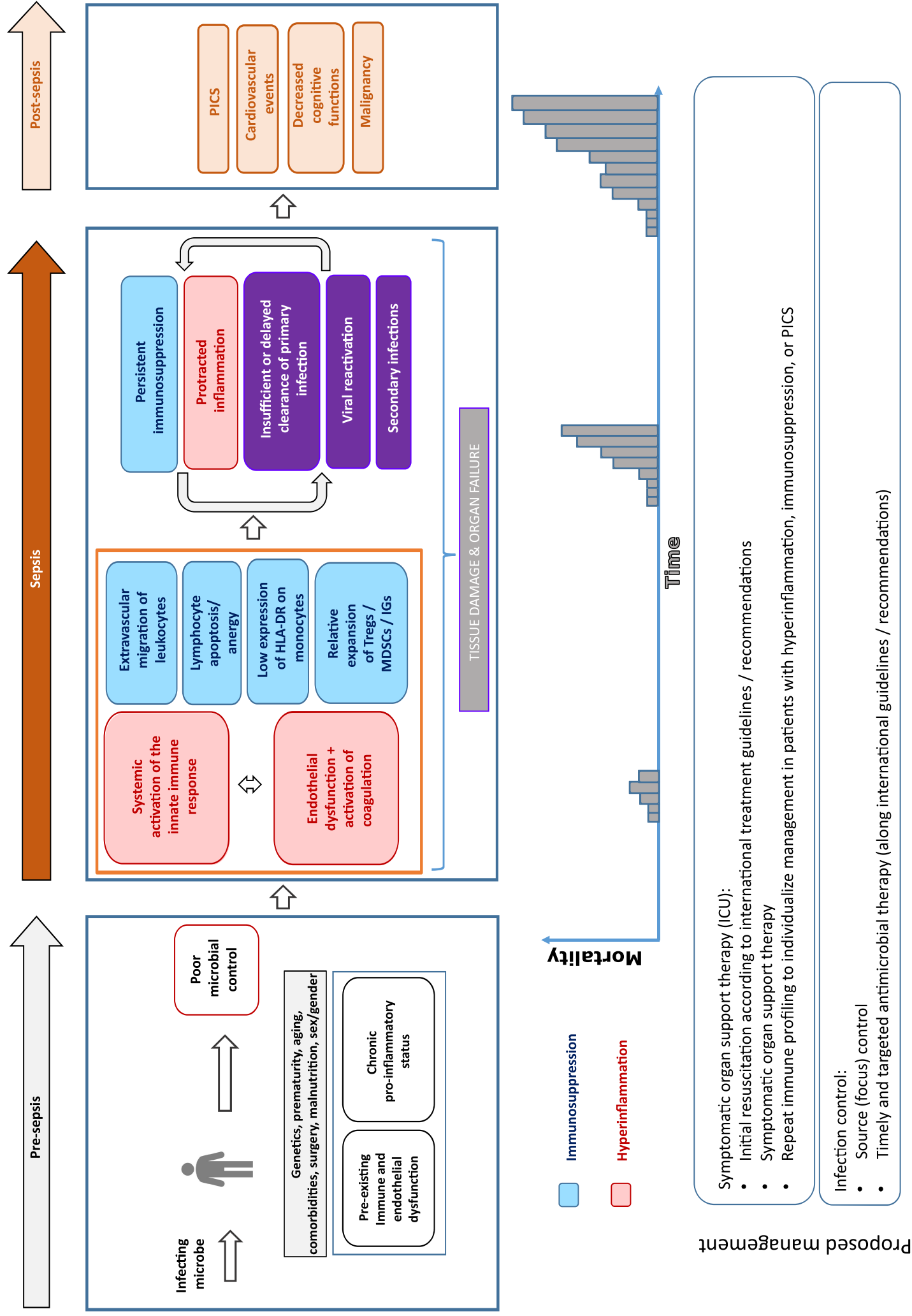
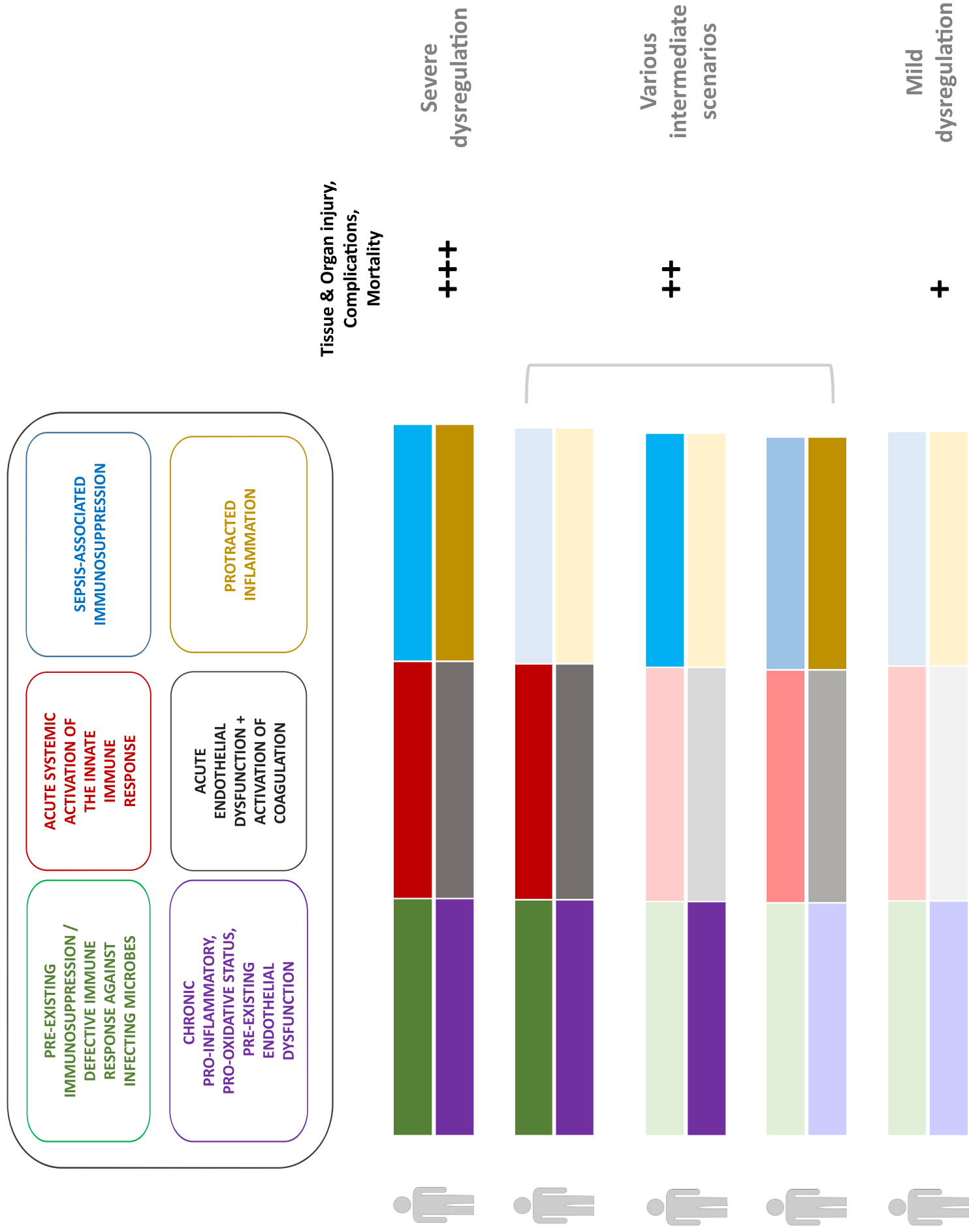


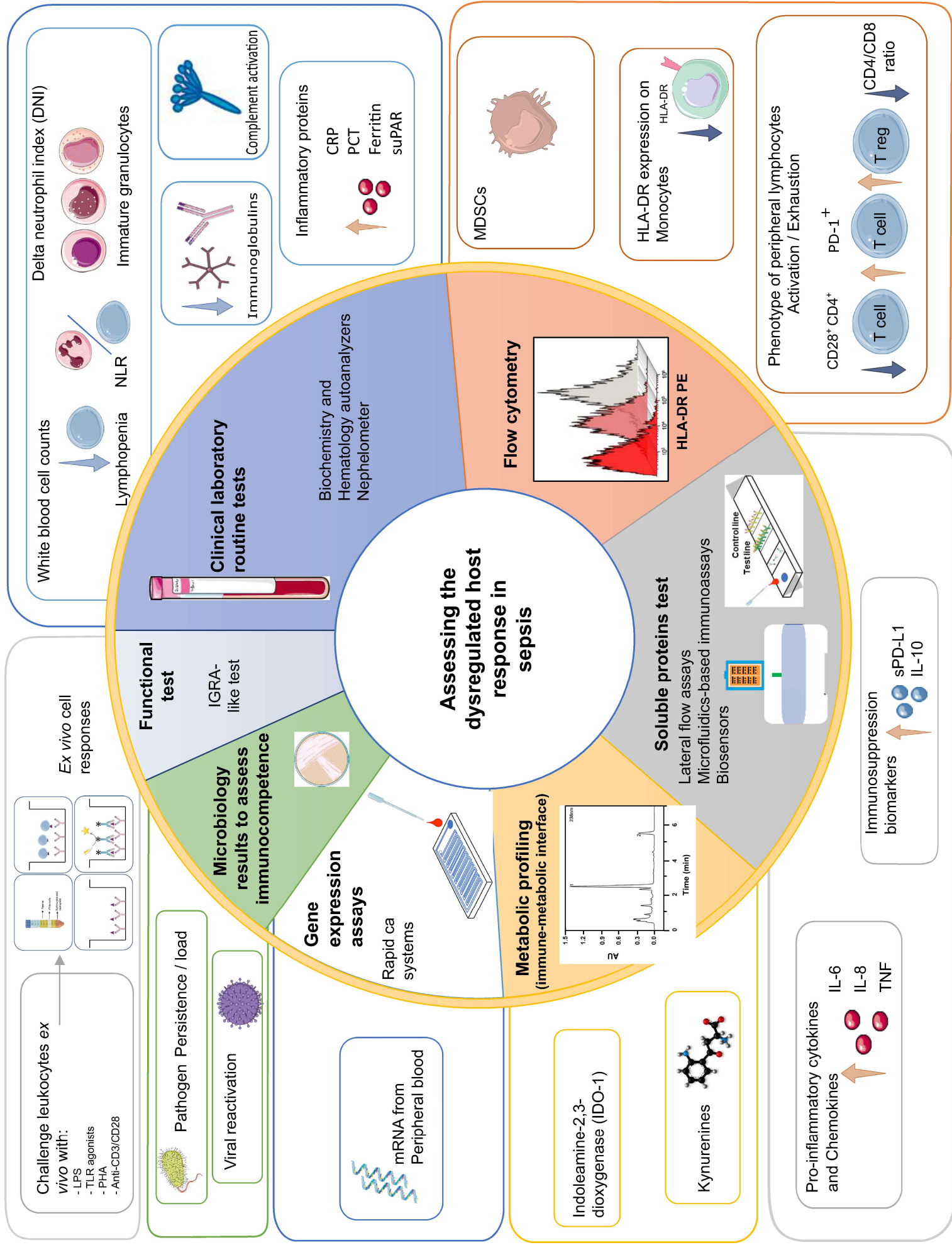


Figure 2

Figure 2



**Figure 3**



## **Appendix**

### **Predisposing risk factors for sepsis**

Several predisposing factors may impair the host's ability to control infection. More than half of children with sepsis show genetic variations linked to inborn errors of immunity<sup>1</sup>. In turn, immaturity of the immune system in preterm infants<sup>2,3</sup> or, at the other extreme, immunosenescence in elderly adults<sup>4,5</sup>, are conditions linked to functional defects in both cell-mediated and humoral immune responses. Cancer and its treatments also induce immunosuppression and may increase the risk for sepsis via functional impairments of T-, B- and NK- and myeloid cells<sup>6,7</sup>. Asplenia can lead to fulminant sepsis, typically caused by encapsulated bacteria, with high mortality<sup>8</sup>. Furthermore, comorbidities including diabetes, chronic obstructive pulmonary disease and hypertension as well as human immunodeficiency virus infection, renal-, and liver disease are accompanied by immunosuppression, chronic inflammation, and endothelial dysfunction, which all predispose towards a dysregulated host response to infection (Figure 1)<sup>9-11</sup>. Surgical trauma elicits an anti-inflammatory response aimed to reduce collateral damage due to tissue injury-induced inflammation, which, when protracted, puts patients at an increased risk of sepsis<sup>12</sup>. Additionally, smoking, alcohol abuse and malnutrition constitute factors that impair immunity<sup>13-16</sup>. Beyond these factors, sex can influence sepsis susceptibility and progression, through genetic and hormonal differences that influence immunity, with a higher risk of poor outcomes in males<sup>17,18</sup>. Also, gender-related aspects, such as differential access to healthcare, can affect the incidence and outcomes of sepsis<sup>18</sup>. These factors underscore the complex nature of predisposing risk factors for sepsis.

## **Biomarker-guided immunoadjuvant treatments in sepsis using extracorporeal therapy and immunoglobulin supplementation**

### *Immunoglobulin supplementation*

In the recent phase II RCT (CIGMA trial), treatment with IgM-enriched immunoglobulin in patients with severe CAP conferred a substantial survival benefit in the subgroup of patients with low levels of IgM at enrollment<sup>19</sup>. The ongoing RCT on immunoglobulin therapy in patients with septic shock (FAT-trial) uses IgM levels at baseline for inclusion as well as daily IgM levels to adjust the dose of immunoglobulins, and will clarify whether IgM should be considered an appropriate therapeutic target<sup>20</sup>. Furthermore, future studies should evaluate whether administration of active polyclonal or monoclonal antibodies against a specific pathogen in patients seronegative for these antibodies translates into clinical benefit, as COVID-19 studies suggest<sup>21</sup>.

### *Extracorporeal treatment in sepsis*

Blood purification devices that aim to remove immunologically active molecules (e.g. pro-/anti-inflammatory mediators, bacterial products/compounds [LPS via polymyxin-B], and/or pathogens) may improve clinical outcomes, although data are inconsistent<sup>22-25</sup>. An early proof-of-concept study demonstrated restoration of mHLA-DR following simultaneous selective removal of C5a, IL-6 and LPS<sup>25</sup>. Patient-tailored strategies and technical advances may be key in the future development and appropriate use of extracorporeal blood purification devices.

**Supplementary Table 1. Current and future markers to profile immune failure in sepsis: Significance and suggested use.**

Name	Significance and suggested use in sepsis
<b>Basic and routinely available biomarkers</b>	
<p><b>CRP</b></p> <p><i>Significance</i></p> <p><i>Suggested use</i></p>	<ul style="list-style-type: none"> <li>• C-reactive protein (CRP) is an acute phase protein produced by the liver in response to IL-6. It is a marker of ongoing inflammation.</li> <li>• Methylprednisolone treatment in patients with community-acquired pneumonia (CAP) and CRP &gt;150 mg/L is associated with lower risk for treatment failure in comparison to placebo<sup>26</sup>.</li> </ul> <ul style="list-style-type: none"> <li>- Clinical routine.</li> <li>- Patient enrichment in sepsis trials with relatively homogenous populations (e.g. CAP) or in combination with clinical parameters or other biomarkers, such as in persisting inflammation, immunosuppression and catabolism syndrome (PICS)<sup>27</sup>.</li> </ul>
<p><b>Leukocyte counts</b></p> <p><i>Significance</i></p> <p><i>Suggested use</i></p>	<ul style="list-style-type: none"> <li>• Total leukocyte counts are often elevated in sepsis, but acute leukopenia may also be an early sign of septic shock.</li> <li>• The white blood cell count (WBC) can be subdivided in neutrophils, eosinophils, basophils, monocytes and lymphocytes.</li> <li>• Lymphopenia at admission is associated with hospital mortality<sup>28,29</sup>. Persisting lymphopenia may indicate sepsis-induced immunosuppression.</li> <li>• Absolute lymphocyte count &lt; 900 cells/<math>\mu</math>L or 1 100 cells/<math>\mu</math>L has been used for patient enrichment in sepsis trials investigating immune checkpoint inhibitors and rhIL-7<sup>30-36</sup>.</li> <li>• Lymphocyte counts are included in the definition of PICS<sup>27</sup>.</li> <li>• Elevated neutrophil to lymphocyte count ratio (NLR) may be used as an early indicator of sepsis in the emergency room and in neonatal sepsis<sup>37,38</sup> but has moderate sensitivity/specificity to differentiate sepsis from a non-sepsis diagnosis in the ICU<sup>39</sup>.</li> <li>• Eosinopenia in the acute disease phase differentiates infectious vs noninfectious etiologies better than CRP<sup>40</sup>.</li> </ul> <ul style="list-style-type: none"> <li>- Clinical routine.</li> <li>- Patient enrichment in trials investigating interventions aimed at enhancing adaptive immune responses.</li> </ul>
<p><b>Ferritin</b></p> <p><i>Significance</i></p> <p><i>Suggested use</i></p>	<ul style="list-style-type: none"> <li>• Ferritin is an acute phase protein.</li> <li>• Highly elevated levels serve as a marker of hemophagocytic lymphohistiocytosis (HLH), or the hyper-inflammatory, (Macrophage Activation Syndrome-like (MALS)-like phenotype in sepsis<sup>41</sup>.</li> <li>• Ferritin &gt; 4 420 ng/mL has been used to guide IL-1 inhibition in sepsis<sup>42</sup>.</li> </ul> <ul style="list-style-type: none"> <li>- Clinical routine.</li> <li>- Patient enrichment in clinical trials investigating anti-inflammatory therapies to treat the (MAS)-like phenotype (MALS).</li> </ul>

<b>Interleukin-6</b>	
<i>Significance</i>	<ul style="list-style-type: none"> <li>• Interleukin (IL)-6 is a pro-inflammatory cytokine that rapidly increases in multiple critical illnesses including sepsis, Acute Respiratory Distress Syndrome and COVID-19.</li> <li>• Early elevations IL-6 are related to a hyperinflammatory phenotype in ICU patients<sup>43-45</sup>.</li> </ul>
<i>Suggested use</i>	<p>IL-6 &gt; 1 000 pg/mL has been used to guide TNF inhibition in sepsis<sup>46</sup>.</p> <ul style="list-style-type: none"> <li>• No clinical trials on IL-6 inhibition in bacterial sepsis have been conducted.</li> </ul> <p>- May have potential in identifying a hyperinflammatory phenotype.</p>
<b>More specific but not routinely available biomarkers</b>	
<b>mHLA-DR</b>	
<i>Significance</i>	<ul style="list-style-type: none"> <li>• Monocyte (m)HLA-DR expression is the most widely used marker of immune suppression in sepsis.</li> <li>• It is independently associated with increased risk of nosocomial infections and death in septic patients<sup>47</sup>.</li> <li>• When measured by standardized flow cytometry methodology, results are expressed as a numbers of antibodies bound per cell (Ab/cell)</li> <li>• Reference values are available for defining normal ranges in healthy controls (13 500 to 45 000 Ab/cell)<sup>48</sup>, immunosuppression (&lt; 8 000 Ab/cell) and marked immunosuppression or “immunoparalysis” (&lt; 5 000 Ab/cell)<sup>49</sup>.</li> </ul>
<i>Suggested use</i>	<ul style="list-style-type: none"> <li>- Guiding initiation of and monitoring of immunostimulatory therapy in individual patients.</li> <li>- Patient enrichment in trials investigating interventions aimed at enhancing innate immune responses.</li> </ul>
<b>Immunoglobulins</b>	
<i>Significance</i>	<ul style="list-style-type: none"> <li>• Therapy with IgM-enriched immunoglobulin in patients with severe CAP showed a substantial benefit in survival rate in the subgroup of patients with low levels of IgM at enrollment<sup>19</sup>.</li> <li>• The ongoing randomized control trial on immunoglobulin therapy in patients with septic shock (FAT-trial) will clarify whether IgM should be considered an appropriate therapeutic target<sup>20</sup>.</li> </ul>
<i>Suggested use</i>	<ul style="list-style-type: none"> <li>- Report IgG and IgM levels in studies on sepsis-associated immunosuppression.</li> </ul>
<b>Lymphocyte subpopulations count</b>	
<i>Significance</i>	<ul style="list-style-type: none"> <li>• In elderly patients with sepsis, the mean CD4/CD8 T-cell ratio was significantly lower in non-survivors than in survivors<sup>50</sup>.</li> </ul>
<i>Suggested use</i>	<ul style="list-style-type: none"> <li>- Report in studies on sepsis-associated immunosuppression.</li> </ul>

<p><b>Lymphocyte subpopulation phenotypes</b></p> <p><i>Significance</i></p> <p><i>Suggested use</i></p>	<ul style="list-style-type: none"> <li>• In sepsis, the percentage of CD4+CD25+Foxp3+ regulatory T cells (T-regs) increases<sup>51</sup>.</li> <li>• A typical hallmark of T-cell exhaustion is a sustained increase in cell-surface expression of the inhibitory immune checkpoint molecule programmed cell death 1 (PD-1) on T-cells.</li> <li>• Increased expression of PD-1 (and PD-L1 on antigen-presenting cells) is associated with progression of infection to sepsis, risk of nosocomial infections, and a more severe disease state<sup>52-54</sup>.</li> </ul> <p>- Patient enrichment in sepsis trials investigating interventions aimed at enhancing adaptive immune responses.</p>
<p><b>Immature myeloid cells</b></p> <p><i>Significance</i></p> <p><i>Suggested use</i></p>	<ul style="list-style-type: none"> <li>• MDSCs mobilize and migrate from bone marrow to peripheral tissues or immune organs.</li> <li>• Low surface expression of CD16 and CD10 on neutrophils is indicative of MDSCs<sup>55-57</sup>.</li> <li>• Increased abundance of these MDSCs is associated with increased risk of death and secondary infections in sepsis<sup>55,58</sup>.</li> <li>• Delta neutrophil index (DN) is the immature granulocyte fraction provided by certain blood cell analyzers. It has been proposed as a useful marker for early diagnosis and prognostication in sepsis, but its value for immune profiling requires further study<sup>59</sup>.</li> </ul> <p>- Report MDSCs proportions/counts in studies on sepsis-associated immunosuppression.</p>
<p><b>Soluble receptors</b></p> <p><i>Significance</i></p> <p><i>Suggested use</i></p>	<ul style="list-style-type: none"> <li>• Soluble PD-1 and PD-L1 (i.e., sPD-1 and sPD-L1) are recognized as possible sepsis biomarkers<sup>60,61</sup>, but no clear links with immune organ failure have been identified yet.</li> </ul> <p>- Report in studies on sepsis-associated immunosuppression.</p>
<p><b>Complement</b></p> <p><i>Significance</i></p> <p><i>Suggested use</i></p>	<ul style="list-style-type: none"> <li>• Links between the complement system and impaired innate immunity have been reported<sup>62</sup>, as well as a relationship between C3 depletion and the expansion of T-regs<sup>63</sup>.</li> <li>• The consecutive release of the C5a anaphylatoxin has been proposed as a therapeutic target<sup>64,65</sup>.</li> </ul> <p>- Report in studies on sepsis-associated immunosuppression.</p>
<p><b>Other cytokines/cytokine panels</b></p> <p><i>Significance</i></p> <p><i>Suggested use</i></p>	<ul style="list-style-type: none"> <li>• Early elevations in IL-8 are related to a hyperinflammatory phenotype in ICU patients<sup>43,66,67</sup>.</li> <li>• Increased levels of IL-10 and TGF-<math>\beta</math> are associated with immunosuppression and implicated to be causally related to the reduced expression of mHLA-DR<sup>68,69</sup>.</li> </ul> <p>- Report in studies on sepsis-associated immunosuppression.</p>

<p><b>Cytokine release assays / Functional tests</b></p> <p><i>Significance</i></p> <p><i>Suggested use</i></p>	<ul style="list-style-type: none"> <li>• Functional tests assess the capacity of a cell population (usually leukocytes) to respond to an <i>ex vivo</i> immune challenge.</li> <li>• For instance, low Tumor Necrosis Factor (TNF) production by <i>ex vivo</i>-stimulated whole blood was used as an inclusion criterion to apply immunostimulatory therapy in pediatric patients with sepsis<sup>70</sup>.</li> <li>• Commercial products are available with tubes containing an immunological stimulus dissolved in culture medium, which draw a specific volume of blood. These can subsequently be incubated and centrifuged, after which the supernatant can be assessed for cytokines or other mediators<sup>71</sup>.</li> </ul> <ul style="list-style-type: none"> <li>- Report in studies on sepsis-associated immunosuppression.</li> </ul>
<p><b>Viral reactivation</b></p> <p><i>Significance</i></p> <p><i>Suggested use</i></p>	<ul style="list-style-type: none"> <li>• Reactivation of latent viruses such as cytomegalovirus, herpes simplex virus, and Epstein-Barr virus is a hallmark of sepsis-associated immunosuppression<sup>72-74</sup>.</li> </ul> <ul style="list-style-type: none"> <li>- Monitoring of high-risk patients with prolonged critical disease.</li> <li>- Report in studies on sepsis-associated immunosuppression.</li> </ul>
<p><b>Metabolic markers</b></p> <p><i>Significance</i></p> <p><i>Suggested use</i></p>	<ul style="list-style-type: none"> <li>• Sepsis-associated immunosuppression is paralleled by marked tryptophan depletion and increased kynurenine levels<sup>75</sup>.</li> <li>• IDO-induced generation of kynurenine is associated with a global decrease in effector T-cell functions, increased T-cell apoptosis, decreased T-cell proliferation/ activation, and increased generation of regulatory T-cells<sup>75</sup>.</li> </ul> <ul style="list-style-type: none"> <li>- Report in studies on sepsis-associated immunosuppression</li> </ul>
<p><b>Damage associated molecular patterns (DAMPs)</b></p> <p><i>Significance</i></p> <p><i>Suggested use</i></p>	<ul style="list-style-type: none"> <li>• DAMPs such as HMGB1 and heat shock proteins may play a role in sepsis-associated immunosuppression.</li> <li>• Soluble urokinase plasminogen activator receptor (suPAR) &gt; 6 ng/mL was used to guide treatment with the IL-1 receptor antagonist anakinra in the SAVE-MORE trial in COVID-19<sup>76</sup>.</li> </ul> <ul style="list-style-type: none"> <li>- May be of value for patient enrichment in sepsis trials investigating interventions aimed at enhancing adaptive immune responses.</li> <li>- Report in studies on sepsis-associated immunosuppression.</li> </ul>



<b>Emerging biomarkers</b>	
<p><b>Microfluidics-based platforms for protein profiling</b></p> <p style="text-align: center;"><i>Significance</i></p> <p style="text-align: center;"><i>Suggested use</i></p>	<ul style="list-style-type: none"> <li>• New microfluidics-based platforms are able to quantify immunological or endothelial proteins in a multiplex format in less than two hours<sup>77</sup>.</li> <li>• Biosensors are emerging to rapidly profile host response proteins, cell surface markers or even functional parameters such as neutrophil motility<sup>78,79</sup>.</li> </ul> <ul style="list-style-type: none"> <li>- In exploratory phase</li> <li>- May in the future be integrated into workable algorithms in combination with soluble markers of immune and endothelial responses, platelet counts or mRNA to generate a decision tree to serve as a prognostic enrichment tool/ decision trees usable by clinicians.</li> </ul>
<p><b>Transcriptomics</b></p> <p style="text-align: center;"><i>Significance</i></p> <p style="text-align: center;"><i>Suggested use</i></p>	<ul style="list-style-type: none"> <li>• Commercially available automated multiplex PCR devices that identify acute infection and suspected sepsis<sup>80,81</sup> are currently being adapted/developed for immune endo/phenotyping<sup>82-84</sup>.</li> </ul> <ul style="list-style-type: none"> <li>- In exploratory phase</li> <li>- May in the future be used for patient enrichment in sepsis trials.</li> </ul>

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